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Doubled haploids of *Coffea canephora*: development, fertility and agronomic characteristics

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Summary

Doubled haploids (DH) of *Coffea canephora* Pierre were developed using haploid embryos which occur spontaneously in association with polyembryony. The frequencies of polyembryonic seeds and haploid embryos varied according to the parental genotypes. However, production of a large number of DH seemed possible from all genotypes. More than 750 DHs produced from various genotypes were grown under field conditions and evaluated for different characters of agronomic importance. Approximately half of DH genotypes did not survive, suggesting a strong, negative effect of homozygosity. Inbreeding depression is particularly severe on general vigor and reproductive aspects. For several characters studied such as leaf shape, leaf rust resistance and hundred bean weight, considerable genetic variations were observed within and between groups of DHs constituted by the DHs produced from the same clone. Despite their low vigor and reduced fertility, the DHs of *C. canephora* offer new possibilities in genetic research and coffee breeding.

Introduction

Haploid plant production followed by chromosome doubling offers the possibility of developing completely homozygous genotypes from heterozygous parents in a single generation. The potential advantages of haploidy in genetic research and plant breeding are well documented (Choo, 1983; Baenziger et al., 1984; Gallais, 1988). However, utilization of doubled haploid (DH) in tree genetic programmes has been very limited and has not been really assessed yet. Considerable efforts are required before doubled-haploid breeding can be used in most woody species. While the ability to produce high number of DH from many genotypes is of importance (Radojevic & Kovoor, 1986; Zhang et al., 1990), the necessity to evaluate the agronomic value of the DHs produced is of equal significance.

Since success in coffee haploid production by *in vitro* culture technique has been limited so far (Lanaud, 1981; Ascanio & Arcia, 1987) use of the spontaneous haploids is the only way presently available to produce DHs in *C. canephora.* The occurrence of spontaneous haploids has been mentioned in many tree species (Winton & Stettler, 1974). In *Coffea canephora* Pierre, the spontaneous development of haploid embryos was first reported by Dublin and Parvais in 1975. The three coffee haploid plants obtained by these authors showed low vigor and did not survive. A method based on grafting of suspected haploid embryos extracted from immature polyembryonic seeds onto young, normal, diploid stock, was proposed in 1982 which could allow the production of a large number of DH (Couturon, 1982; Couturon & Berthaud, 1982). Haploid embryos are of maternal origin as deduced from inheritance of morphological and enzymatic markers (Couturon, 1982; Valverde, pers. com.).

The purpose of this study was to evaluate the possibility to produce a large number of doubled haploids in a tropical crop tree species of agricultural importance such as *C. canephora,* and to investigate their agronomic performance.

Materials and methods

Production of DH plants of *C. canephora* was carried out from 1980 to 1985, and was based on the haploid plants occurring spontaneously among polyembryonic seedlings. A large number of immature seeds from genotypes of various origins were collected at the coffee breeding stations of Divo and Man (Ivory Coast) and, after dissection, polyembryos were removed. The joined embryos were unattached and the smallest were grafted on four months-old diploid seedlings according to Couturon & Berthaud (1979). Haploid plantlets were identified, and chromosome doubling induced by colchicine treatment as previously described (Couturon, 1982). The DHs were cloned by cuttings and were grafted by using different heterozygous clones as rootstock in the nursery. On average, three one year-old plants per DH genotype were transplanted in the field. From preliminary results, grafting of DHs onto heterozygous clones was strongly recommended, and was systematically used in this study. In that way, more than 750 DH genotypes (about 2000 trees) were grown under field conditions at the coffee breeding station, Man, Ivory Coast.

