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I. Coulibaly · J. Louarn · M. Lorieux · A. Charrier
S. Hamon · M. Noirot

Pollen viability restoration in a *Coffea canephora* P. and *C. heterocalyx* Stoffelen backcross. QTL identification for marker-assisted selection

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Abstract Male fertility of interspecific hybrids was analysed in one F1 and two backcrossed progenies originating from a cross between *Coffea canephora* and *Coffea heterocalyx*. Male fertility was tested using pollen stainability with acetic carmine. The results showed a marked decline in fertility at the F1 level, and fertility was almost fully restored after two backcrosses. The computed broad-sense heritability represented 47% of the variance. Quantitative trait loci (QTLs) locations and effects on pollen viability were estimated using an amplified fragment length polymorphism (AFLP) genetic linkage map constructed in the segregating BC1 population. Three significant QTLs (LOD>3 and $p < 0.001$ by ANOVA) were detected for pollen viability, two of which were responsible for the bimodal distribution of pollen viability in the segregating population. One QTL was involved in fertility variations among fertile BC1 plants. Fertility inheritance is discussed in relation with previously demonstrated chromosomal sterility in *Coffea* hybrids and the effect of detected QTLs. The potential use of genetic markers to overcome sterility in interspecific hybrids is also discussed.

Keywords *Coffea canephora* · Interspecific hybrid · Sterility · Pollen viability · QTL

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I. Coulibaly · M. Lorieux · S. Hamon · M. Noirot (✉)
Centre IRD, 911 Avenue Agropolis B.P. 5045,
34032 Montpellier Cedex 1, France
e-mail: noirot@mpl.ird.fr
Tel.: +33-4-67-41-62-43, Fax: +33-4-67-41-62-22

A. Charrier
ENSA de Montpellier, 2 Place Pierre Viala,
34060 Montpellier Cedex 1, France

J. Louarn
Station IRD de Génétique des Caféiers, B.P. 434 Man,
Côte-d'Ivoire

Introduction

In several plant genera, interspecific hybridization has played a key role in the creation of modern varieties. In coffee trees, *Coffea heterocalyx* (HET) (Stoffelen et al. 1996) is the only wild diploid autogamous species and it was selected as a gene donor to introgress self-compatibility in the allogamous diploid cultivated species *Coffea canephora* (CAN) (Louarn 1992; Coulibaly et al. 2002). F1 hybrids and backcrossed BC1 and BC2 generations are currently available at the IRD Coffee Genetic Station of Man (Côte-d'Ivoire).

Unfortunately, the most described feature of interspecific hybrids is their sterility. This classically includes genic sterility (negative combinations between parental genes), chromosomal sterility (lack of structural homology disturbing meiotic pairing), and cytoplasmic sterility (unfavorable interactions between nuclear genes and foreign cytoplasm) (Grant 1971). Consequently, full-fertility recovery is an important prerequisite for the agronomic use of these hybrids. This is particularly important in coffee trees where the presence of two beans per fruit is a must to obtain a commercially acceptable bean shape. Peaberries containing only one bean have a lower commercial value. Backcrossing has been commonly performed in attempts to overcome such hybrid sterility.

Pollen viability (PV) is an accurate and fast male fertility estimator. Previous investigations on coffee pollen fertility showed that cytoplasm-staining tests, using acetic carmine and *Alexander* dye or the vital staining test (tetrazolium salt), gave results that were closely correlated with pollen in vitro germination tests on agar (Grassias 1980; Owuor and Vossen 1981). In our study, a quantitative approach was applied to assess pollen viability in the F1, BC1 and BC2 in order to forecast the possibility of full male fertility restoration. In addition, a QTL analysis based on an AFLP linkage map was carried out on the BC1 progeny.

