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POLLEN-GRAIN INTERACTIONS IN A NEOTROPICAL *COSTUS*: EFFECTS OF CLUMP SIZE AND COMPETITORS

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INTRODUCTION

The physiological and biochemical processes involved in pollen germination and pollen-tube growth have received considerable attention,[1-3] but the ecological and evolutionary implications of pollen characteristics have not. Interest in the potential relationship between pollen genotype, pollen-tube growth and progeny performance have motivated several studies which examine the fitness consequences of post-pollination, pre-fertilization interactions among male gametophytes. Experiments by Mulcahy and Mulcahy,[4] and Ter-Avenesian[5] suggest that gametophytic competition may influence sporophyte quality. Haldane[6] suggested that any mutation which enhanced pollen-tube growth would spread rapidly through a population. Thus intrapopulation variation in pollen-tube growth may influence the fertilization success of pollen parents, and the fitness of the sporophytes produced.

At present there are insufficient experimental data from natural populations to evaluate the role of pollen growth characteristics in the reproductive biology of higher plants. An analysis of the evolutionary impact of variation in pollen growth requires a complete decomposition of the sources of variation in pollen germination and pollen-tube growth, including (1) paternal genotype, (2) paternal environment, (3) pollen genotype, (4) the population effect, i.e., the number and genetic composition of pollen grains deposited, (5) maternal genotype, (6) maternal environment, and (7) the interactions among these components of variation. If pollen-tube growth is a character with heritable variation, we expect a response to selection, but the rate of evolution may be reduced as the proportion of the phenotypic variation in growth that is attributable to environment increases. In this context, the population effect may be particularly important. Several studies[7,8] have indicated a mutual stimulation effect among pollen grains *in vitro*, and others[9,10] have demonstrated the same phenomenon *in vivo*. The magnitude of the population effect may be sufficient to influence the evolution of floral characters related to pollinator foraging and pollen deposition.

The research presented here represents an attempt to quantify some of the sources of variation in pollen germination and pollen-tube growth. Experiments were conducted to answer the following questions: (1) Is there significant variation among individual plants in pollen germination and growth *in vitro*? (2) Does the number and/or genotypic composition of pollen grains influence germination and growth *in vitro*? (3) Does the number of pollen grains deposited on a stigma influence the probability of fertilization, and fruit maturation *in vivo*? We emphasize that the results presented here are preliminary, and require cautious interpretation, particularly in light of the rapid advances being made on the physiology of fertilization in higher plants.

The study organism was *Costus guanaiensis*, a neotropical herb of the family Zingiberaceae. Details of plant and floral biology are given in Schemske.[11]

MATERIALS AND METHODS

All plants used in this study were the greenhouse-grown progeny of plants hand-pollinated in Panama. To examine pollen germination and pollen-tube growth *in vitro*, pollen was germinated on an agar medium consisting of 15% Sucrose, 0.5% Agarose, .03% Calcium Nitrate and .02% Boric Acid, in 100 x 15 mm petri dishes. Incubation was for 6 hrs at 28°C. Pollen-tube growth was halted by spraying with chilled FAA and exposure to -15°C for 15 min. All measurements were made with a binocular scope at 15X, and an ocular micrometer with 1 unit equal to 80μ. Germination was defined as a pollen grain with an emerging pollen tube ≥ the pollen grains' diameter.

The effect of pollen number on pollen germination and pollen-tube growth was determined by constructing groups of 1, 4, 16, and 64 pollen grains. Each replicate consisted of an equal sample of 64 pollen grains per group (= 64 "clumps" of 1 grain, 16 of 4 grains, 4 of 16 grains and 1 of 64 grains). Groups were distributed on individual plates, with maximum physical contact among pollen grains in a given group. The effects of clump size (pollen grains/group), genotype and the clump size X genotype interaction were quantified by a standard ANOVA, with flowering date as a covariate. The ANOVA, and other analyses of clump size effects included only those plants which were ½-sibs, or unrelated.

To quantify potential competitive effects between pollen grains, *in vitro* experiments were conducted utilizing pollen from parents with varying degrees of relatedness. Pollen germination and pollen-tube growth were examined for treatment and control clumps of 4, and 16 pollen grains. The competition treatment represented a 50:50 mixture of pollen from 2 parents which were unrelated, ½-sibs, or full sibs. To allow identification of pollen grains after germination, grains from different parents were arranged on opposite sides of a clump. Two controls, representing single-parent clumps for each of the parents in the treatment, were incubated on the same plates as the treatment. To test for competitive interactions, frequency distributions of pollen-tube lengths were compared for treatments and controls by a Kolmogorov-Smirnov Two-Sample Test.

