

Full Length Research Paper

# Hereditary proof of formative segments of parthenocarpy in Apomictic MUSA species

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Apomixis and parthenocarpy are broadly botanically defined as embryo and seed, and fruit development respectively, in the absence of fertilization. The objectives of this study were to genetically elucidate the development of apomictic seed and parthenocarpic fruit development in *MUSA*. Borneo and Calcutta 4 plants were vegetatively propagated and were assayed in replicates of 2 and 6, respectively at Onne and Ibadan, Nigerian locations. Each mother and ratoon was assayed over 2 flowering cycles, resulting in a total of 16 inflorescences analyzed. Pollination barrier procedures were performed involving bagging of the inflorescences, isolating them from natural pollinators. Scoring for apomixis was based on seed-set in isolated (bagged) inflorescences without pollination. Parthenocarpy was determined by seedless pulp development in open pollinated or as artificial pollination by Calcutta 4, using standard *MUSA* crossing procedures. Floral and fruit developmental traits indicated that Borneo was monoecious, comprising traits of apomixis and vegetative parthenocarpy. Borneo × Calcutta 4 cross, a P1-lacking, P2 and P3 modelled *MUSA*, suggests that Borneo may be genetically modelled to comprise P1 but lack P2 and/or P3. Such modifier genes appear to correlate with rates of genome by environment dependent seed development regimes and if not synchronized with fruit development regimes, results in dehiscence, death of developing fruit, and non-parthenocarpic expression.

**Key words:** Apomixis, banana, parthenocarpy, pollination, genotype, environment, *Musa*, modelling, genetics, Nigeria.

## INTRODUCTION

Apomixis is the natural ability of more than 400 plant species to reproduce asexually through seed (Koltunow, 1993; Grossniklaus et al., 2001). While sexual embryos result from the union of male and female gametes, which produce genetically varied offspring, apomictic embryos are formed directly from gametophytic or sporophytic ovarian tissue without paternal contribution. Apomixis may be obligate or facultative, resulting in either only apomictic produced embryos and seed, or a combination of apomictic and sexually-derived embryo and seed,

respectively (Crane, 2001; Hanna and Bashaw, 1987; Koltunow, 1993; Grossniklaus et al., 2001). Apomixis has been reported in more than 300 species and at least 35 different families, and commonly may be identified in the Gramineae, Compositae, Rosaceae, Euphorbiaceae and Rutaceae, but it has never been identified before in the Musaceae (Bashaw, 1980; Hanna and Bashaw, 1987; Koltunow, 1993; Ogburia and Adachi 1996; Ogburia et al., 1997; Grossniklaus et al., 2001). Vegetative parthenocarpy *Sensu stricto* is widely botanically defined as fruit development in the absence of fertilization (Mapelli et al., 1978; Mazzucato et al., 1998; Mazzucato et al., 1999; Robinson et al., 1971; Rotino et al., 1997; Spena et al., 1999; Spena and Rotino, 2001).

In *Musa*, both parthenocarpic and non parthenocarpic

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plant forms exist, often with some genotypes exhibiting both forms with the parthenocarpic forms preferred due to their: 1) non-requirement for fertilization leading to more stable fruit yield; 2) increased fruit pulp quantity and quality; and 3) seedless characteristics (Dodds and Simmonds, 1948; Ortiz, 1995; Ortiz, 2000; Ortiz et al., 1997; Ortiz and Vuylsteke, 1995a; PBIP, 1995; PBIP, 1997; Vuylsteke et al., 1997). In non-parthenocarpic *Musa*, unfertilised ovaries typically dehisce and die and do not produce mature fruit in a genotype-dependent developmental regime. Up to months later, unfertilised ovules dehisce and die inside such non-parthenocarpic ovaries. A current genetic model for the expression of vegetative parthenocarpy has been described, that in part explains the variable expression of vegetative parthenocarpy in *Eumusa Musa acuminata* interspecific hybrids, and *M. acuminata/Musa balbisiana* interspecific hybrids as a quantitative trait that is primarily the result of a major gene P1, and two minor modifier genes P2 and P3 (Ortiz, 1995; Ortiz and Vuylsteke, 1995a). As reported by Ortiz and Vuylsteke (1995), this current genetic model of vegetative parthenocarpy in *Musa* suggests that: 1) parthenocarpic expression is oligogenically inherited in the *Eumusa* species comprising A (*M. acuminata*) and B (*M. balbisiana*) genomes, primarily through expression of a dominant major gene allele P1 that is necessary and sufficient for parthenocarpy expression, and through two additional modifier gene loci which expression is necessary but not sufficient for parthenocarpic expression; and 2) that a *M. acuminata* subspecies *Burmannicoides* accession Calcutta 4 may be genetically modelled to comprise homozygous P2 and P3 loci that are sufficient but not necessary for parthenocarpy expression, but lacks a dominant P1 allele that is necessary and sufficient parthenocarpy expression.

