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*Full Length Research Paper*

# Ploidy variation of *Musa* hybrids from crosses

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**Plantain and banana (*Musa* spp) breeding involves crossing 3x (triploid) landraces to 2x (diploid) accessions as female and male parents, respectively, selecting 4x (tetraploid) and 2x primary hybrids from the 3x - 2x progenies, and crossing 4x - 2x hybrids to produce secondary 3x hybrids. In these crosses, complex ploidy and genome arrays occur frequently making it difficult to predict the products of crosses. The objective of the study was (a) to determine the ploidy of progenies of 2x – 2x, 2x – 4x and 4x – 2x crosses, and (b) to assess the agronomic characteristics of diploid and triploid progenies recovered from the same or similar crosses. The breeding population involved progenies of interploidy crosses field established in a randomized complete block design (RCBD) with three replications. Ploidy was determined using Flow cytometry analysis of nuclear DNA content and chloroplast characteristics. Results indicated that progenies of 2x–2x crosses were predominantly diploid (99.7%), those of 2x–4x crosses were mainly diploid (96.2%), while the 4x–2x crosses produced predominantly triploid progenies (94.1%). Very highly significant differences (P<0.001) among and within ploidy groups were observed for mean number of chloroplasts and the most frequent number of chloroplasts in stomatal guard cells of progenies. Diploid hybrids recovered from crosses had shorter days to flowering, plant height and bunch weight compared to triploid hybrids.**

**Key words:** Genetic improvement, *Musa* species, ploidy, unilateral sexual polyploidization.

## INTRODUCTION

Plantains and bananas (*Musa* spp.) are giant perennial herbs that are cultivated in the tropical and sub-tropical regions of the world where they serve as staple and cash crops. The genus *Musa* has 22 chromosomes in wild species and 22, 33 and 44 chromosomes in cultivated forms, with a basic haploid number of 11 chromosomes. Majority of cultivated varieties (Simmonds and Shepherd, 1955; Simmonds, 1995a) are triploid (2n=3x=33), that are derived from intra-specific crosses within *M. acuminata* Colla (A genome) and inter-specific crosses between *M. acuminata* and *M. balbisiana* Colla (B genome). The remainder are mostly diploid, while tetraploid clones are naturally rare.

A major problem of plantain and banana landraces is their susceptibility to black Sigatoka, a defoliating fungal disease caused by *Mycosphaerella fijiensis,* Morelet (Wilson and Buddenhagen, 1986). If unchecked, this disease induces leaf decay thereby reducing the photosynthetic area, and causing a reduction in yield (Mobambo et al., 1993). Chemical control of the disease is costly and hazardous to the environment, thus genetic improvement through host plant resistance becomes the most practical option for sustainable control of the disease. The genetic improvement of plantains and bananas is therefore aimed at developing triploid hybrids that are disease resistant and with sufficient desirable characteristics of the landraces.

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Breeding for resistance to *M. fijiensis* at the International Institute of Tropical Agriculture (IITA) attempts to mimic the evolutionary development of the *Musa* species complex (Rowe and Rosales, 1996; Ortiz, 1997) from inter-specific hybridisation and polyploidy-zation involving *Musa acuminata* Colla and *M. balbisiana* Colla. Thus, female fertile triploid landraces of plantain (AAB genomes) are crossed to diploid accessions of *M. acuminata* or *M. balbisiana* that are resistant to black Sigatoka. Euploid and anueploid progenies are produced from the 3x-2x crosses. Among these, agronomically desirable and disease-resistant diploid and tetraploid hybrids are selected and intermated via 4x-2x crosses, in an attempt to produce high yielding 3x hybrids.

