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# Evaluation of the Polycross Mating Technique in Producing Musa Seed to Accelerate and Decentralize Breeding

**WILSON** Victoria [1,*] and **TENKOUANO** Abdou [2]

[1] Department of Plant Science and Biotechnology, Rivers State University, Port Harcourt, Rivers State, Nigeria; *victoria.wilson@ust.edu.ng.; victoriawilson.2005@gmail.com*

[2] Executive Director of CORAF /West and Central African Council for Agricultural Research and Development, Dakar Senegal

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*Correspondence*:

***Website:***
*victoria.wilson@ust.edu.ng.;*

***E-mail:***
*victoriawilson.2005@gmail.com*

***Phone:***
*+234 807 338 5776*
*+234 806 483 3492*

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## Abstract

This study was conducted at the International Institute of Tropical Agriculture, High Rainfall Station, Onne, Rivers State, Nigeria. The objective was to produce viable triploid Musa seeds that can be easily distributed to researchers and farmers to accelerate and decentralize Musa breeding in developing economies using the polycross mating technique. Four 4x females, TMP4x 1658-4, TMP4x 7152-2, TMP4x 2796-5 and TMP4x 5511-2, a diploid male plantain TMP2x 2829-62 and 2 diploid male bananas TMB2x 5105-1 and SH 3362 were cultivated in two polycross blocks of 4x - 2x to yield the desired 3x seeds. The RCB experimental design was replicated twice. Data, comprising total number of bunches and seeds obtained in 3 crop cycles, were analyzed by ANOVA at P = 0.05 and means assessed using LSD at P = 0.05. Ploidy status of the offspring was determined by flow cytometry. Cumulatively and significantly, TMP4x 2796-5 yielded the highest number of seeds, followed by TMP4x 1658-4 with TMP4X 7152-2 yielding the least. Ratoon crop 1 produced the highest number of seeds, followed by the plant crop. Ratoon crop 2 had the fewest seeds but all its offspring were triploids. Overall, of the 5,795 well-formed seeds, 4,997 (86%) were triploids. This study showed the valuable contribution in the use of a 4x – 2x polycross mating technique for producing viable 3x seeds in Musa. Seed production schemes should be established in Musa producing countries using this mating technique to distribute Musa seeds to researchers and farmers to accelerate and decentralize breeding.

**Keywords:** Accelerating and Decentralizing Musa Breeding, Polycross Mating Technique; Ploidy Status; Tetraploids; Triploids; Diploids