DH genotypes produced from the clones IF A25, IF 160, IF 200, and IF 420 were evaluated in detail. Each individual tree was observed from 1989 to 1992, and the following characters were recorded: (1) Plant vigor scored on a 1–6 scale with 1 = sub-lethal, 6 = vigor comparable to a heterozygous plant; (2) Leaf area and (3) Leaf shape. The leaf area (0.88 × length × width at the broadest portion) was the mean in cm^2 of twenty leaves of comparable age selected from four or five years-old trees; the leaf shape was the mean ratio width:length; (4) Leaf rust (*Hemileia vastatrix*) susceptibility scored by repeatedly observing tree on a 1–5 scale with 1 = resistant, 5 = highly susceptible; (5) Fruit set estimated for two branches per tree as the percent of flowers which set fruit 6 months later; (6) Pollen viability evaluated as the percent of pollen uniformly coloured by aceto-carmine staining on random sampling of 200 pollen grains; (7) Cherry yield scored over two years on a 0–4 scale with 0 = no production, 4 = production comparable to a heterozygous plant; (8) Fruit filling estimated on a sample of 300 cherries by the coefficient = number of seeds/600; (9) Peaberries determined as the percent of berries containing only one round bean; (10) Hundred bean weight deduced from the dry weight at 0% humidity of a sample of 250 beans.

Value of a genotype for the different characters, was determined by the mean of all trees of the same DH genotype. Analyses of variance were performed using NDMS (ORSTOM, France) statistical computer package. Broad-sense heritabilities were estimated [$h^2_L = \sigma^2_G/(\sigma^2_G + \sigma^2_e)$] for plant vigor, leaf area, leaf shape and cherry yield using the appropriate expected mean squares (Gallais, 1990) calculated by performing a one way analysis of variance for an experiment with two individual trees per genotype. Genotypes were considered to be random effects, and genotypes and environments effects were assumed to be independent. Spearman's rank correlation coefficients between pairwise combinations of characters were obtained according to the procedure outlined in Dagnelie (1970). A t-test was used to determine whether the coefficient was different from zero. For comparison of the mean value of DH families (i.e. group of DHs produced from the same parental clone) with their respective parental clones, clone-values were estimated by the observation of a minimum of four plants.

Results

Haploid plant production

The occurrence of polyembryonic seeds was investigated. A large number of genotypes representing the main groups of diversity characterized in *C. canephora* (Berthaud, 1986) were tested. Although considerable variation in frequency of seed with polyembryony was observed between genotypes, the mean frequencies of genetic groups including respectively West African forms (i.e. Guinean), Central African forms (i.e. Congolese) and intermediate forms (i.e. hybrid) did not differ significantly (Table 1). The overall frequency of seed with polyembryony was 0.39% in this study.

DHs were obtained from 38 different genotypes, and results on DH production from the 10 most intensively screened genotypes are summarized in Table 2. Large differences in polyembryonic seed frequency as well as in the ratio of haploid plants among the plantlets obtained from polyembryos after grafting were observed between parental genotypes. The frequency of seed with polyembryos varied between 0.1% and 1.0%, while the proportion of haploid plantlets varied between 6% and 32%. Success in the grafting process was also found to be dependent on the genetic origin. The frequency of plantlets obtained per 100

Table 1. Frequency of seed with polyembryo in different genetic groups

Genetic group	Number of genotypes studied	Frequency (%) of polyembryonic seeds		F test
		Mean	Std. Dev.	
Guinean (G)	58	0.38	0.36	0.09[a]
Congolese (C)	8	0.36	0.27	
Intermediate (I)	16	0.42	0.41	

[a] P = 0.91.

Table 2. Production of doubled haploid (DH) plants from different clones by polyembryo grafting

Clone (genetic group)	No. of seed examined	Polyembryonic seed		Plantlets obtained after grafting		Haploid plants			Surviving DH genotypes in field conditions	
		No.	Per 1000 seeds	No.	Per 100 embryos grafted	No.	Per 100 plantlets	Per 1000 seeds	No.	Per 100 haploid plants
IF A25 (C)	150217	556	3.7 bc	338	61 cd	44	13 bc	0.29	34	77 b
IF KM27 (C)	122618	462	3.8 bc	302	65 bc	18	6 d	0.15	16	89 ab
IF 444 (C)	87473	84	1.0 e	53	63 bcd	4	7 d	0.05	3	–
IF 149 (I)	26858	118	4.4 bc	65	55 d	19	29 a	0.71	8	42 cd
IF 160 (I)	112962	238	2.1 d	178	75 a	35	20 ab	0.31	34	97 a
IF 200 (I)	387630	3895	10.0 a	2271	58 d	497	22 a	1.29	240	48 c
IF 232 (I)	9229	76	8.2 a	41	54 de	13	32 a	1.41	5	38 cd
IF 420 (I)	91026	861	9.5 a	569	66 b	127	22 a	1.40	99	78 b
02 040 (G)	39321	135	3.4 c	57	42 e	6	11 bcd	0.15	4	–
02 0128 (G)	29558	130	4.4 b	54	42 e	16	30 a	0.54	3	19 d