To investigate the influence of number of pollen grains, i.e., clump size, on pollination success, hand pollinations were made using 1, 4, 16 and 64 grains/clump. Pollen donors and pollen recipients were either unrelated or ½-sibs. There was no significant difference in fruit production due to genetic relatedness between pollen and seed parents, so this aspect of the study will not be discussed further. Pollination success was expressed as the percent of pollinated flowers maturing fruit.

RESULTS AND DISCUSSION

The results of the analysis of variance and multiple comparison of means are given in Table 1 for the following three characters: (1) the % of pollen grains which did not germinate, (2) the % of grains with a pollen-tube $\geq$ 41 ocular divisions in length (≈ 3.3 mm), and (3) the % of grains with pollen tubes $\geq$ 81 ocular divisions (≈ 6.5 mm). There was no significant effect of flowering date for any of the three characters, but there were significant parent and clump effects for all characters. The parent X clump interaction was significant for the two pollen-tube length characters. For single grains, 59% did not germinate, as compared to 40% for 4-grain clumps, 21% for 16-grain clumps and only 16% for 64-grain clumps. The % of pollen grains with long tubes was greatest for the largest clump sizes. For clumps of 16 or 64 pollen grains, the % of grains with tubes $\geq$ 81 ocular divisions was 50 times greater than that of single grains. These data indicate that pollen parentage and pollen clump size significantly influence pollen germination and growth *in vitro*.

Frequency distributions of pollen-tube length for individual pollen parents based on pollen growth in groups of 16 pollen grains were compared by Kolmogorov-Smirnov Two-Sample Tests. Of the 10 possible comparisons, 7 distributions were significantly different ($p < 0.05$). Thus, as indicated previously by the analysis of variance (Table 1), there is significant among-plant variation in pollen germination and pollen-tube growth.

Given the marked positive effect of pollen clump size on germination and pollen-tube growth (Table 1), we asked if the kinship of pollen grains within a clump had any effect on pollen growth. That is, does pollen from a single individual or from closely related individuals produce a population effect different in magnitude or direction from that if pollen clumps are comprised of pollen from unrelated or distantly related parents? The competition experiments provide an *in vitro* examination of this question. Specificity of the population effect at the level of kin groups would add another component to the observed variation in pollen germination and pollen-tube growth, and has implications for the evolution of pollen dispersal units[12,13] and the genetic consequences of long-distance pollen flow.

TABLE 1

ANOVA AND MULTIPLE COMPARISONS OF MEANS (DUNCAN'S MULTIPLE RANGE TEST). MAIN EFFECTS IN THE ANOVA INCLUDE POLLEN PARENT (PARENT), NUMBER OF POLLEN GRAINS PER CLUMP (CLUMP), AND THE PARENT * CLUMP INTERACTION. FLOWERING DATE (DATE) IS A COVARIATE. THE BODY OF THE ANOVA TABLE CONSISTS OF F-VALUES. FOR THE MULTIPLE COMPARISONS OF MEANS, VALUES FOLLOWED BY THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT (SIG. LEVEL = 0.05). ALL STATISTICAL ANALYSES WERE CONDUCTED ON THE ARC-SINE TRANSFORMATION OF PERCENTAGE DATA, BUT THE MEANS PRESENTED HERE ARE UNTRANSFORMED.

	ANOVA				Multiple Comparison of Means			
					Pollen Grains per Clump			
Character	Date (1)[a]	Parent (3)[a]	Clump (3)[a]	Parent Clump* (9)[a]	1	4	16	64
% ungerminated (82)[b]	0.0	13.0****	39.8****	1.4	59.2_k	40.2_l	20.5_m	16.0_n
% of grains with pollen tubes $\geq$ 41 divisions[c] (82)[b]	0.7	11.3****	35.4****	6.1****	4.7_n	16.7_m	25.5_k	17.8_l
% of grains with pollen tubes $\geq$ 81 divisions[c] (82)[b]	0.2	5.1**	26.7****	2.8**	0.1_m	3.0_l	5.2_k	5.1_k

[a,b] Degrees of freedom for numerator, and denominator, mean square, respectively.

[c] Measured on an ocular micrometer; 1 division = 0.08 mm.

