

## The Impact of Pollen Donor on Flavor in Cocoa

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### ABSTRACT.

Past studies have shown evidence of pollen parent effects on yield, bean size and pod characteristics but its effect on flavor attributes is not clearly understood. An incomplete diallel mating design involving five cacao cultivars (West African Amelonado (WAA), Imperial College Selection (ICS) 1, Iquitos Mixed Calabacillo (IMC) 67 and two Trinidad Selected Hybrids (TSH) coded as CCL 200 and CCL 201) with widely differing flavor attributes were used to investigate the magnitude of female and male parent effects on key intrinsic flavor attributes. The seeds derived from pods arising from these pollinations were fermented, dried and made into cocoa liquor according to standardized methods. Flavor evaluations were carried out by a trained sensory panel for nine flavor attributes with five repetitions and hidden flavor reference controls. The study was conducted over two cocoa crop years. The results failed to detect dominant xenia effects for important ancillary flavor attributes i.e., cocoa flavor, acidity, fruitiness and floral flavors but showed significant female parent effects for cocoa and floral flavors. Small but inconsistent male parent effects were seen for astringency. Lack of xenia effect for the major flavor attributes implies that the flavor quality of cocoa beans is determined principally by the genotype of the female parent.

### INTRODUCTION

Cacao (*Theobroma cacao* L.) possesses an unusual incompatibility mechanism, first discovered by Pound (1932), which exhibits features of both sporophytic and gametophytic systems. Many studies have investigated this incompatibility system (Cope, 1939; Posnette, 1944, 1945; Voelcker, 1936) in cacao and its effect on planting systems and yield (Lockwood, 1977; Warren et al., 1995). Over the years, extensive work has been done on pollination biology of cacao (Dias and Kageyama, 1995; Dos Santos Dias et al., 2003; Lanaud, 1987, 1988; Young, 1986, 2007; Young et al., 1987; Yong Tan, 1990). Past studies have highlighted the many differences in fruit set, bean size, shape, color and quality of the beans both within and between, particular cacao cultivars under natural and artificial pollination (Enriquez and Soria, 1968; Falque et al., 1995, 1996; Glendinning, 1963; Iwaro et al., 2003; Lachenaud, 1994, 1995).

A number of studies have described the specific effect of pollen parent on yield and some pod characteristics (Iwaro et al., 2003; Jacob and Toxopeus, 1969; Lockwood and Edwards, 1980). Although, Smulders et al. (2008) were able to differentiate the pollen donor contribution to particular pods from a known location using 15 microsatellite markers and were able to trace it to the chocolate made from these pods, they acknowledged that a link to flavor and pollen donor was needed but was outside the scope of their study. The only previous works attempting to link flavor to pollen donor effects in cocoa were Clapperton et al. (1994a, 1994b) and Lockwood and Eskes (1996). These studies reported lack of pollen donor effect on astringency involving cacao cultivars with markedly different levels of astringency. Lockwood and Eskes (1996) expressed surprise at the lack of xenia effect on flavor since xenia effects have been reported on cotyledon color. They concluded that more work needed to be done in this specific area to arrive at a clear answer, but also recognized that experiments to investigate this phenomenon posed “*formidable technical difficulties*”. The objective of the present study was therefore to investigate if xenia effects exist for the various flavor attributes of cocoa over a range of crosses as part of a broader investigation examining factors possibly contributing to “*terroir*” in cocoa (Guittard, 2005; Nesto, 2010; Sukha et al., 2014).

## MATERIALS AND METHODS

### Tree selection and experimental design

The cacao cultivars chosen for this study were West African Amelonado (WAA), Imperial College Selection (ICS) 1, Iquitos Mixed Calabacillo (IMC) 67 and two Trinidad Selected Hybrids (TSH) coded as CCL 200 and CCL 201. Selection was based on the availability of sufficient numbers of verified true-to-type trees (determined by morphological characterization and simple sequence repeat (SSR) molecular marker analysis) and their widely differing flavor attributes (Sukha et al., 2008). Each of the five parents was selfed where possible and mated with the other four parents in all possible combinations analogous to a 5 × 5 diallel mating design. Selfing was possible only with WAA and ICS 1 as the other cultivars were self-incompatible. The study was conducted over two cocoa crop years, as two trials. Selected crosses were done over both crop years to validate the repeatability of results (Table 1).