In this study, we present the first scientific report on the expression of apomixis in *Musa*, while giving the evidence on the involvement of genetic components that may partially account for the parthenocarpic gene expression *sensus stricto* in an environmental dependant manner. The ability to identify and predict a banana cultivar's potential of seedlessness across environments by examining developmental cues may be a valuable tool to a banana breeder. Herein, we describe tools to screen and identify apomictic gene expression, and to identify developmental cues that might be predictable across environments. This may allow geneticists and breeders to counter-select for a trait of apomixis and select for increased expression of parthenocarpy, with the identification of apomixis even in the presence of masking traits such as non.parthenocarpy.

## MATERIALS AND METHODS

### Plant materials and ecoregional field sites

The plant genotypes utilized in this study were *M. acuminata* subspecies *Microcarpa* accession Borneo and a *M. acuminata*

subspecies *Burmannicoides* accession Calcutta 4 established in 2003 in the *Musa* Field Genebank, of the International Institute of Tropical Agriculture (IITA) Onne and Ibadan stations in Nigeria. Plants were vegetatively propagated by desuckering, and were assayed in replicates of 2 and 6 respectively in 2004/2005 in both stations. The Calcutta 4 accession has been characterized by Ortiz and Vuylsteke (1995a), as a non-parthenocarpic *Musa* lacking a necessary and sufficient dominant P1 allele for parthenocarpy that appears to be necessary and sufficient for the expression of vegetative parthenocarpy in *Eumusa*. Calcutta 4 accession is typically used in many breeding programs around the world. Each mother sucker planted mat was assayed over 2 flowering cycles each (that is, a mother and daughter sucker), resulting in a total of 16 inflorescences analyzed overall, between the two locations. The coastal Onne planting site is geographically located at 4°51' N, 7° 03' E at 10 m above sea level (masl) and comprises an Ultisol derived from coastal sediments that is well drained but poor in nutrient availability and high extractable phosphorous; while the inland Ibadan site is located 7° 31' N, 3° 54' E and at ~210 masl, comprising an Alfisol that is slightly degraded and acidic but relatively nutrient rich, having been produced for this study, by the clearing of a small forested location of deciduous trees. In addition, the range of site variations include: 1) single modal (Ibadan) to bimodal (Onne) rainfall pattern with an annual rainfall total of ~1300 mm (Ibadan) to ~2,500 mm, most of which falls at both locations between the months of May and September; 2) average daily temperature range variations between 20 and 35°C; and 3) fluctuating but relatively constant radiation at ~5285 MJ/m<sup>2</sup>/year, reflective of both sites proximity to the equator.

### Scoring assays for the expression of apomixis and vegetative parthenocarpy traits

Pollination barrier procedures were performed involving bagging of the inflorescence buds, isolating them from natural pollinators. Scoring for apomixis was based on seed-set in isolated (bagged) inflorescence without pollination while parthenocarpy was determined by seedless pulp development in open pollinated or as artificial pollination by Calcutta 4, using standard *Musa* crossing procedures. Because some flowers emerged at night, possibly subjecting the first whorl of flowers of the inflorescence to possible pollination by night time pollinators such as bats, scoring for apomixis and parthenocarpy did not include any scoring of the first hand.

## RESULTS

Observations and scoring of floral and fruit developmental traits indicated that Borneo is monoecious, and comprises both a trait of apomixis and vegetative parthenocarpy like trait. In *Musa* several flower bracts replace leaves at the transition from the vegetative to the floral stage, and an inflorescence comprising a rachis and associated inflorescent bud comprised of tightly overlapping sets of bracts separating whorls of flowers (hands) emerge (Figure 1A). On the date of rachis and inflorescence emergence, pollination barrier procedures were performed involving bagging of the inflorescence buds, isolating them from natural pollinators, while anthesis of successive flowers occurred along with internodal growth of the rachis, with whorls of female flowers (hands) emerging between overlapping bracts derived from the inflorescence bud (Figure 1B).