The above process is slow due to limited under-standing of the genetic organization and meiotic behaviour of the species (Ortiz and Vuylsteke, 1996). For example, variation in genome size (ploidy) and structure is observed across and within generations (Tenkouano et al., 1998a) making it difficult to predict the products of crosses. This implies that the 4x–2x crosses may also give rise to progenies of multiple ploidy levels, and the ploidy of individual plants determines to a great extent their performances in field trials. Ploidy influences morphological characters of plants in *Musa* species (Tezenas du Montcel et al., 1995). It also influences some phenology and yield para-meters as plant height, bunch weight and its compo-nents. In addition, ploidy affects the generation time of an organism since the quantity of DNA influences the rate of mitosis (Bennet, 1972). It is therefore necessary to determine the ploidy status of breeding genotypes from the onset and ensure that only individuals of the desired utilization classes are field evaluated. The knowledge of the ploidy of hybrids would also be very important for its classification into genomic groups.

The ploidy status of breeding genotypes is traditionally determined by chromosome counting (Osuji et al., 1996), a technique which is laborious and not suitable for large-scale screening of segregating populations as in *Musa*. Ploidy determination may also be based on morpholo-gical criteria, although in *Musa* populations, phenotypic differences are expectedly greater within than between ploidy levels (Dessauw, 1988). Ploidy estimation by the analysis of pollen and chloroplast characteristics (Tenko-uano et al., 1998) or more accurately and rapidly by using flow cytometry analysis of nuclear DNA content (Dolezel, 1997) have been advocated. Ploidy determi-nation using chloroplast characteristics is based on the observation that chloroplast number in stomatal guard cells increases as cell size increases in response to increased ploidy state. Flow cytometry analysis of nuclear DNA content is the most accurate method of ploidy deter-mination (van Duren et al., 1996; Dolezel, 1998), especially for plant populations with genome size variations. It is convenient, rapid, does not require dividing cells, is not destructive and is capable of detecting small variation in DNA (Dolezel, 1997). The present study was to (a) determine the ploidy of progenies of 2x – 2x, 2x – 4x and 4x–2x crosses, and (b) assess the agronomic character-ristics of diploid and triploid progenies recovered from the same or similar crosses. This is necessary in order to elucidate the pattern of ploidy segregation and hence the pattern of inheritance that may be expected in *Musa* populations. Knowledge of these issues would provide a more deterministic approach to the process of improving *Musa* species and would be useful to breeders for proper manipulation of the plantain genome as well as establishing the status of new cultivars.

## MATERIALS AND METHODS

### Study site and genetic material

The study was carried out at International Institute for Tropical Agriculture ([IITA), High Rainfall Station, Onne. IITA Onne Station is located in Southeastern Nigeria (lat. 4° 43' N, long. 7° 01' E 10 m altitude above sea level) in the densely populated Niger Delta region of Rivers State. The station is located in the secondary centre of plantain diversification in the humid lowland rainforest of West Africa. Detailed characteristics of the station have been described elsewhere (Ortiz et al., 1997).

*Musa* populations used in this study were developed by IITA at the study site. Four female-fertile triploid plantain landraces (*Musa* spp., AAB group) and one male-fertile triploid banana landrace (*Musa* spp., AAA group) were crossed to 7 diploid accessions (AA) to obtain aneuploid and euploid progenies. The AAB accessions were 'Bobby Tannap', 'Obino l'Ewai', 'Ntanga' and a soma-clonal French reversion mutant of 'Agbagba', while the AAA accession was 'Padri'. The AA accessions include *M. acuminata* sub sp. burmannicoides 'Calcutta 4', *M. acuminata* subsp. *malacensis* 'Pisang lilin', 'Heva', 'Long Tavoy', 'Manang', 'Tjau Lagada' and the FHIA hybrid SH3362. Eight tetraploid (4x) and nineteen diploid (2x) progenies selected from these crosses (Vuylsteke et al., 1993a and 1993b; Vuylsteke and Ortiz, 1995) were intermated in 2x–2x, 2x–4x and 4x–2x crosses to produce secondary hybrids evaluated in this study. Hybrids and parental clones were field established in a randomized complete block design (RCBD) with 3 replications. Cultural practices were similar to those used by Swennen (1990) under alley cropping with multispecies hedgerows. Agronomic data were collected on field established hybrids.