Values followed by the same letter are not significantly different at the 0.05 probability level as determined by 't test' of arcsin transformed data.

grafted embryos varied between 42% and 75%; the average was 58%.

The number of haploid plants per 1000 examined seeds ranged from 0.1 to 1.4. The best results were obtained from genotypes (clones IF 200, IF 232 and IF 420) combining a high rate of polyembryo production and large proportion of haploids among the plantlets obtained after grafting. However, no simple relations were observed between the factors. For instance, the clone IF 160 showed a high proportion of haploid plantlets, a good aptitude for embryo grafting, but a low frequency of polyembryos.

Most of DH genotypes showed a low vigor. As a consequence, a significant proportion of DH genotypes did not survive in field conditions (Table 2). The frequency of surviving DH genotypes varied in relation to parental genotypes from 19% to 97%; the average was 49%. The classification of parental genotypes according to the frequency of plantlets obtained per 100 grafted embryos, and the frequency of surviving DH genotypes in field conditions appeared similar, suggesting a possible relationships between the two variables.

Doubled haploid observations

DHs presented large variations for the different agronomic traits and fertility characters observed. The relative importance of genetic and environmental effects was estimated for some agronomic traits using the DH family produced from the clone IF 200. Results from the analyses of variance are given in Table 3. There were highly significant differences between DH geno-

Table 3. Mean square values for agronomic traits of doubled haploid genotypes produced from the clone IF 200 (two replications for each genotype)

Traits	Source of variation	df	Mean square	Estimates of heritability (Broad sense)
Plant vigor	Genotype	119	0.92***	0.42
	Error	120	0.38	
Leaf area	Genotype	65	871.40***	0.83
	Error	66	80.95	
Leaf shape	Genotype	65	0.00265***	0.88
	Error	66	0.00017	
Cherry yield	Genotype	110	2.74***	0.57
	Error	111	0.76	

*** significant effect at $P < 0.001$.

types for: plant vigor, leaf area, leaf shape and cherry yield. Heritabilities (broad sense) were high for leaf area and leaf shape, and fair for plant vigor and cherry yield indicating that the variability observed in this study was due primarily to genetic variance.

The frequency distributions of DH genotypes produced from different genotypes (clone) are illustrated in Fig. 1 for plant vigor, susceptibility to leaf rust, and cherry yield. X^2-tests showed that the different DH families did not have the same frequency distribution. In relation to plant vigor, DHs produced from IF 200 tended to have a lower vigor, whereas DHs produced from IF 420 tended to have a higher vigor. For all families, the frequency of DHs exhibiting a vigor comparable to a heterozygous tree (score 6) was very low (mean frequency of 5%). With regard to susceptibility to leaf rust, the distribution of DHs produced from the resistant clone IF 160 is skewed towards resistant levels. In contrast, the distribution of DHs produced from the moderately susceptible clone IF 200 is skewed towards susceptible levels. The distributions of DHs produced from the clones IF 420 and IF A25 are centred on the parental clone values (moderately resistant). For cherry yield, the frequency distributions show that most of DHs had a very low production. No DH genotype had a cherry yield comparable to a standard heterozygous plant.

In addition to cherry yield, characteristics relating to gamete viability and fruit development were observed. The frequency distributions of DH genotypes produced from different clones are illustrated in Fig. 2 for pollen viability and fruit set. Despite the notable exception of DHs produced from the clone IF A25, most of DHs showed a fair level of pollen viability (> 50%) as estimated by aceto-carmine staining. This result was confirmed by the high success rate of crosses using DH genotypes as male parent (data not shown). The fruit set of most DH genotypes was poor (< 30%). In comparison, fruit set of a standard heterozygous plant ranged between 40–60% depending on genotypes and environmental conditions.