Materials and methods

Plant material

The plant materials were the same as those analysed by Coulibaly et al. (2002). *C. canephora* P. (CAN) and *C. heterocalyx* Stoffelen (HET) were hybridized using CAN as the female parent, due to the unilateral incompatibility between the two species. Seven interspecific F1 hybrids were obtained, one of which was backcrossed, as female parent, with a bulk of CAN progenitors. About 100 BC1 individuals were obtained. The second backcross generation (BC2) was obtained from three controlled pollinations between self-compatible BC1 parents (as female) and CAN pollinators: D160436 × IF181 (11 plants); D160437 × IF444 (6 plants); and D160538 × IF444 (13 plants).

Pollen viability evaluation

Male fertility was estimated on four CAN, four HET, seven F1 hybrids, 40 BC1 hybrids and 30 BC2 hybrids.

Pollen viability (PV) was evaluated according to Belling's acetic carmine 2% coloration test (Grassias 1980). The day before anthesis, three flower buds were harvested per tree and isolated overnight in a Petri dish. The pollen was extracted by crushing stamina in 2% acetic carmine. Pollen viability was scored as the percentage of stained pollen grains in all of the pollen counted (at least 300).

QTL identification

The procedure for obtaining AFLP markers was described in Coulibaly et al. (2002). The MAPMAKER/Exp. version 3.0b software program was used to determine linkage groups and to order loci. Analysis were performed with a LOD score threshold of 5.0 and a maximum recombination value of 30% for grouping and ordering markers. The mapped population was larger than the one we used for fertility inheritance, and comprised 74 BC1 plants.

Single marker analysis (ANOVA) was first performed using the Statistica software package to determine genome regions involved in male fertility variation with a probability level of $p < 0.001$. QTL mapping by interval analysis was also done with a LOD score threshold of 3 by using the MAPMAKER/QTL 2.4 software program; linkage groups were scanned at the 2-cM-interval test statistic. In order to minimize the type-I error (i.e. to avoid false-positive QTLs), only QTLs identified by both methods were retained. Genetic effects and the percentage of phenotypic variation attributable to QTLs were estimated at the LOD peak.

Other statistical analyses

One-way ANOVA was used to compare between-species pollen viability (p = 2 species, n = 4 trees per species). Within each species, a one-way ANOVA was applied to test between-plant variation. When between-tree variation was significant, genetic variance σ^2_g and residual variance σ^2_e were estimated as $(MS_g - MS_r)/n$ and MS_r, respectively (MS = mean square). Broad-sense heritability $(h^2_{s.l.}) = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$ was then calculated.

A one-way ANOVA was also performed to test between-hybrid differences in each generation (F1, BC1 and BC2) and to compare BC1 and BC2 generations.

A nested ANOVA model was carried out to test between-family differences and between-hybrid variation within families (p = 3 families, q = 6 hybrids per family and n = 4 flowers per hybrid). Between-family, between-hybrid and residual variances were calculated using $\sigma^2_f = (MS_f - MS_h)/nq$, $\sigma^2_h = (MS_h - MS_e)/n$, $\sigma^2_e = MS_r$, respectively. Genetic effects represented $\sigma^2_f + \sigma^2_h$, and their contribution to the phenotypic variance was defined as $(\sigma^2_f + \sigma^2_h)/(\sigma^2_f + \sigma^2_h + \sigma^2_e)$. Between-family contributions to genetic effects were formulated as $\sigma^2_f/(\sigma^2_f + \sigma^2_h)$.

Other tests used were: a two-way ANOVA with interaction to test digenic interactions between QTLs; a chi^2 test to compare observed segregations to expected Mendelian distributions in the BC1.

In addition, the simplex method (Nelder and Mead 1965; O'Neill 1971) was used to estimate QTL effects.

Statistical analyses were carried out using the Statistica software package (5.1 version, 1997 for Microsoft Windows), except for the simplex method that was performed using a program that we developed.

Results

Pollen viability (PV) in parental species

PV was 81% and 89% in four CAN and HET trees, respectively, with no significant differences ($F_{1,6} = 0.93$; $p = 0{,}37$). No variations were recorded within HET ($F_{3,8} = 0.93$; $p = 0.47$), as expected from plants rising from a self-pollinated homozygous tree. In contrast, highly significant differences were observed between trees (62 to 98%) within CAN ($F_{3,8} = 27.4$; $p < 0.001$), representing important genetic effects ($h^2_{s.l.} = 90\%$).