**Table 1. The five cacao cultivars selected for the study arranged in a 5 × 5 diallel mating design for pollinations.<sup>z</sup>**

		Male parent				
		WAA	ICS 1	IMC 67	CCL 200	CCL 201
Female parent	WAA	1,2 <sup>y</sup>	1,2	1,2	1,2	1,2
	ICS 1	1,2	1,2	1,2	1	1,2
	IMC 67	1	1	SI	1	1
	CCL 200	1,2	1	1	SI	1
	CCL 201	1,2	1,2	1,2	1,2	SI

<sup>z</sup>The cultivars West African Amelonado (WAA), Imperial College Selection (ICS) 1, Iquitos Mixed Calabacillo (IMC) 67 and two Trinidad Selected Hybrids (TSH) coded as CCL 200 and CCL 201) were selfed where possible and mated with the other four parents in all possible combinations analogous to a 5 × 5 diallel mating design carried out over two crop years, trial 1 and 2, to study the effect of male and female parent effects of flavor attributes in cocoa.

<sup>y</sup>1,2 = Crosses done in both trial 1 and 2; 1 = Crosses done in trial 1 alone; SI = self-incompatible types.

### Pollinations, sample preparation and sensory evaluation

Trees selected for this study were pruned and fertilized 3 months before hand pollination to increase flowering and tree yield. These trees were sprayed with a broad-spectrum pyrethroid insecticide and copper fungicide 1 week before hand pollination to remove ants (family Formicidae) and other crawling insects that could cause unwanted open pollinations and to eliminate any moss covering flower cushions. Flower buds on trees selected as pollen donors and pollen recipients that would open within 24 h were identified and covered with pollination hoods and secured to the tree with 25 mm long stainless steel pins.

Hand pollinations were done between the hours of 0700 and 1200 HR daily using the method described by Doodnath (1996). Pollinated flowers were monitored daily and a successful pollination was considered to have occurred when after 3 d the flower was in a “swollen ovary” state. This was the first visible sign that the ovules had been fertilized. Flowers usually abscised and fell off the tree within 24 to 48 h with unsuccessful pollinations.

Trees in this study were kept in an optimal physiological state by irrigation and application of a foliar fertilizer (7N–9.6P–5.8K) to minimize the incidence of cherville wilt. After 50 to 70 d when the susceptible period for cherville wilt had passed, the immature pods were covered, where possible, with wire cages to prevent attacks by neotropical red squirrels (*Sciurus granatensis*) and orange-winged parrots (*Amazona amazonica*). Immature pods were checked weekly and sprayed with copper fungicide in two heavy applications (30 g m<sup>-2</sup>) mixed with a sticker solution (0.5 mL m<sup>-2</sup>) during the 6-month growing period to protect against black pod disease (*Phytophthora* spp.)

Fully mature disease-free pods from successful pollinations were harvested and both primary (fermentation and drying) and secondary (bean roasting and liquor preparation) processing were conducted using the methods

described in Sukha et al. (2008). Sensory panel training as well as flavor evaluations were also carried out according to the methods described in Sukha et al. (2008).

### Data analysis

Individual flavor attribute scores from the two trials were entered into Microsoft Excel (Microsoft, Redmond, WA) for a pooled analysis. Analysis of variance (ANOVA), paired *t* tests, Tukey-Kramer test and linear regression analysis were carried out on the sensory data, as well as, female parent  $\times$  pollen donor interactions determined from ANOVA data.