Rank correlation coefficients were calculated between plant vigor and fertility characters (Table 4), and between pollen viability and female fertility characteristics (Table 5). Although no strong relationship was demonstrated, plant vigor was positively correlated with cherry yield. The frequency of peaberries appeared also positively correlated with plant vigor but only in the DH family produced from IF 200. Fruit set, fruit filling, and pollen viability did not show significant correlation with plant vigor. Regarding the relation between male and female fertility characteristics, pollen viability showed a positive correlation with fruit filling and a negative correlation with the occurrence of peaberries.

For various characters, mean values of DH families were compared with the values of their respective parental clones (Table 6). Mean performances of DH families for leaf area, pollen viability, fruit filling, and hundred beans weight appeared significantly lower. No significant difference was observed for leaf shape, and frequency of peaberries was significantly higher in the DH families. The ratio, DH family mean: parental clone value, expressing importance of the differences, fluctuated according to the characters, but it appeared relatively constant from one clone to another.

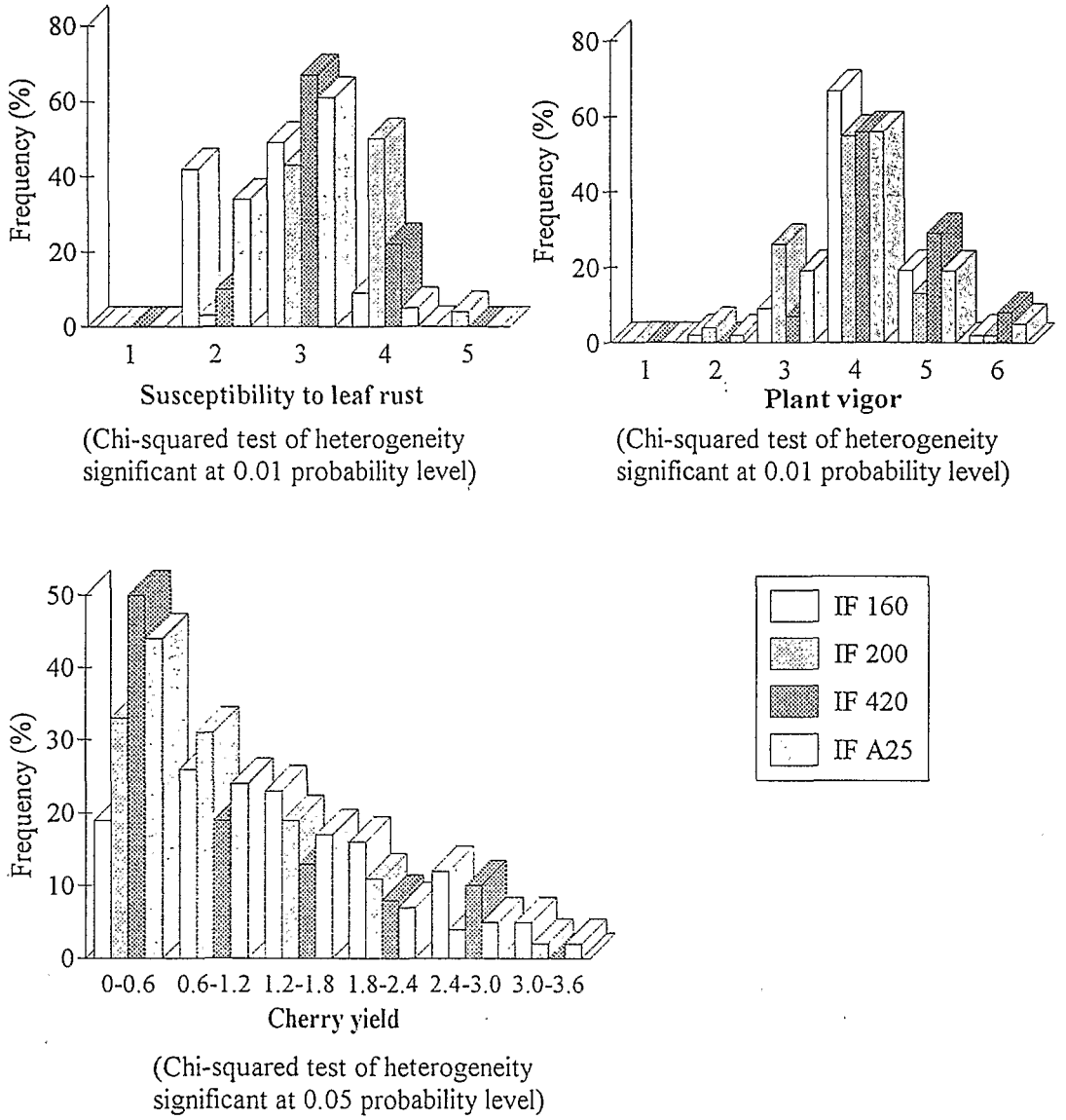

Fig. 1. Frequency distributions of doubled haploid genotypes produced from different clones (IF 160, IF 200, IF 420, IF A25) for plant vigor (score), susceptibility to leaf rust (1 = resistant, 5 = susceptible), and cherry yield (class score).

Discussion

Wide utilization of DHs in genetic and plant breeding programmes requires the production of large numbers of DHs from almost any genotype. The spontaneous occurrence of haploid plants in association with polyembryony seems an attractive way for DH development in *C. canephora.* Although the frequencies of occurrence of polyembryos and haploids are under genetic control, no genotype seemed recalcitrant to the method; DHs were produced from genotypes representing a large genetic diversity. Low aptitude of some genotypes for polyembryo and haploid development can be balanced by a high number of seeds examined. The overall frequency of polyembryonic seeds observed in this study is comparable to the result

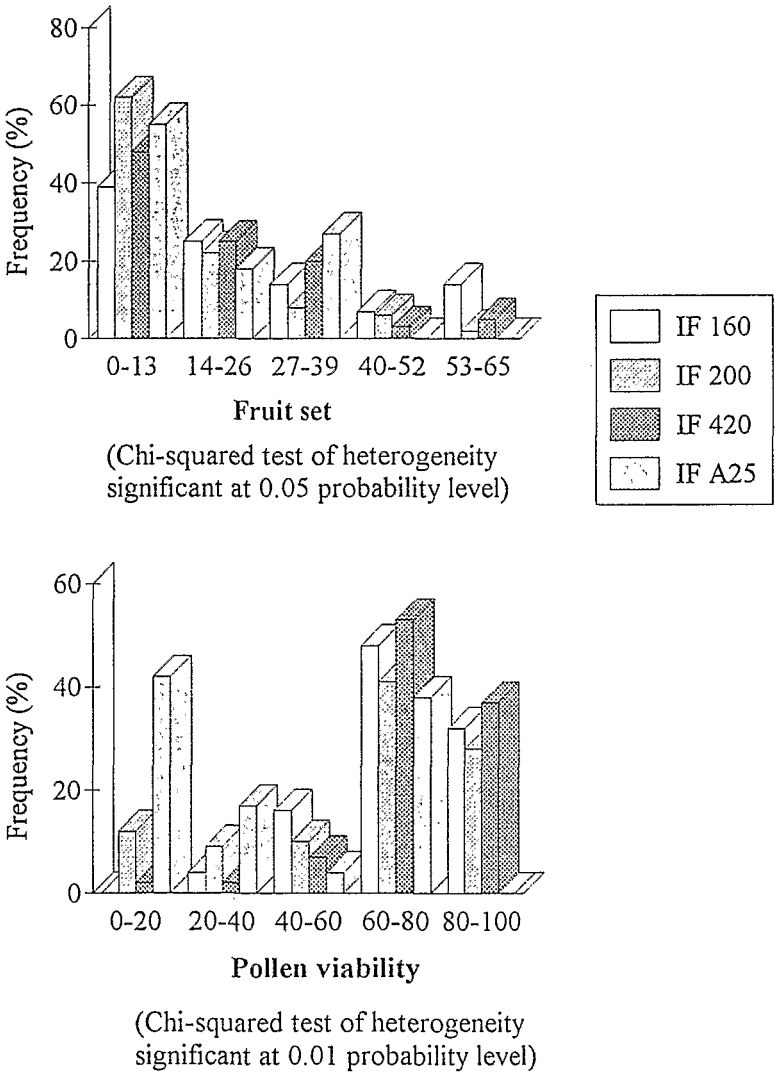

Fig. 2. Frequency distributions of doubled haploid genotypes produced from different clones (IF 160, IF 200, IF 420, IF A25) for pollen viability (%) and fruit set (%).