Pollen viability restoration

PV was substantially reduced in F1 hybrids, and four of the seven F1 hybrids did not flower or else presented floral abnormalities. They were excluded from the analysis. No significant PV difference was noted between the other plants ($F_{2,6} = 4.29$; $p = 0.07$), and their PV (mean 18%) represented 20% of the CAN one (Table 1).

In the BC1 progeny, PV was a mean 3.2-fold (58%) higher than that observed in the F1. Highly significant differences were recorded between BC1 hybrids ($F_{42,85} = 23.9$; $p < 0.001$). The BC1 PV distribution ranged from very low values (8%) up to full fertility (90%), with overlapping of both F1 and parental variation (Fig. 1a). Two groups could be defined (Fig. 1a): a group of 12 weak fertile plants (BC1-1), with a PV ranging from 8% to 44% (mean 23.9%); and a group of 31 fertile plants (BC1-2) with a PV ranging from 50% to 90% (mean 71.2%). PV did not differ in the BC1-1 and F1 groups ($F_{1,14} = 0.085$; $p = 0.775$).

This observed 12–31 segregation ratio did not differ significantly from a 1–3 ratio, suggesting Mendelian inheritance with two major and independent loci (A and B) and two alleles per loci (A and a, B and b, respectively, where the capital letter indicates a CAN allele and the lower-case letter indicates a HET allele). Four genotypes (AABB), (AaBB), (AABb) and (AaBb) theoretically exist in BC1. Since pollen viability in the F1 was identical to that of the BC1-1 subgroup, the two types of hybrids would have the same genotype (AaBb). And then the three genotypes (AABB), (AaBB) and (AABb) would lead to the same phenotype (PV = 71.2%), corresponding to the BC1-2 subgroup.

In BC2, the PV distribution was unimodal, ranging from 52% to 95% (Fig. 1b), with a mean of 84%, which

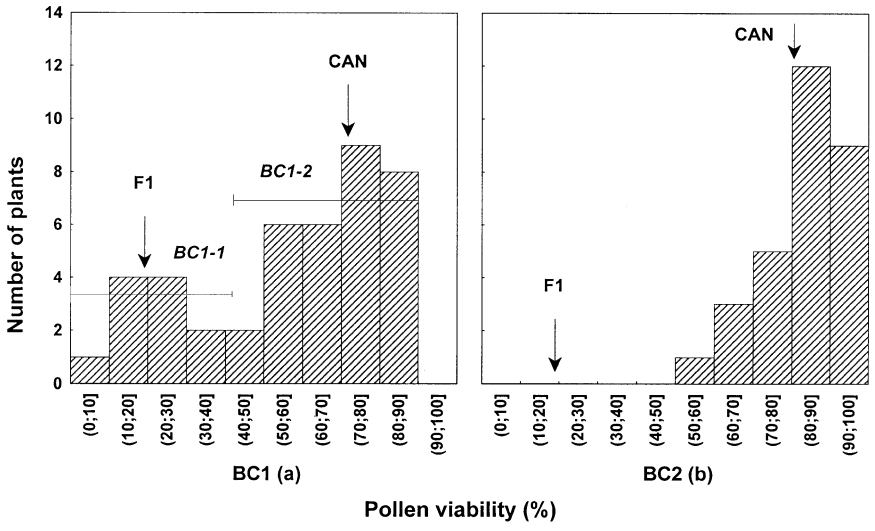

Fig. 1 Pollen viability distribution for the BC1 population (**a**) and BC2 population (**b**) *Arrows* indicate means for CAN and F1 hybrids

Table 1 Mean performance and range of pollen viability (%) of the parental species, and the F1, BC1 and BC2 hybrid progenies. Minimums and maximums are indicated in parenthesis