### Flow cytometry (FCM) analysis

Leaf samples were collected from the cigar (emerging tightly rolled leaf), or youngest fully expanded leaf of field-grown plants and immediately stored in ice packs. About 50 mg of mid-rib tissue was chopped with a sharp razor blade in a petri dish with 0.5 ml ice-cold Otto 1 buffer (0.5 M citric acid monohydrate, 0.5% Tween 20) to release cell nuclei. Another 0.5 ml Otto 1 buffer was added to the suspension, which was filtered through a 50 µm nylon mesh and kept at room temperature. The suspension of released cell nuclei was stained by addition of 2 ml Otto 11 buffer (0.4 M anhydrous $Na_2HPO_4$) containing 4 µg $ml^{-1}$ DAPI (4-6-diamidino-2-phenylindole).

Fluorescence detection was carried out with a Partec PAS 11 flow cytometer (Partec GmbH, Germany) whereby relative fluorescence intensities were translated into histograms corresponding to the relative DNA content, hence ploidy status, of

**Table 1.** Ploidy frequency and percentage composition of progenies from 2x – 2x, 2x – 4x and 4x – 2x crosses in *Musa* populations.

| Ploidy of hybrids | 2x - 2x | | 2x - 4x | | 4x - 2x | |
|---|---|---|---|---|---|---|
| | Number recovered | % | Number recovered | % | Number recovered | % |
| 2x | 1234 | 99.7 | 332 | 96.2 | 17 | 3.0 |
| 3x | 4 | 0.3 | 13 | 3.8 | 542 | 94.1 |
| 4x | 0 | 0 | 0 | 0 | 15 | 2.6 |
| 5x | 0 | 0 | 0 | 0 | 2 | 0.3 |
| **Total** | **1238** | **-** | **345** | **-** | **576** | **-** |

**Table 2.** Analysis of variance on the effect of ploidy on number of chloroplast in stomatal guard cells in *Musa* populations.

| Source | DF | Frequency of chloroplasts per stomatal guard cell pair | |
|---|---|---|---|
| | | Mean | Mode |
| Ploidy | 2 | 33554.2*** | 31971.3*** |
| Clone(Ploidy) | 120 | 1327.2*** | 1252.0*** |
| Error | 4995 | 57.0 | 74.6 |
| **Variance Components (%)** | | | |
| Ploidy | - | 56.0 | 52.5 |
| Clone(Ploidy) | - | 21.4 | 18.3 |
| Error | - | 22.6 | 29.2 |

*** indicates significant F-test at P < 0.001.

tested samples (Dolezel, 1997). Two reference accessions of known ploidy level, 'Calcutta 4' (diploid) and 'Obino l'Ewai' (triploid), were used as internal standards and the analytical instrument was calibrated so that the $G_1$ peak of nuclei isolated from the control diploid plant was on channel 50, while that of the triploid was on channel 75 (Pillay et al., 2000). This setting was kept constant during analysis of samples prepared from the breeding population to compare their peak or histogram to that of the reference plants. Thus, peaks appearing on channels 50, 75 and 100 corresponded to diploid, triploid and tetraploid plants, respectively (Pillay et al., 2000). Peak records were used to construct the frequency distribution of ploidy classes for 2x–2x, 2x-4x and 4x-2x families.

#### Determination of chloroplast density in stomatal guard cells

The chloroplast density in stomatal guard cells of breeding genotypes was determined using the procedure of Compton et al. (1996) with minor modifications. Leaf samples were collected from the second fully expanded leaf. Three sections evenly distributed along the middle part of the leaves were made. The lower epidermis of the sections were removed with forceps, transferred to a microscope slide and immersed in one drop of 2% iodine solution. The preparation was covered with a cover glass and allowed to stain for 5 min. All slides were observed under bright field illumination using a Leitz Diaplan binocular microscope at x 400 magnifications. Ploidy was estimated by counting the number of chloroplast per guard cell pair for 10 stomata of each of the three leaf sections giving a total of 30 cell pairs per plant. The total number of chloroplast, mean and mode were recorded and analysed.

## RESULTS

The results of the FCM analysis showed the frequency of individuals of different ploidy classes recovered from crosses (Table 1). In 2x–2x crosses, progenies were predominantly 2x (99.7%), while very few (0.3%) were 3x. Similarly, individuals recovered from 2x–4x were mainly 2x (96.2%), while 3.8% of the progenies were 3x. The 4x-2x crosses produced predominantly 3x progenies (94.1%). About 3.0% of hybrids were 2x, 2.4% were 4x, while 0.3% were 5x (pentaploid).