previously reported (Dublin & Parvais, 1975). Differences observed between genotypes in frequency of spontaneous haploid obtained after grafting could be either inversely related to the frequency of lethal and sub-lethal genes in the haploid source stocks as postulated by Chase (1969), or resulted from genetic factor controlling haploid embryo initiation and development as reported in maize (Lashermes & Beckert, 1988). The lack of evident relation between the frequency of polyembryo and the proportion of haploids among the plantlets obtained after grafting suggests the existence of different genetic factors affecting the overall production of haploids. The mean production of 0.6 haploid plants per 1000 seeds observed in this study is rather low, but increases in the production might be obtained by genetic and technique improvement. From some DH genotypes, production as high as 5 haploid plants per 100 seeds has been obtained. Environmental factors during flowering time seem important, and applications of physical or physiological treatments

Table 4. Rank correlation coefficients between plant vigor and fertility characters for different doubled haploid families (no. of genotypes analyzed is indicated in parenthesis)

Fertility characters	DH family			
	A25	160	200	420
Fruit set	0.12 (22)	0.15 (31)	0.07 (100)	0.12 (39)
Fruit filling	0.06 (19)	0.01 (32)	- 0.13 (112)	0.17 (52)
Peaberries	0.07 (19)	0.18 (32)	0.30** (112)	0.02 (52)
Pollen viability	- 0.10 (22)	0.18 (27)	- 0.09 (50)	0.15 (59)
Cherry yield	0.37* (41)	0.34* (44)	0.24** (184)	0.17 (96)

*, ** Significant value at the 0.05 and 0.01 probability levels respectively.

Table 5. Rank correlation coefficients between pollen viability and characteristics of female fertility for different doubled haploid families (no. of genotypes analyzed is indicated in parenthesis)

Female fertility characteristics	DH family			
	A25	160	200	420
Fruit set	0.33 (19)	- 0.02 (26)	0.02 (33)	0.19 (30)
Fruit filling	0.13 (15)	0.51* (20)	0.44* (32)	0.17 (37)
Peaberries	- 0.21 (15)	- 0.36 (20)	- 0.48** (32)	- 0.37* (37)
Cherry yield	0.35 (22)	- 0.13 (27)	- 0.04 (50)	0.15 (59)

*, ** Significant value at the 0.05 and 0.01 probability levels respectively.

such as delayed pollination should be considered. In spite of the embryo grafting process, it is reasonable to assume that a large part of haploid embryos did not survive, and a technique such as *in vitro* embryo rescue may be of value.

Haploids and DHs of *C. canephora* show a low vigor. Vegetative organs of DHs are particularly susceptible to anthracnosis due to *Colleotrichum coffeanum* which is, under normal conditions, only spread to weak and dying trees. Special care was given to the DH growing and modified cultural practices (e.g. pruning, important quantity of fertilizer) were required. Nevertheless, approximately half of the DH genotypes did not survive in field conditions although several plants (2 to 4) per genotype were transplanted, suggesting a strong, negative effect of homozygosity on plant development. *C. canephora* presents a large genetic diversity. Cultivated clones are only a few generations removed from their wild ancestors, and a high level of heterozygosity is maintained by a self-incompatibility system (Berthaud, 1986). In consequence, the genetic load is assumed to be high, and the strict homozygosity of DHs may lead to the frequent expression of lethal or sub-lethal genes. The large difference in survival rate observed between DH families could result from differences of the parental clones in frequency of recessive deleterious alleles.