Item	CAN	HET	F1	BC1	BC2
Pollen viability (%)	81.4 (62–98)	88.8 (85–95)	18.2 (15–25)	58.2 (8–90)	83.6 (59–95)

Table 2 Pollen viability in BC2 progenies according to the BC1 parent

BC1 female parent	PV of the BC1 parent	PV of the BC2 progeny
D160436	58% *b*	73.1% *b*
D160437	84% *a*	91.1% *a*
D160538	85.3% *a*	86.9% *a*

was significantly higher than that of BC1 ($F_{1,70}$ = 28.4; $p < 0.001$). Its range spanned both the within-CAN and within-BC1-2 variations. Significant differences ($F_{2,15}$ = 5.17; $p = 0.019$) were noted between the three BC2 families (D160436 × IF181, D160437 × IF444 and D160538 × IF444) and also between hybrids within families ($F_{15,54}$ = 15.4; $p < 0.001$). Genetic effects (between-families and between-trees within families) accounted for 83% of the phenotypic variance, whereas between-family variation contributed to 47% of the genetic effects: consequently the family PV increased with the parental PV (Table 2).

A model explaining pollen viability restoration changes from F1 to BC2

Assuming that univalent chromosomes from the same pair segregate randomly ($p = 0.5$) between daughter-cells at anaphase-I, and that only euploid gametes are viable, a relationship between the univalent chromosome number (UCN) and PV can be theoretically expressed as PV = $(0.5)^{UCN/2}$. Hence, pollen viability should exponentionally decrease as UCN increases, and vice versa.

In a backcross scheme, UNC should be twofold lower at each generation. With the female parent of all BC1 hybrids having PV = 25%, four UNCs were expected for this parent, leading to the prediction of UNC = 2 and PV = 50% in BC1: the expected PV was close to the 58% observed. BC1 tree # D160436 had a PV of 58% and its UCN should be 1.6. Consequently, the expected PV in its BC2 offspring should be 76%. Similarly, the expected PV in BC2 progenies of BC1 trees # D160437 and # D160538 should be 92%. In all cases, this was close to observed values (Table 2), showing that the chromosomal sterility model can forecast PV restoration.

Identification of pollen viability QTLs in BC1

Three PV QTLs were detected on two linkage groups LG2 and LG13 (Fig. 2; Table 3), called *pv1*, *pv2 and pv3*, respectively.

Pv1 and pv2 QTLs and bimodality of the BC1 population

Pv1 and *pv2* were both on LG2, and 80-cM-distanced. They accounted for 45% and 36% of the PV variance, respectively. *Pv1* was localized at one end of LG2, 6 cM from the AAGCAA14 marker, while *pv2* was in a cluster, 2 cM from the AAGCAC13 marker. The two AFLP