It was not possible to use the entire dataset at the same time for ANOVA, due to the presence of self-incompatible crosses in the diallel mating design and because the study was conducted as two trials over two crop years. To address this, a number of smaller diallels on combined trial 1 and trial 2 data were created from the crosses in the main  $5 \times 5$  diallel design (Data not presented).

General linear model (GLM) ANOVA using MINITAB (release 14; Minitab, State College, PA) was carried out on data from each smaller diallel for trial 1 and trial 2 to determine the significance of female parent, pollen donor and female parent  $\times$  pollen donor interaction effects over the five repetitions of flavor evaluations per sample. The F test values from the GLM ANOVA were used to gauge the relative magnitude of the effects.

Paired *t* tests gave better resolution to the ANOVA results by focusing on the difference between paired sets of reciprocal crosses and presenting the probability that the actual mean difference was consistent with zero. Comparisons of reciprocal crosses allowed examination of significant effects on flavor with a specific combination of crosses and involved the same pair of accessions where each accession has the opportunity to be the female parent and pollen donor in turn.

## RESULTS

The ANOVA results from the pooled trial 1 and trial 2 data (Table 2) showed a general trend indicating that the female parent effects were significant ( $P \leq 0.001$  to  $P \leq 0.05$ ) in majority of the diallels for cocoa, acid, floral, nutty, raw/beany/green and other flavor attributes, whereas the female parent effects for astringency and fruity flavors were significant only in one diallel, each. In contrast, the male parent effects were not significant for all flavor attributes in vast majority of the diallels, except nutty and other which showed significant male parent effects in some diallel designs. Significant interaction effects ( $P \leq 0.001$  to  $P \leq 0.05$ ) were evident for nutty and other flavor attributes in several diallels. Even when the interaction was significant in two diallels for cocoa flavor and one diallel for floral, the magnitude of the F value was smaller than the female parent effects. Bitterness was the only flavor attribute that did not show significance ( $P \leq 0.001$  to  $P \leq 0.05$ ) for male parent, female parent, or male parent  $\times$  female parent interaction effects.

Paired *t* test between reciprocal crosses revealed that the greatest significant ( $P < 0.001$ ) effect was observed for floral flavor. This effect was strongest (greatest difference in average scores) when CCL 200 was the female parent in the cross. The largest difference in floral flavor occurred when crosses ICS 1  $\times$  CCL 200 and CCL 200  $\times$  ICS 1 were compared to each other. Their average scores for floral were 0.55 and 5.78, respectively on the 10-point attribute scale. Other reciprocal crosses with big differences in floral flavor occurred with CCL 201  $\times$  CCL 200 and CCL 200  $\times$  CCL 201 as well as WAA  $\times$  CCL 200 and CCL 200  $\times$  WAA (0.98 and 6.15; 0.35 and 5.14, respectively).

Significant ( $P \leq 0.001$  to  $P \leq 0.05$ ) reciprocal effects, albeit with smaller differences between reciprocals, were observed for cocoa flavor, astringency and other flavors, whilst no significant reciprocal effects ( $P > 0.05$ ) were observed for acid and fruity flavors. Whilst reciprocal crosses do not specifically identify female parent or pollen donor effects they do allow examination of significant effects on flavor, with a specific combination of crosses. These effects occurred in reciprocal crosses involving CCL 200, CCL 201 (cocoa flavor and astringency) and WAA, CCL 200 (other flavors). Similarly, significant ( $P \leq 0.01$ ) reciprocal effects were observed for nutty flavor when WAA, ICS 1; WAA, CCL 200 and CCL 200, CCL 201 were the parents in reciprocal crosses.