From the observation of surviving DHs, the injurious effects of inbreeding appear character-dependent. Inbreeding depression is particularly severe on general vigor and reproductive aspects including cherry yield but limited, for instance, on leaf area (- 20%), hundred beans weight (- 20%) and resistance to leaf rust. Furthermore and in spite of inbreeding depression, for all characters studied, genetic variations were observed among DH families, indicating the presence of allele segregations, and the relative importance of additive and (additive × additive) genetic variance.

Genetic studies on the resistance of *C. canephora* to leaf rust have been limited but indicate complex inheritance (Berthaud & Lourd, 1982; Bettencourt & Rodrigues, 1988). The continuous variation from resistance to higher susceptibility observed within the different DH families suggest a polygenic resistance

Table 6. Mean performance for various characters of different doubled haploid families and their respective parental clones

Character		IF A25	IF 160	IF 200	IF 420
Leaf area (cm^2)	Clone	–	–	123	166
	DH family	–	–	103	133
	t test			**	**
	DH/clone			0.84	0.80
Leaf shape (w/l)	Clone	–	–	0.41	0.40
	DH family	–	–	0.41	0.41
	t test			ns	ns
	DH/clone			1	1.03
Pollen viability (%)	Clone	94	89	87	93
	DH family	35	72	62	74
	t test	**	**	**	**
	DH/clone	0.38	0.81	0.71	0.79
Fruit filling (%)	Clone	84	87	82	86
	DH family	66	71	64	65
	t test	**	**	**	**
	DH/clone	0.79	0.82	0.78	0.76
Peaberries (%)	Clone	24	21	31	17
	DH family	55	44	57	51
	t tet	**	**	**	**
	DH/clone	2.29	2.10	1.84	3.00
Hundred bean weight (g)	Clone	10.0	13.1	16.8	18.6
	DH family	8.5	11.2	12.8	14.9
	t test	**	**	**	**
	DH/clone	0.85	0.85	0.76	0.80

ns Not significant

** Significant at the 0.01 level of probability.

with quantitative expression, and a high heterozygosity level of the parental clones for the genes involved in resistance mechanisms. However, more precise observations in controlled conditions of infection would be necessary.

The low cherry yield of DHs is for a large part due to a reduced fruit set. Independently of the plant vigor, a high proportion of flowers 'fell down' and did not produce fruit. This phenomenon, usual in *C. canephora*, is notably pronounced in the DHs, and may be related to physiological deficiencies. In addition, the DHs present a high frequency of peaberries and empty berries resulting in a low fruit filling coefficient. Low fruit filling is generally the consequence of fertilization failure or endosperm abortion (de Reffye, 1974). The significant correlations which were found between frequency of peaberries, fruit filling coefficient and pollen viability of DHs, indicate a relationship between male- and female gamete fertilities. In recent observations (unpublished result), progenies of crosses between DHs with low fertility were found to have normal fertility. So, a hypothesis presenting the partial fertility of DHs as a consequence of the colchicine treatment or chromosome abnormality can be excluded. Similar observations have been reported on DHs of cocoa (Lanaud, 1987).

Although their progeny are not affected, the reduced fertility of DHs may raise a problem concerning their use as progenitors for the production of hybrids. However, this limited pollen fertility did not significantly reduce the success rate of crosses, and

most DHs produced enough seed for experimental purposes.

Evidence from this study indicates that large number of DHs of *C. canephora* can be effectively produced. The method used, although time-consuming, does not require any particular facilities and production of DHs could be achieved in any coffee research station. Despite the inbreeding depression, DHs have a great potential in genetic and coffee breeding programmes. Only via haploidy can inbred lines be developed in a self-incompatible diploid crop with a long juvenile period such as *C. canephora*. DHs produced from the same clone present large genetic variations and genetic analysis of important characters (e.g. incompatibility system, leaf rust resistance, caffeine content) can be undertaken. DHS are particularly well suited to detect the additive effects of quantitative trait loci (QTL) via linkage to genetic markers (Knapp et al., 1990; Lashermes et al., 1993a, 1993b). Regarding coffee breeding, the DHs provide the possibility to develop F_1 hybrid varieties (Charrier & Berthaud, 1988). However, this application which could have a considerable impact, requires extensive experiments and further assessments (Lashermes et al., 1994).

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