**$p < 0.01$; ****$p < 0.0001$.

The results of the competition experiments are given in Table 2. Pollen germination and pollen-tube growth were greater for 16-, as compared to 4-grain clumps, indicating the consistency of the population effect. There were *no* significant differences between treatments and controls in pollen-tube lengths for any kinship class, in 4- or 16-grain clumps. Thus, the general conclusion from both sets of experiments is that the genetic relatedness among pollen grains has *no* effect on pollen germination and growth. These results are preliminary, however, and warrant further investigation. In particular, the greatest heterogeneity in germination behavior was observed in the unrelated class for 16-grain clumps, with significant differences ($p < 0.05$, proportion test) for % germination between treatments and controls in 3 of the 4 comparisons.

TABLE 2

EFFECTS OF GENOTYPIC COMPOSITION ON POLLEN GERMINATION AND GROWTH *IN VITRO*. THE "TREATMENT COMPOSITION" COLUMN OF THE FORM X → Y REFERS TO THE GERMINATION AND GROWTH OF POLLEN FROM PARENT Y IN (1) THE PRESENCE OF POLLEN FROM PARENT X (= TREATMENT, T), OR (2) MONOCULTURE (= CONTROL, C). ENTRIES UNDER "POLLEN-TUBE LENGTH" ARE THE % OF POLLEN GRAINS IN A GIVEN CATEGORY.

Kinship	Treatment Composition (X → Y)	T or C	4 Grains/Clump					16 Grains/Clump					
				Pollen Tube Length[b]					Pollen Tube Length[b]				
			N[a]	0	1-40	41-80	81-120	N[a]	0	1-40	41-80	81-120	>120
Unrelated	A → B	T	40	15	51	28	5	72	28	45	16	11	0
		C	60	13	73	13	0	112	17	61	17	5	0
	C → B	T	32	25	53	19	3	64	13	59	11	16	0
		C	56	27	50	11	13	112	26	53	15	6	0
	B → C	T	32	16	72	13	0	64	14	65	17	5	0
		C	68	19	70	10	1	112	5	67	23	5	0
	B → A	T	40	51	46	3	0	72	17	63	14	7	0
		C	72	51	44	1	3	128	34	57	5	5	0
½-sibs	A → C	T	68	13	75	10	1	96	5	77	16	1	1
		C	116	16	70	12	2	176	5	78	15	2	0
	A → D	T	32	9	85	6	0	32	3	77	17	3	0
		C	32	6	85	9	0	48	5	80	16	0	0
	C → A	T	68	47	41	10	2	96	36	53	9	2	0
		C	92	50	47	3	0	176	33	59	8	1	0
	D → A	T	32	50	47	3	0	32	35	59	6	0	0
		C	32	41	60	0	0	48	34	59	7	0	0
Full-sibs	C → D	T	90	23	71	4	0	96	14	73	13	0	0
		C	124	14	83	3	0	192	11	79	10	0	0
	D → C	T	88	26	66	8	0	96	15	71	13	1	0
		C	120	20	72	7	1	176	7	78	13	2	0

[a] Number of pollen grains.

[b] In divisions from ocular micrometer; 1 division = 0.08 mm.

The results of the *in vivo* hand pollination experiments are illustrated in Table 3. There is a highly significant effect of pollen number on the probability of successful fertilization and seed production ($\chi^2 = 105$; $p < 0.0001$). Pollination with single grains produced no fruit in 79 crosses, while the frequency of fruit production increased successively to 68% for 64-grain pollinations. These data, in conjunction with the evidence for a positive effect of pollen number on germination and growth *in vitro*, suggest that interactions among pollen grains may have a significant effect on fertilization. This interpretation requires further investigation, as population effects *in vitro* may be a function of media composition, particularly calcium content (J. Heslop-Harrison, *pers. comm.*). However, pollen number has been experimentally demonstrated to influence pollen germination[9,10] and fruit set[9] *in vivo*. The genotype of the maternal parent is also an important factor.[9,10]

TABLE 3

FRUIT SET (%) FOLLOWING HAND POLLINATION

Number of Pollen Grains			
1	4	16	64
0 (79)[a]	3.1 (63)[a]	48.7 (39)[a]	68.1 (47)[a]

[a] Number of flowers pollinated.

Although these results are preliminary, we suggest that pollen-grain interactions may be an important factor influencing the evolution of plant characters related to pollination. Further research is needed to determine (1) the heritability of pollen germination and pollen-tube growth, (2) the processes that maintain genetic variation for pollen germination and growth, and (3) the mechanisms and evolutionary consequences of pollen-grain interactions *in vivo*.

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