Table 2. Summary of significant effects across nine flavor attributes for cacao female parent, pollen donor and female parent × pollen donor interactions from general linear model (GLM) analysis of variance (ANOVA).<sup>z</sup>

Diallel	Female parent effects																	
	Cocoa		Acidity		Astringency		Bitterness		Fruity		Floral		Nutty		R/B/G <sup>y</sup>		Other	
	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.	F value
1	NS	2.72	*	4.90	NS	0.24	NS	2.28	NS	2.81	**	7.78	NS	1.71	**	8.02	***	13.35
2	***	13.14	NS	1.39	NS	1.09	NS	0.33	NS	1.81	NS	1.11	***	6.28	*	3.12	***	5.29
3	***	51.06	***	13.96	*	5.14	NS	1.37	NS	3.30	***	111.88	***	14.7	---	---	NS	0.48
4	NS	1.95	NS	1.98	NS	0.57	NS	2.89	NS	0.59	NS	0.18	NS	2.17	***	9.89	NS	3.09
5	***	15.47	*	2.97	NS	0.42	NS	1.73	NS	2.60	***	103.09	***	7.51	*	2.82	***	6.76
6	***	9.47	NS	0.34	NS	0.37	NS	1.25	*	4.55	***	10.32	***	6.84	***	8.88	***	17.68
	Pollen donor effects																	
1	NS	1.95	NS	1.29	NS	1.81	NS	0.8	NS	1	NS	1.91	***	5.58	NS	1.08	*	2.96
2	NS	0.51	NS	1.29	NS	0.56	NS	0.99	NS	0.61	NS	0.59	**	7.06	NS	0.74	NS	0.75
3	NS	0.07	NS	2.57	NS	0.62	NS	0.97	NS	0.42	NS	0.72	NS	0.23	---	---	NS	1.29
4	NS	2.39	NS	0.02	NS	1.0	NS	0.21	NS	1.57	NS	1.51	NS	3.03	NS	2.01	NS	0.01
5	NS	0.39	NS	1.35	**	5.1	NS	0.27	NS	1.95	NS	0.25	**	5.35	NS	1.16	NS	1.65
6	NS	2.33	NS	0.46	NS	1.23	NS	1.2	NS	0.18	NS	1.13	**	5.23	NS	1.42	*	3.78
	Female parent × Pollen donor interactions																	
1	NS	1.28	NS	2.32	NS	0.71	NS	1.93	NS	1.35	NS	2.41	***	5.73	NS	1.85	NS	2.14
2	**	4.09	NS	1.31	NS	1.48	NS	1.90	NS	1.32	***	50.48	***	7.22	NS	0.70	***	7.06
3	NS	0.66	NS	0.01	NS	0.77	NS	0.19	NS	0.22	NS	0.33	NS	0.71	---	---	*	3.59
4	NS	0.33	NS	2.56	NS	1.54	NS	0.64	NS	0.18	NS	0.21	*	4.59	*	3.18	NS	0.24
5	**	3.60	NS	1.72	NS	1.97	NS	1.69	NS	1.06	NS	1.43	***	3.99	NS	0.70	**	3.24
6	NS	1.99	NS	1.53	NS	0.91	NS	1.89	NS	1.19	NS	1.40	***	5.67	NS	1.79	**	3.32

<sup>z</sup> Analysis of variance carried out on data from each smaller diallel for trial 1 and trial 2 (listed in Table 2) over five repetitions of flavor evaluations per sample. The F test values were used to gauge the relative magnitude of the effects.

<sup>y</sup>R/B/G = raw/beany/green flavors.

\*, \*\*, \*\*\* significant differences by GLM ANOVA at  $P \leq 0.05$ ; 0.01 and 0.001 respectively; NS = nonsignificant; --- = Analysis of variance not possible due to zero scores.

Linear regression analyses from pooled trial 1 and trial 2 sensory data was carried out to determine whether the average score for each cultivar over all crosses as male or female parent was related to the score of the actual cross. The independent variable in the linear regression was obtained from the average scores for all possible combinations of crosses, repetitions and trials in the overall diallel mating design (Table 1) for each female parent in turn. Averages for similar combinations were also calculated for each pollen donor in turn. The dependent variable was derived from the average score for a particular flavor attribute, for each individual cross.