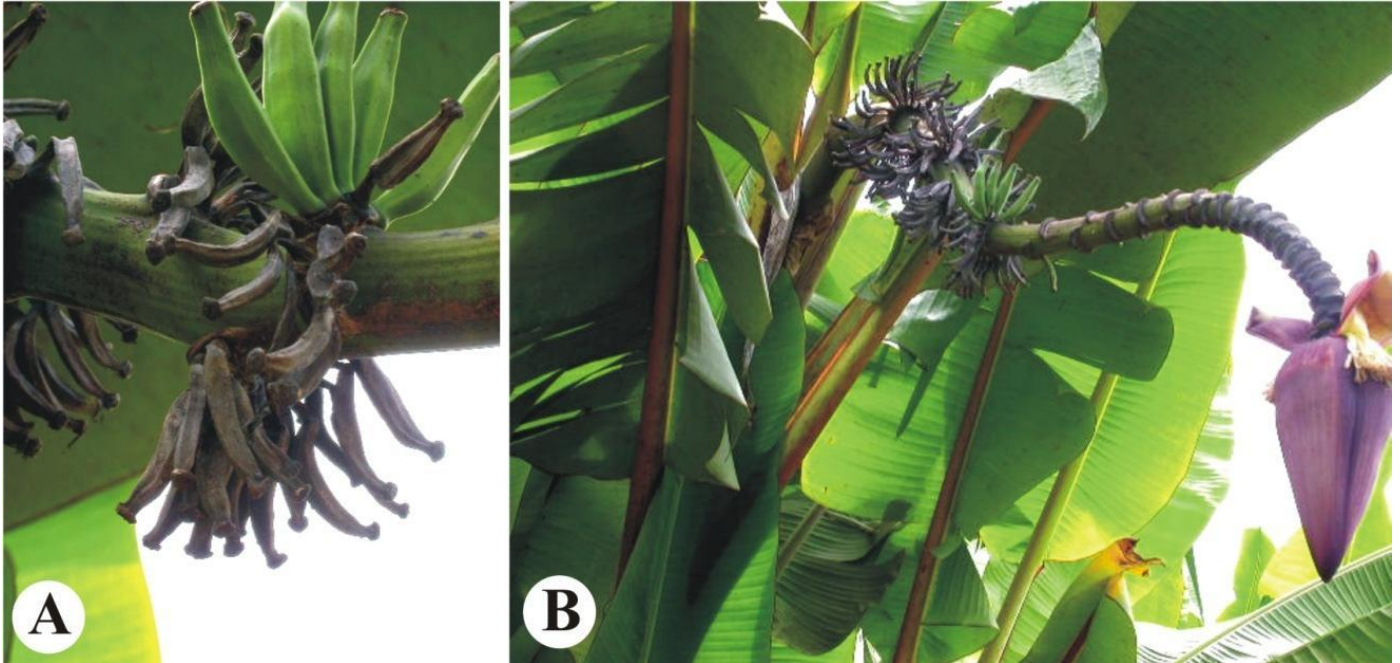
**Figure 1.** Inflorescence bud emergence in *Musa acuminata* subspecies *Microcarpa* Borneo (A) and an external pollination barrier bagging assay (B).

Results of the examination of the emerging flowers of inflorescences indicated that Borneo was monoecious, with female and male flowers developmentally isolated, as observed in most *Musa* genotypes. Although the female flowers appeared to be developmentally isolated from male flowers, and did not appear to produce any mature anthers or pollen, in some instances anthers were removed to determine if visually undetectable pollen formation was occurring that could have any potential effect on our scoring for variability of apomixis or parthenocarpy. Since some genotypes of *Musa malaccensis* are recognized as comprising hermaphroditic flowers, this analysis was necessary as a control. The results of apomictic and parthenocarpy scoring of the initial flowers of Hands 2 and greater, of female flowers with and without their apparently infertile male floral parts, exhibited no clearly detectable difference in scoring for apomixis or parthenocarpy: 1) each of both the de-anthered and anther comprising flowers produced apomictic embryos without change; and 2) parthenocarpic-like expression in both treatments produced hands of mixed parthenocarpic and

non-parthenocarpic scored fingers, and produced apomictic seed in parthenocarpic fingers.