## INTRODUCTION

Plantains and bananas (*Musa* spp.) are one of the most important crops representing the staple food and source of income of many small holder farmers and homestead gardeners in several developing economies of the tropics and are cultivated in over 135 countries (Brown *et al,*. 2017). Scientists and farmers in many of these developing economies grow them by vegetative propagation using suckers, slips, corms, etc. (Wilson and Tenkouano, 2019a) rather than seeds as in cereals like rice and maize. The attendant perishability, bulkiness, transportation cost and pathogen /pest infestation in such planting materials is a challenge for *Musa* breeding and production (Almekinders *et al*., 2019). Challenges slowing genetic improvement of *Musa* include low reproductive fertility, irregular meiotic behaviour, slow propagation, the long time span between generations, large area requirement for field testing (6m$^2$ per plant) and a low rate of *in vivo* propagation, etc. (Ortiz, 2013; Ortiz and Swennen, 2014). Generally, it still takes a long time (7-15 years) by conventional breeding to release new varieties (Ceccarelli and Grando, 2007; Brown *et al.,* 2017) except when a specific disease or pest resistance is involved and breeding time is substantially shortened (Vuylsteke *et al.* 1993; Ortiz *et al*., 1997). However, for varieties that truly meet most farmers' and consumers' needs this could be up to 27-30years in the case of *Musa* (Tenkouano *et al.,* 2019). To be successful, it is essential that cross-breeding of *Musa* should produce true seeds by sexual hybridization. Currently, breeding efforts in *Musa* depend mostly on hand pollination for seed production, yet it takes about 1,000 seeds, obtained after over 1,000 hand pollinations of 200 plants (0.12 ha), to produce a particular tetraploid plantain-banana hybrid per year (Ortiz, 2013). The alternative embryo rescue and tissue culture, constitute additional labour and cost and requires specialized high-tech facilities that are unavailable or limited in many developing economies. The “Polycross” describes a pollination scheme centred on naturally occurring and unplanned mating of various genotypes requiring no emasculation or hand crossing; cultivated jointly in solitary blocks that make them land, labour, and cost-efficient and suitable for devising mating experiments (Varghese, 2015).. To accelerate breeding work, a *Musa* polycross of 4x female and 2x male parentage with improved tolerance and resistance to abiotic and biotic factors, can become an efficient seed producing system to create a process for concurrent introgression of requisite genes into any population for varietal improvement (Tenkouano, 2000). The progeny arising from this polycross mating technique is predominantly of the desired triploid ploidy level (Oselebe *et al*., 2006; Wilson *et al*., 2019). This will also promote breeding for precise adaptation using a decentralized process of selection in a specific environment (Ceccarelli and Grando, 2007). Additionally, it simultaneously boosts more efficient multi-location trials because the seeds are easy to handle and inexpensive to transport over several locations (Ortiz and De Cauwer, 1999), whilst also addressing the challenge of genotype by environment interactions when breeding for broad adaptation of varieties. Moreover, farmers will be able to join the breeding program earlier than in present research station-based programs in which they join the research near the tail-end of the program. This combines decentralized selection plus farmers' participation in a more participatory approach that could accelerate breeding and encourage better adoption and "ownership" of released varieties by farmers (Ceccarelli and Grando, 2007; Ceccarelli, 2015). Currently there are no formal or informal *Musa* seed production schemes in most *Musa* producing countries (Awah *et al*., 2009). The major purpose of this study was to show the efficacy of the polycross mating technique to produce improved viable triploid *Musa* seeds and contribute to the development of an efficient scheme for accelerating and decentralizing *Musa* breeding in developing economies with limited access to the expensive embryo rescue and tissue culture facilities. Benefits include promoting early on-farm participation and "ownership" by farmers in evaluating improved varieties in their environment as well as easy seed multiplication and distribution.

## MATERIALS AND METHODS

**Study area:** The experiment was conducted at the International Institute of Tropical Agriculture (IITA) Onne station Rivers State, Nigeria (4°51'N, 7° 03'E, 10m above sea level). At the experimental site the soil is loamy and siliceous iso-hyperthermic Typic Paleudult with a pH of 4.6. Weather conditions during the cropping cycles recorded an annual average rainfall of 2400mm and mean minimum and maximum relative humidity values of 78% and 89%. The mean annual minimum and maximum temperatures were 25 ºC and 27 ºC respectively, while solar radiation was an average of 4 hours daily

**Experimental Materials**: Four female tetraploid plantain hybrids TMP4x 2796-5, TMP4x 5511-2, TMP4x 7152-2 and TMP4x 1658-4 served as seed parents and the three diploid hybrid males or pollen parents were the plantain-derived diploid hybrid TMP2x 2829-62, and two banana hybrids TMB2x 5105-1 and SH 3362.

**Experimental Design, Treatments and Field Layout**: The experiment was laid out in a randomized complete block design (RCBD) with 2 replicates (Gomez and Gomez 1984; Ortiz and Vuylsteke, 1995; Ortiz 1998; Atanda *et al*.; 2015) in two polycross blocks (PCB1 and PCB2). One of the polycross blocks covering 620m$^2$ was located in the southern and the other in the eastern extremities of the station, isolated from all plantain and banana fields by a distance of 200m and 270m respectively to prevent unwanted pollen from contaminating the plants. The polycross blocks were further surrounded by triploid plantain cultivars (Obino l'ewai and Bobby Tannap). Two-month old seedlings of experimental materials raised in the nursery were transplanted from the nursery to the polycross blocks. The PCB1 consisted of two primary tetraploid plantain hybrids TMP4x 2796-5 and TMP4x 5511-2 as female parents, while PCB2 comprised TMP4x 1658-4 and TMP4x 7152-2 also as female parents, replicated at 12 plants per clone. Each polycross block had 31 plants of each of the three male parents. Plant spacing was 3m x 2m with the 4x female and 2x male parents positioned in the field such that eight male plants surrounded each and every female plant to enhance floral synchrony and increase the chances of pollination between male and female plants. After the plant crop was established, the next 2 followers or daughter plants were selected to continue the next cycle of production as ratoons 1 and 2 and other suckers were thinned out. The plants were grown for three consecutive crop cycles - plant crop, first ratoon (ratoon 1) and second ratoon (ratoon 2).