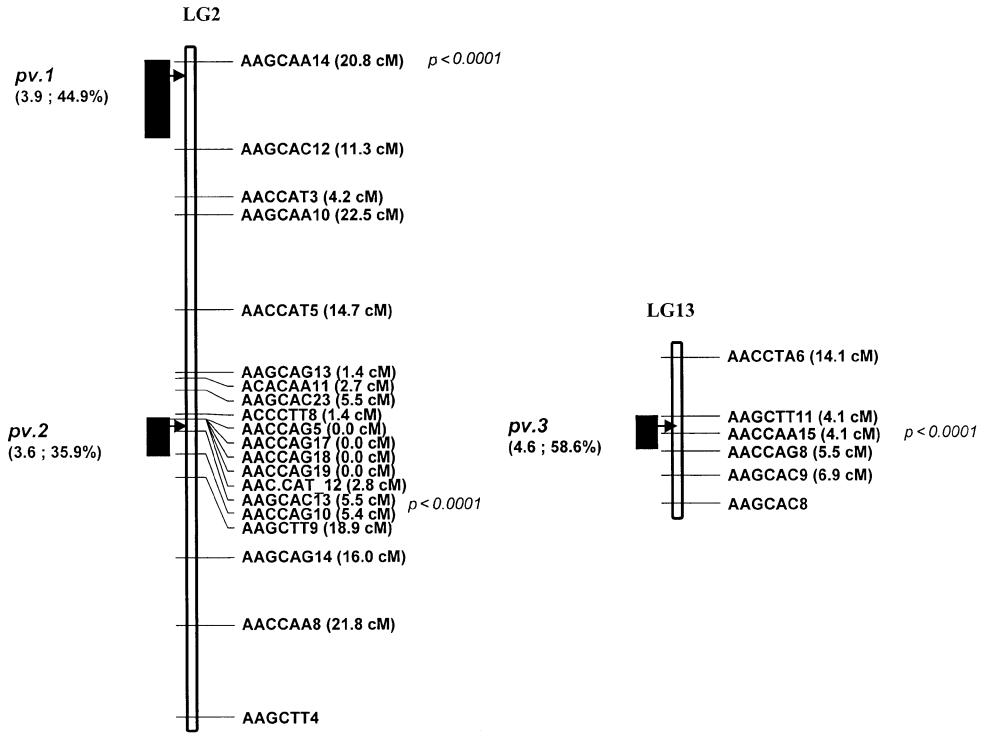

Fig. 2 Linkage map based on AFLP markers showing locations of pollen viability (*pv*) putative QTLs (LOD > 3.0 and $p < 0.001$) determined by both interval mapping and ANOVA analysis. Markers are named, on the right side of the linkage group, the combination of the three selective nucleotides of the *Eco*RI primer and the *Mse*I primers, followed by a number symbolizing the size of the marker. The genetic distance in cM (Kosambi) is indicated on the same line at the right of the locus name. Putative QTLs are presented as hatched bars to the left side of the linkage group, and the length of the bar represents the 1-LOD support interval from the peak LOD indicated by an *arrow*. The numbers in parenthesis, under the trait name, represent the peak LOD and variance explained by individual QTLs, respectively. The *p* value determined by ANOVA is indicated in front of the nearest QTL marker

markers did not show segregation distortion: the AAGCAA14 marker showed 36:36 segregation, corresponding to the expected Mendelian segregation in BC1. For AAGCAC13, the observed segregation was 43:29 and also did not differ from the 1:1 ratio (Table 3).

AAGCAC13 and AAGCAA14 markers were taken into account to analyse the QTL effects of *pv1* and *pv2*. No interactions were detected between these two markers. For each marker, we defined two alleles, C1 and c1 for AAGCAC13, and C2 and c2 for AAGCAA14; where CAN is C1C1C2C2 and HET is c1c1c2c2. Homozygous hybrids at both loci (C1C1 C2C2) displayed the strongest fertility with PV = 77% (Table 4), while double heterozygous hybrids (C1c1 C2c2) had the lowest PV (36%). Heterozygous hybrids at one locus and homozygous at the other (C1c1C2C2 and C1C1C2c2) both had PV = 54%, suggesting similar effects for C1 and C2, and c1 and c2. To determine the QTL effects, we cannot use the classical additive approach because PV was expressed as a proportion, i.e. resulting from a Bernouillian process.

Based on the assumption of chromosomal sterility, a model can be developed where the genotype of the hybrid determines its pollen viability. Let P_D be the probability of producing viable pollen for a homozygous hybrid (C1C1 or C2C2) and P_d be the respective probability for heterozygous hybrids (c1C1 or c2C2), then the expected pollen viability of C1C1C2C2, C1c1C2C2, C1C1C2c2 and C1c1C2c2 genotypes would be P_D^2, P_DP_d, P_DP_d and P_d^2, respectively. Using the simplex method, we estimated $P_D = 0.876$ and $P_d = 0.606$. The expected hybrid pollen viability would then be 0.768, 0.531, 0.531 and 0.367 for C1C1C2C2, C1c1C2C2, C1C1C2c2 and C1c1C2c2 genotypes, respectively. This probability model, applied to QTL effects, fitted well with the observed data presented in Table 4.