Expression of apomixis in Borneo appears to be completely penetrant, but a variably activated genetic lesion in a maternal effecting developmental pathway that is restricted to unfertilized ovarian and fruit development, results in the variable expression of a trait of vegetative parthenocarpy as botanically defined *S. stricto*. In the total of 16 inflorescences sampled (2 flowering cycles each of 2 plant mats at the Onne site and 6 plant mats at lbadan site) all fingers scored for apomictic seeds at 2, 3, 4, and 8 weeks post emergence of their inflorescence and were identified as comprising immature apomictic seeds. Immature apomictic seeds in nonparthenocarpic trait expressing fingers often aborted in death as their apparent non-parthenocarpic developing host fruits dehisced and died, at approximately 4 weeks (Figures 2 A and B).

Results of analysis of the parthenocarpy-like expression showed that in all of the 16 inflorescences assayed at least one finger exhibited a parthenocarpic-like trait, with most rachises exhibiting both



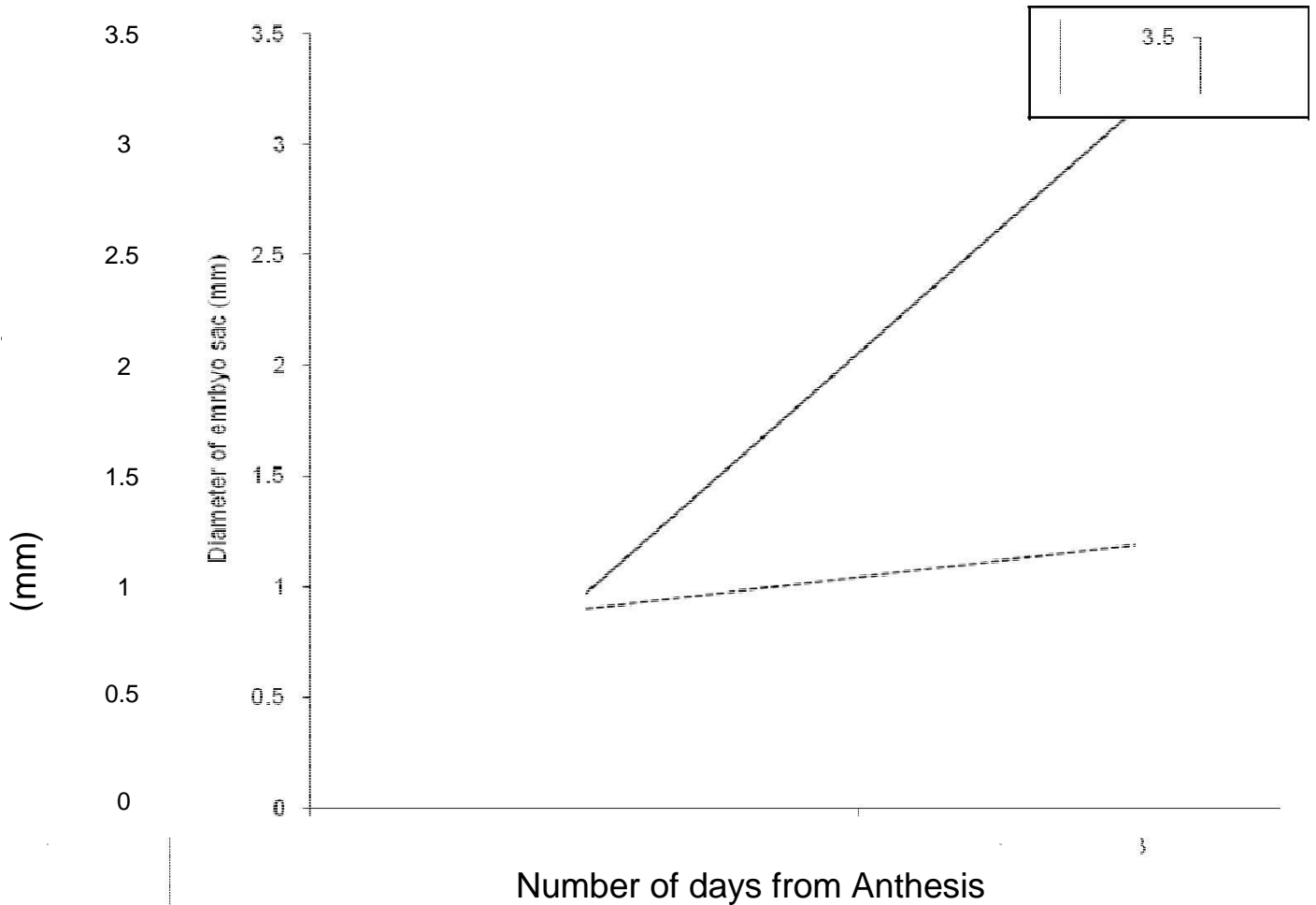
**Figure 2.** Variable expression of vegetative parthenocarpy in unpollinated *Musa acuminata* subspecies *Microcarpa Borneo* after pollination barrier bagging assay. (A) illustrates a close-up view of a hand of parthenocarpic and nonparthenocarpic expression and (B) illustrates a typical 6 week post-emergence.