A summary of significance of linear relationships between ( $P \leq 0.001$  to  $P \leq 0.05$ ) female parent and progeny and pollen donor and progeny are presented in Table 4 using the values of slope and the significance of these values, as well as, magnitude of these effects (given by the  $t$  statistic values) from linear regression analyses. There were significant ( $P \leq 0.001$  to  $P \leq 0.05$ ) female parent effects for all flavor attributes except astringency, indicated by slopes significantly ( $P \leq 0.001$  to  $P \leq 0.05$ ) larger than 0. The slopes were particularly large for floral and cocoa flavors (0.72 and 0.96, respectively) and significantly different from zero indicating a strong dependency of the female parent for floral and cocoa flavors (Table 4). The coefficient of determination for the

regressions were 0.72 and 0.97 for cocoa and floral flavors respectively. In contrast the male parent effect on bean quality were largely not significant except for astringency which was significant at  $P < 0.05$ .

## DISCUSSION AND CONCLUSIONS

The objective of this study was to examine the effect of pollen donor on the flavor attributes of selected cacao cultivars to determine if there was a xenia effect on flavor and the diallel mating design provided an effective framework to investigate this premise. Pollen donor effects have been studied in other crops, such as apple and cherry, for other quality attributes such as firmness and acid content (Davarynejad et al., 1994) and fruit size and total soluble solids (Ansari and Davarynejad, 2008) but rarely on flavor.

The optimized organoleptic evaluation methods (Sukha et al., 2008) provided robust data which were able to provide statistically valid results. This highlights the effectiveness of the sensory design in being able to provide data from which we are able to resolve significant effects from small differences in scores. The results showed that flavor of cocoa beans was determined largely by the genotype of the female parent and with a possible marginal effect of pollen donor on flavor, confined mainly to astringency, nutty and other flavors. Due to the inconsistency of significance of male parent effects over crosses, and the relatively small effects one cannot preclude type II error. However, F test values and *t* statistic values were presented where possible for each flavor attribute to gauge the relative magnitude of the male and female parent effects observed.

The possibility of the pollen donor contributing to bean flavor originates from the fact that the endosperm of the seed is determined by male and female gametes. However, this appears to be superseded by the strong influence of the seed coat and mucilage (pulp), that is entirely maternally derived since the integument of the matured ovule develops into the seed coat which shares the same diploid genotype of the mother tree (Fang et al., 2014). Fresh cocoa seeds are characterized by an astringent and bitter taste due to the high content of phenolics, especially anthocyanins (Jinap et al., 2005) and because they do not contain the necessary aroma precursors (Ziegleder and Biehl, 1988).

Many authors including Andersson et al. (2006) have highlighted the kinetics of bean acidification during fermentation, on flavor development. The maternally derived seed coat and pulp is degraded by yeasts, lactic and acetic acid bacteria resulting in lactic and acetic acid formation as a result of exothermic reactions. The acids permeate through the differentially permeable seed coat into the seed tissue and, together with the heat evolved during fermentation, cause seed death. The storage proteins and carbohydrates are subsequently degraded by seed enzymes yielding aroma precursors which are peptides, free amino acids and reducing sugars (Afoakwa et al., 2008; Andersson et al., 2006; Kadow et al., 2013; Schwan and Wheals, 2004; Ziegleder and Biehl, 1988).

In addition to the endogenous formation of flavor and aroma precursors from fermentation and drying, there is also mounting evidence for the exogenous influence of the pulp directly on aroma and flavor development. Eskes et al. (2007, 2012) discuss the direct permeation of aroma components from the pulp into the seed tissue during fermentation that may be retained during the drying process.

The study therefore failed to detect xenia effects for most of the important flavor attributes i.e., cocoa flavor, acidity, fruitiness and floral flavor which agrees with the preliminary work of Clapperton et al. (1994a, 1994b) and Lockwood and Eskes (1996) suggesting a general lack of xenia effect on flavor in cacao and implies that the flavor quality of cocoa beans is determined principally by the genotype of the female parent.

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