The results of the analysis of variance on the mean number of chloroplasts and the most frequent number of chloroplasts in stomatal guard cells of progenies revealed very highly significant differences (P<0.001) among and within ploidy groups (Table 2). The partitioning of variation in the data set into its components using the REML option in SAS showed a preponderance of interploidy than intraploidy differences among the progenies for all the parameters studied. Interploidy differences accounted for more than 52% of total variation for all the parameters. In all, more than 70% of the total variation in the data set was due to genetic effects.

A consistent trend was observed for the parameters measured. The mean number of chloroplasts per stomatal guard cell pair was 16.9 in 2x clones; 33.5 in 3x clones and

**Table 3.** Ploidy effects on chloroplast characteristics of *Musa* progenies from crosses involving 2x – 2x, 2x – 4x and 4x – 2x.

| Ploidy class | Number of chloroplast per stomatal guard cell pair | |
|---|---|---|
| | Mean | Mode |
| 2x | 16.9 | 16.4 |
| 3x | 33.5 | 32.3 |
| 4x | 36.3 | 35.9 |
| LSD (0.05) | 2.1 | 2.4 |

**Table 4.** Comparative field performance of diploid progenies of 2x – 4x and triploid progenies 4x – 2x crosses grown during 1999-2001 at Onne.

| Plant characteristics | 2x - 4x (465) | 4x - 2x (353) | LSD $_{(P = 0.05)}$ |
|---|---|---|---|
| Plant height (cm) | 246.1 | 271.9 | 39.4 |
| Number of days to lowering | 237.8 | 335.1 | 65.8 |
| Bunch weight (kg) | 1.6 | 3.5 | 1.9 |
| Fruit length (cm) | 7.3 | 10.3 | 2.3 |
| Fruit circumference (cm) | 5.4 | 7.4 | 1.5 |

36.3 in 4x clones (Table 3). Similarly, the most frequent number of chloroplasts per stomatal guard cell pair was 16.4 in 2x clones, 32.3 in 3x clones and 35.9 in 4x clones. The values decreased with decrease in ploidy with the 2x clones having the lowest values. The mean number of chloroplasts in 3x and 4x individuals was 1.98 and 2.15 times greater than that of the diploids.

Analysis of the agronomic data of the field established hybrids indicated that diploid hybrids from 2x–4x crosses or 4x–2x crosses had shorter days to flowering, plant height and bunch weight compared to triploid hybrids recovered from the same or similar crosses (Table 4). The higher bunch weight of the triploids was attributed to the fruit size and not the number of fruits, which was higher in the diploids.

## DISCUSSION

The aim of breeding schemes involving 4x and 2x *Musa* hybrids is to produce 3x progenies that have superior agronomic and post-harvest characteristics. However, complex ploidy and genome arrays occur in such crosses and make it difficult to predict the products of 2x–2x, 2x–4x or 4x–2x breeding. Earlier reports on analysis of microsporogenesis in 4x and 2x breeding lines (Oselebe et al., 2001) indicates meiotic irregularities leading to the generation of gametes with differing chromosome sets. Predominantly, 1x pollen was recovered from the diploid and tetraploid male parents. This implies that crosses between 4x and 2x parents would predominantly produce 3x progenies in the 4x–2x direction and 2x progenies in the 2x–4x direction, assuming that macrosporogenesis is normal. 2x–2x crosses would also produce predominantly 2x progenies.

The present result confirmed the expected ploidy of progenies from the different crosses. 4x-2x crosses produced predominantly 3x progenies, whereas 2x–4x crosses gave predominantly 2x progenies. About 5.7% of progenies recovered from 4x–2x crosses were 2x, 4x and 5x individuals. Similarly, 0.3% of progenies recovered from 2x–2x crosses were triploids.