parthenocarpic and nonparthenocarpic fingers, as shown in Figure 2. In one of the three replicate plant mats grown in Ibadan, a first emerged unpollinated rachis and inflorescence produced a combination of both parthenocarpic like and non parthenocarpic expression from its female flowers, while a second successive rachis and inflorescence produced only parthenocarpic-like expression. Although the nature of the variability of the expression of the vegetative parthenocarpy trait cannot be reliably determined given the small sample size (14 inflorescences from 5 clonal mats) of Borneo assayed, the expression of the trait suggests that expression of the trait is the result of variable expressivity of the trait within individuals due to genome by environment interactions, rather than incomplete penetrance of the trait between individuals.

To test whether the nonparthenocarpy trait expression observed was or was not the result of a genetic lesion in a both sexual and asexually derived fruit development regimes, or was restricted to unfertilized fruit, Hands 1 and 3 from two flowers of different mats at the Ibadan site were pollinated with pollen from a *M. acuminata* subspecies *Burmannicoides* accession Calcutta 4, with the remaining hands of the inflorescences of these plants unpollinated. Calcutta 4 is a non parthenocarpic *Musa* accession, genetically modeled as lacking a P1 dominant major allele necessary and sufficient but comprising homozygous P2 and P3 modifier alleles necessary but not sufficient for parthenocarpic expression in *Musa* (Ortiz, 1995; Ortiz and Vuylsteke, 1995a). The results

from scoring of these four fertilized hands, indicated that every flower fertilized produced a fully develop fruit from each flower fertilized, while the unpollinated fingers from surrounding hands exhibited variable expression of parthenocarpy. Furthermore, in all 14 inflorescences assayed, the apparent expressivity of non-parthenocarpic trait expression was temporally and developmentally systematic. Non-parthenocarpic fingers of all 14 inflorescences exhibiting this trait, regardless of location or time of year of flowering, all systematically dried up and died on the rachis at ~4 weeks after rachis and inflorescence bud emergence. These results may be most logically interpreted to indicate that although the heritability of initiation of a non parthenocarpic event is comparatively low, after initiation of a non-parthenocarpic developmental event, that the heritability of the developmental program of dehiscence and abortion of fruit development is quite high and temporally and systematically controlled at a genotypic level.

Calcutta 4 pollinated sexual *versus* apomictic immature seed growth patterns indicates that the relative rates of seed growth in comparison to the developmental regime of fruit, might constitute a developmental genetic link to expression of parthenocarpy. Evidence collected from floral bud bagging assays indicated that all unfertilized Borneo female flowers produced young apomictic embryos and immature seed, but that the survival of these embryos and seed to a stage of mature seed development is dependent upon the incomplete penetrant expression of a non-senescent fruit phenotype that