Two models can thus be used to explain pollen viability variation: a Mendelian model with loci A and B, and

Table 3 Parameters of QTLs explaining male fertility detected by single point analysis (ANOVA), and simple interval mapping (SIM) in the whole BC1 progeny and in the BC1-2 sub-progeny

Segregating population	QTL	LG	Nearest marker	ANOVA	SIM	
				p	*LOD*	R^2 (%)
BC1	*Pv1*	2	AAGCAA14	<0.0001	3.9	44.9
	Pv2	2	AAGCAG13	<0.0001	3.6	35.9
BC1-2	*Pv3*	13	AACCAA15	<0.0001	4.6	58.6

Table 4 Mean pollen viability performance for genotypic groups defined at the loci of AAGCAA14 and AACCAG10, which are the *pv.1* and *pv.2* nearest markers, respectively. C (capital) indicates an allele inherited from CAN. c (small) indicates an allele inherited from HET. The number in parenthesis indicates the genotypic class size

Genotypes at the nearest QTL marker		Pollen viability (%)
AAGCAA14	AAGCAC13	
C1//C1	C2//C2	76.6 (17)
	C2//c2	53.7 (4)
C1//c1	C2//C2	53.7 (6)
	C2//c2	36.3 (11)

a QTL model with loci C1 and C2. A relationship was actually noted between loci A and C1, on one hand, and loci B and C2, on the other hand, based on the following facts: (1) among the 12 BC1-1 hybrids (AaBb), eight were C1c1C2c2; (2) among the 24 mapped BC1-2, only two are C1c1C2c2; while (3) 16 of the 18 C1C1C2C2 belonged to the BC1-2 group.

Pv3 QTL and the within-BC1-2 variation in pollen fertility

Pv3 was detected only when the analysis focused on the BC1-2 group. This QTL was localized on the LG13 linkage group, 2 cM from the non-distorted AACCAA15 AFLP marker.

This marker allowed identification of two hybrid groups within the fertile BC1 class: homozygous C3C3 and heterozygous C3c3. The C3C3 group PV was 79.6%, whereas the C3c3 group PV was 62.7%. In BC1-1, there were no differences between C3C3 and C3c3 genotypes (29.3% vs 31.9%, respectively).

Discussion

Pollen viability in C. canephora

The variability in pollen viability that we observed within *C. canephora* was similar to Louarn's (1992) results in terms of the mean and range. The PV heritability *sensu lato* was high (90%), but our results were based only on one flowering, and climatic conditions are also known to influence PV – the best viability is recorded when anthesis occurs just after the wet season (Louarn 1992). The heritability *s.l.* could be lower in more unsettled environmental conditions.

Pollen viability restoration and chromosome unpairing

In *Coffea* F1 hybrids, PV ranged from less than 5% in the *C. racemosa* × *C. canephora* cross to 80% in *C. canephora* × *Coffea congensis*. Under our conditions, CAN × HET F1 hybrids appeared to be among the least-fertile diploid hybrids (Louarn 1992).

Chromosomal sterility is the common rule in *Coffea* interspecific F1 hybrids: PV is related to the percentage of pollen mother cells (% PMC) having 11 bivalents (Louarn 1975, 1992). In CAN × HET F1 hybrids, the % PMC explains 74% of the pollen viability (computed from Louarn's 1992 data).

In *Viola*, *Nicotiana, Tragopogon* and *Triticum*, this strong sterility is followed by fertility recovery in successive backcrossing generations (Stebbins 1952). By the model we developed, PV variations from F1 to BC2 were clearly explained by the decrease in univalent number. In addition, it could be noticed that the mean PV in F1 hybrids was 18% and the expected UCN was 4.95, which is close to the UCN observed in F1 hybrids (5.3) (Louarn 1992).

Pollen viability restoration in BC2 was facilitated by selection of the most-fertile BC1 hybrids as female parents for the next generation, and also by the low number (two) of loci – or chromosomal regions – involved in pollen viability variation. In *Coffea arabusta* hybrids (*Coffea arabica* × *C. canephora*), selection for pollen viability allowed fertility restoration in some plants after more than two backcrosses (Owuor and Vossen 1981).