Ploidy polymorphism may be attributed to abnormal meiosis (Asker, 1980) which leads to the formation of 2n gametes due to failure of first or second meiotic division (Vuylsteke et al., 1993a). This explains the generation of 3x hybrids via 2x-2x crosses, especially where one of the 2x parents produces 2n pollen (Oselebe et al, 2001), or the generation of 4x hybrids from 4x–2x crosses. This process is termed unilateral sexual polyploidization (USP). Similarly, selective affinity of the chromosomes of the 'A' and 'B' genome may lead to ploidy polymorphism in hybrids. Highly divergent chromosomes are indicative of little pairing at meiosis resulting in the formation of univalents (A or B), bivalents (AA), trivalents (AAA) or quadrivalents at meiosis. Disjunction and cytokinesis in this case results in the formation of a range of possible gametes (monoploid, haploid, triploid etc) and thus multi-ploidy progenies in crosses. This submission is further strengthened by the report of Ude et al. (2002) that identified three putative genetic subspecies of *M. acuminata*, while *M. balbisiana* contained two forms. On assumption that there are A1, A2 and A3 genomes, which may be totally divergent, several outcomes should be possible.

Ploidy polymorphism in progenies of 2x–4x crosses is attributed to complex microsporogenesis in the 4x parent

leading to the formation of gametes with varying chromosome constitutions. Predominant diploid hybrids may have resulted from double reduction in the 4x parent, or a differential survival rate of unbalanced pollen (i.e. 1x gamete) against 2x gametes in the tetraploid pollen donor (Oselebe et al., in Press). This result highlights the importance of microsporogenesis in the determination of ploidy of progenies from interploidy crosses in *Musa*. The choice of male parents is therefore, very critical in the genetic improvement of the species and may determine the crossing strategy to obtain progenies of the desired ploidy level. It further clarifies the appropriate crosses to be made in triploid breeding: 4x–2x instead of 2x–4x crosses.

The result obtained from the count of the number of chloroplast in stomatal guard cell pairs further strengthened the FCM result on ploidy variation of progenies derived from crosses. Significant differences among and within ploidy groups were recorded among the progenies with a preponderance of interploidy, rather than intraploidy differences. Since 70% of the total variation in the data set was due to genetic effects, it implies that chloroplasts number per stomatal guard cell pair might determine ploidy status of breeding genotypes in *Musa* populations. A consistent trend was observed for all the parameters measured, with the mean number of chloroplasts per stomatal guard cell pair decreasing with decrease in ploidy status. This conforms to the submission of Tenkouano et al. (1998b) that the number of chloroplasts in guard cells should increase proportionally with increase in sizes of cells in response to increase in ploidy. The mean number of chloroplasts in 3x and 4x individuals was 1.98 and 2.15 times greater than that of the diploids. However, a major difficulty was in the determination of ploidy of individual plants, since the result was population dependent.

The diploid and triploid hybrids from 2x–4x and 4x–2x crosses had agronomic characters that were related to their ploidy level, suggesting that ploidy determined the inheritance of quantitative characters in *Musa.* Diploid progenies had lower number of days to flowering but with smaller bunches compared to triploid progenies. The higher bunch weight of the triploids was attributed to higher values for fruit length and fruit circumference and not the number of hands. Thus, higher order epistatic interactions may be quite important in determining the actual performance of hybrids.

In conclusion, *Musa* breeders have made considerable progress in breeding for disease resistance and improved agronomic qualities. Improved tetraploid (4x) and diploid (2x) cultivars with disease resistance have been developed which are crossed to produce secondary 3x hybrids. Further progress in *Musa* breeding is dependent on understanding the pattern of ploidy segregation in crosses involving 4x and 2x individuals aimed at generating 3x hybrids. The knowledge of this issue will be fundamental to designing appropriate crosses that would lead to the recovery of hybrids of the desired ploidy level. The critical role of male parents (i.e. 2x or 4x lines) in the genetic improvement of *Musa* species is highlighted, thus clarifying the appropriate crosses to be made in triploid breeding, 4x–2x crosses instead of 2x–4x crosses. An alternative includes the generation of 3x hybrid using 2n gamete in 2x–2x crosses via unilateral sexual polyploidization.

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