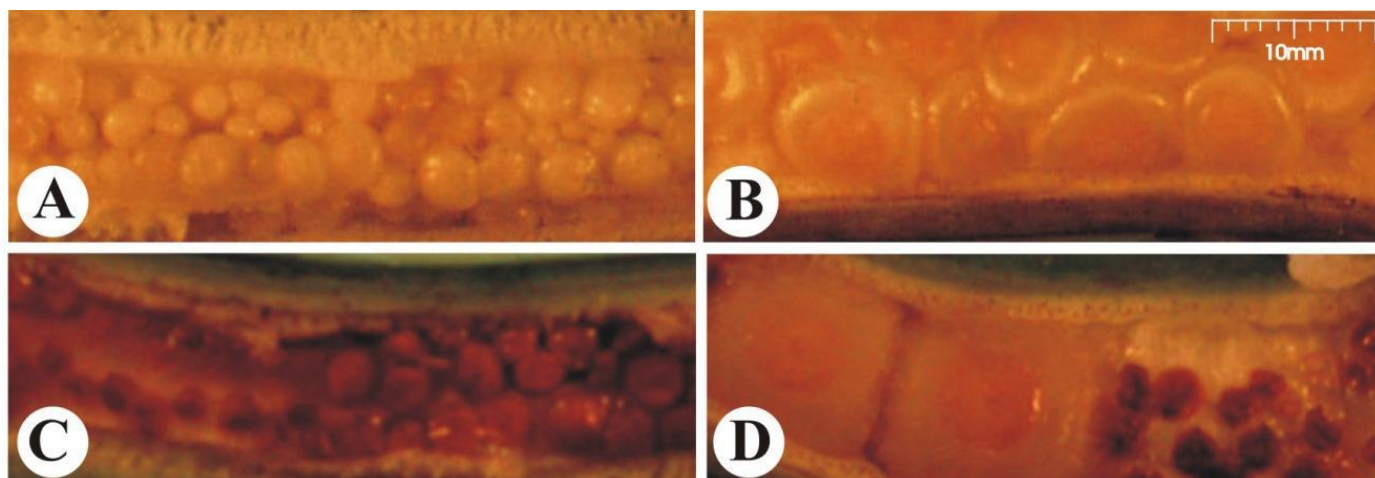
**Figure 3.** Immature seed growth rates of Borneo unpollinated hands.

resembles vegetative parthenocarpic expression. Immature seeds were thus measured for their growth rates in the Borneo plants pollinated with a P1 deficient Calcutta 4, versus an unpollinated plant. Results indicated that both the pollinated versus unpollinated ovaries grew, but that on the average, that mixture of immature apomictic and apomictic seed in the pollinated ovaries grew faster on average, compared to the apomictic seed, and could be reliably identified as early as 13 days post anthesis/pollination (Figures 3 and 4; and Table 1). Although the minimum value of immature seed in the pollinated treatment clearly demonstrates an overlap of seed growth range that may be reflective of uneven fusion of gametes in the facultative apomictic fruit. As shown in Figure 3, analysis of the temporal growth pattern differences translated into the identification of significantly different growth rates of immature seed in pollinated and unpollinated ovaries, with the most logical interpretation being that sexually derived seed appeared to develop faster than apomictic seed.

## DISCUSSION

### The relationship of the observed parthenocarpic *S. STRICTO* to current genetic models of parthenocarpic in *MUSA*

Assuming an extension of the current genetic model of parthenocarpic in *Musa* (Dodds and Simmonds, 1948; Ortiz, 1995; Ortiz, 2000; Ortiz et al., 1997; Ortiz and Vuylsteke, 1995a); in which a P1 major dominant gene is necessary and sufficient for parthenocarpic expression and P2 and P3 allelic loci are necessary but not sufficient for parthenocarpic expression, the most logical interpretation is that Borneo would be interpreted as comprising at least one copy of P1, but lack one or more copies of the P2 and/or P3 modifier alleles as predicted by the model. One logical interpretation from this analysis, is that of a genetic model by which seed development to a certain size, in relation to a temporal developmental regime of fruit might be required, in which expression of parthenocarpic *S. strict* is dependent.



**Figure 4.** Seed and fruit development of *Musa acuminata* subspecies Microcarpa Borneo from unpollinated hands, and a hand pollinated with *Musa acuminata* subspecies Burmannicoides Calcutta 4. Fruit cross sections shown are: unpollinated fingers at 13 day. (A) 34 day. (C and D) post-anthesis; and pollinated fingers at 13 day post anthesis (C), with pollination performed at anthesis.