Identification of pollen viability QTLs in BC1

Pv1 and *pv2* could be the two expected Mendelian loci (or chromosomal regions) involved in the BC1 bimodal distribution. The two QTLs are physically linked but genetically independent in terms of the recombination rate (80 cM). According to a chromosomal sterility hypothesis, this means that for genotypes (AABb), (AaBB) and (AABB), partial homology of LG2 would be sufficient to reduce univalent emergence. This is in accordance with the non-occurrence of segregation distortion at C1 and C2 loci and the assumption of sterility arising from aneuploid gametes having, or missing, two chromosomes.

There is not full agreement between the Mendelian model involving loci A and B and the QTL model involving loci C1 and C2. In the first model, genotypes AABb, AaBB and AABB give the same phenotype, whereas in the second model, genotypes C1C1C2C2, C1c1C2C2 and C1C1C2c2 lead to two different phenotypes. The first model should be retained, except if we consider the two modes of the BC1 distribution as a sampling artefact. Consequently, QTL markers would not match loci A and B, and the discrepancy would result from the QTL identification method based on an additive model. Nevertheless, no absolute co-localization did not mean complete independence of the two models: loci A and B should be localized on the same LG2 and probably not very far from selected markers AAGCAC13 and AAGCAA14.

Concerning *pv3*, this QTL could explain the slightly lower viability (72%) of the sub-fertile group (BC1-2)

with regards to the parental mean value (81%). The absence of segregation distortion suggests that a lack of fertility due to *pv3* could also be of chromosomal origin (unpairing).

Two points should be discussed about *pv3*: (1) why did it not show any effect in the BC1-1 population; and (2) why was it detected only within the BC1-2 population, although the two points are not independent. The lack of detection in BC1-1 could be explained by the low sample size (12 hybrids), coupled with the lower effect of *pv3* (the C3c3/C3C3 PV ratio was 79%). The absence of detection of *Pv3* using the whole BC1 population could also be artefactual and related to the QTL method. Indeed, with an additive model, C1c1C2C2 and C1C1C2c2 hybrids presented pollen fertility close to that of the C3c3 group.

Lastly, the lower effect of *pv3* relative to *pv1* and *pv2* could have resulted from the presence of only one point of unpairing on linkage group 13, leading to a higher probability of bivalent emergence at metaphase-I.

Impact on breeding

As often reported, the sterility of coffee interspecific hybrids has limited the use of diploid genetic resources of wild species for cultivated *C. canephora* improvement (Capot 1972a, b; Yapo et al. 1991; Louarn 1992). The present investigation highlighted two major points: (1) fertility might be improved by selection, and (2) molecular markers could potentially be used in interspecific breeding schemes for fertility restoration. Indeed, selection at the plantlet stage within BC1 of the C1C1C2C3C3 genotypes could lead to field planting of only hybrids with high pollen viability.

Nevertheless, selection for pollen viability should not have negative effects on the efficiency of the autogamy introgression process. As the S locus is localized on the LG11 linkage group (Coulibaly et al. 2002), pollen viability appears to be independent of the S locus. Hence, using AFLP markers, including that of the S locus, hybrids could be selected at the plantlet stage for the four independent *loci*.

Prospects

The present study should be complemented by: (1) an investigation to identify univalent chromosomes using an in situ hybridisation method during meiosis. This would require pre-identification of some repeat sequences involved in chromosome differentiation between CAN and HET; (2) a follow-up of female fertility restoration in relation to male fertility restoration during F1, BC1 and BC2 generations. Indeed, pollen viability restoration does not imply hybrid fertility restoration. Although pollen viability could be restored as early as the second backcross, female fertility is still known to be weak (Louarn 1992; Coulibaly, unpublished data). This indicates that chromosome unpairing would have a greater effect on female fertility than on pollen viability. Why? In addition, are the same QTLs responsible for variations in female fertility?

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