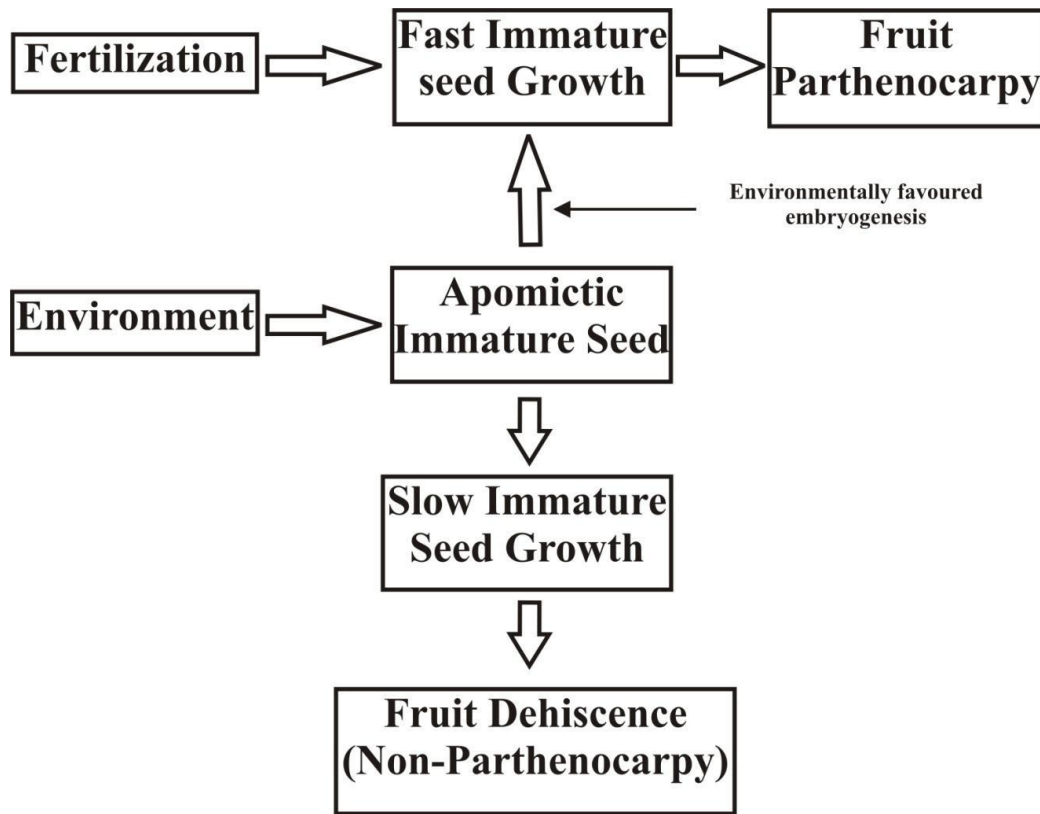
**Table 1.** Analysis of variance (ANOVA) of Borneo embryo sacs and immature seed sizes in micrometers at 13 day .Post-anthesis in ovaries unpollinated and pollinated at anthesis with Calcutta 4 pollen. Least square difference (LSD) ANOVA (A) and associated t-test parameter values (B) demonstrating significance are shown.

A. Least square difference (LSD) ANOVA. LSD value is 0.1956						
Treatment	N	Mean $\pm$ standard deviation	Minimum	Maximum	Grouping	
Unpollinated	30	1243.33 130	1100	1600	A	
Pollinated	30	2003.33 980	1000	3600	B	

**B. t-test parameter values**  
H0:LSMean1  
Diameter Standard H0: LSMEAN=0 =LSMean2  
Treatment LSMEAN Error Pr > |t| t Value Pr > |t|  
Unpollinated 1243.33333 69.05254 <.0001 -7.78  
<.0001  
Pollinated 1003.33333

As shown in Figure 4A and B, and Table 1A, the growth measurements of pollinated and unpollinated immature seed are statistically significantly different as early as 13 days post-anthesis in pollinated and unpollinated hands, but both comprise a common minimum size range. This overlap in size distribution can be most logically interpreted as indicative that the minimum size range for both pollinated and unpollinated forms might be accommodated by unequal sexual fertilization of ovules in the pollinated fingers, with facultative apomixis also occurring in the pollinated fingers. As shown in Figure 4A, C and D, nonparthenocarpic fingers at 34 days (Figure 4C) post anthesis exhibited no identifiably enlarged apomictic seeds compared to 13 days (Figure 4 A), while parthenocarpic fingers at 34 days post anthesis (Figure 4D) exhibited identifiably enlarged apomictic seeds. Under the current genetic model for parthenocarp in

*Musa*, Calcutta 4 is interpreted as lacking a P1 allele but is homozygous for P2 and P3. Therefore, if this genetic model accurately describes the genetic mechanism behind the detailed biology, then it is logically predictable that the functional activity of the expression of P2 and P3 alleles as modifiers in part might be that they contribute to faster immature embryo and seed growth. This may provide a signal for parthenocarpic development with regulation of signal of genotype by environment (G  $\times$  E) in accordance with a G  $\times$  E model as illustrated in Figure 5A natural conclusion of such modeling would be that the most parsimonious explanation for the expression of the variable expressivity of parthenocarp in Borneo might be that it comprises at least one dominant P1 allele, but exhibit either epistatic effects due to heterozygosity of modifier P2 and/or P3 loci, or lack of one or both of these alleles. To test this model, further genetic studies will be



**Figure 5.** A Model of genotype by environment ( $G \times E$ ) interaction that putatively explains the nature of the developmental genetic component of the  $G \times E$  dependence of the variable expression of vegetative parthenocarpy *Sensu stricto* in a facultative apomictic *Musa* plant.

further required, as well as a detailed understanding of the  $G \times E$  effects that can adjust the phenotypic outcomes of scoring of progeny for elucidation of such a model.

The variable expressivity of parthenocarpy *S. stricto* in Borneo exhibits  $G \times E$  dependence. If the assumption that a physical source of a physiological signal transduction occurs by transmission of developing immature seed to immature fruit is involved in the activation of a genetic pathway of parthenocarpy *S. stricto* or a fruit abortion developmental regime, depending on one's perspectives of what is the default developmental pathways. The most logical interpretation of our data is that environmental signals or environmental conditions that favor more rapid seed development in apomictic seed may be viewed as the major environmental components of a  $G \times E$  model that can largely account for variable expression of its parthenocarpic expression patterns. The inland Ibadan site has many differences to the Onne coastal site, that have previously been documented as accounting for genotype by location ( $G \times L$ ) interactions resulting in variable expression of plant height and girth, height of the tallest sucker at flowering, bunch weight, numbers of fingers, fruit weight, black sigatoka disease tolerance,

and variable expression of parthenocarpy (Ortiz and Vuylsteke, 1994a; Ortiz and Vuylsteke, 1994b; Ortiz and Vuylsteke, 1995a; Ortiz and Vuylsteke, 1995b; PBIP 1995; PBIP, 1997; Vuylsteke and Ortiz, 1995; Vuylsteke et al., 1993; Vuylsteke et al., 1997). In addition, pollen viability for several *Musa* accessions has been demonstrated to correlate with dry season in Nigeria between Ibadan and Onne locations (Ortiz, 1997). Given that flowering from a initially planted sucker can only occur at minimum at 9 to 13 months intervals, any statistically valid examination of correlations of  $G \times E$  dependent environmental components involved in the expression of parthenocarpy will require a valid design for required randomization of flowering to different times of year, and a substantively much larger sample size of clones for validation of results, indicating that a valid means of design and an identification of environmental signals appears to be possible.

Extensive surveys of the literature indicate that this is the first scientifically verified discovery of apomictic trait expression in the genus *Musa*. Herein we report not only the first report of apomixis in the genus *Musa*, but a simple methodology to screen for apomixis that may allow breeders and geneticists to counter select for a trait of apomixis, even in the presence of masking traits such

as a variably expressing trait derived from a genetic lesion in parthenocarpic expression. Apomixis, *S. stricto*, is defined as the production of embryo sacs, embryos, or seed in the absence of fertilization; and parthenocarpy *S. stricto* is defined as fruit development in the absence of fertilization. For both Borneo and for apomictic clonal plants derived from it, the survival of the apomictic embryos to a stage of mature seed development can be expected to be dependent upon the completion of a fruit developmental regime. Because an ability to identify and predict the ability of a cultivar's degree of seedlessness across environments may be a valuable tool to a *Musa* breeder, our description of the developmental biology associated with these processes, in part also has value in that the genetic scoring and screening means provided may allow for counter selection of apomixis, even when such expression occurs in the presence of other masking traits such as fruit nonparthenocarpy.

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