**Bunch Harvest and Seed Extraction**: After fruit maturity, bunches from each of the 12 female plants were harvested, ripened and seeds extracted followed by counting of the well-formed hard seeds to determine seed yield for each female parent, the total number of seeds obtained in each crop cycle and the cumulative total over the three crop cycles..

**Ploidy Analysis of Offspring***:* Seeds extracted from the four female tetraploid parents were germinated in seed trays and later planted in perforated nursery bags containing topsoil and poultry manure in a ratio of 7:1 and grown in the screen house nursery for 3 months. At 3 months, ploidy status of offspring from the 4x - 2x polycross mating technique was ascertained using flow cytometry method to find out the proportion of diploids, triploids, and tetraploids, etc., in each crop cycle. Leaf samples were taken from the cigar leaf (emerging tightly rolled candle leaf) or youngest fully expanded leaf and stored immediately in ice-packs. To routinely separate the nuclei, 70 mg of midrib tissue of each offspring was sliced into bits using a sharp razor blade into a glass petri dish containing 1mL of ice cold Otto I buffer (0.5 M citric acid monohydrate, 0.5%Tween–20). An extra 0.5 mL of the Otto I buffer was added and the suspension mixed and filtered through a 50 µm nylon mesh and stored at room temperature. The suspension of released cell nuclei was stained by the addition of 2mL Otto II buffer (0.4 M anhydrous Na2HPO4) containing 4 *µ*g mL-1 DAPI (4-6- diamidino-2-phenylindole). Fluorescence detection was carried out with a Partec Ploidy Analyzer PA-II (Partec GmbH, Münster, Germany), and the relative fluorescence intensities were translated into histograms corresponding to the relative DNA content, showing the ploidy status of each sample. To establish the ploidy level of the offspring internal standards as controls were included. These comprised three reference *Musa* species of known ploidy levels - 'Calcutta 4' (diploid banana), 'Obino l'Ewai' (triploid plantain) and a hybrid tetraploid plantain were used as internal standards with the analytical instrument calibrated so that the G1 peak records of nuclei isolated from the controls were as follows: the diploid plant was on channel 50, the triploid was on channel 75 and the tetraploid was on channel 100. The setting was kept constant during analyses of samples prepared from the offspring to compare their peak or histogram to that of the control reference plants. Thus, peaks appearing on various channels corresponded to diploid, triploid, tetraploid and pentaploid offspring respectively. Peak records were used to construct the frequency distribution of ploidy status of the offspring. In addition, when in doubt, chromosome counts were also used to confirm ploidy status of any offspring (Dolezel *et al.*, 1997; Karamura *et al.*, 2016)

**Data Collection and Statistical Analyses***:* The following data were collected in each crop cycle (i) the number of bunches produced by each of the 12 females were harvested at maturity and counted (ii) the number of seeds produced by each female were extracted from the mature and ripened bunches and counted (iii) the ploidy status of the offspring were ascertained as described earlier by flow cytometry method. All data collected were evaluated by ANOVA at ($P = 0.05$) using the SAS (Statistical Analyses Software) version 9.3 to assess significant differences. Where F-test was significant the means were compared by LSD at ($P = 0.05$).

## RESULTS

### Bunch Yield

Generally, all females produced more bunches in the second crop cycle (ratoon crop 1) and next highest in the third crop cycle (ratoon crop 2) with the exception of TMP4x 7152-2 which had its highest bunch yield in the first crop cycle (plant crop) and TMP4X 1658-4 having its highest bunch yields in ratoon crops 1 and 2 as shown in Fig. 1. Cumulatively over the 3 cropping seasons, TMP4x 5511-2 produced significantly ($P = 0.05$) the highest number of bunches than all other females, whereas TMP4x 7152-2 produced significantly ($P = 0.05$) the lowest number of bunches.

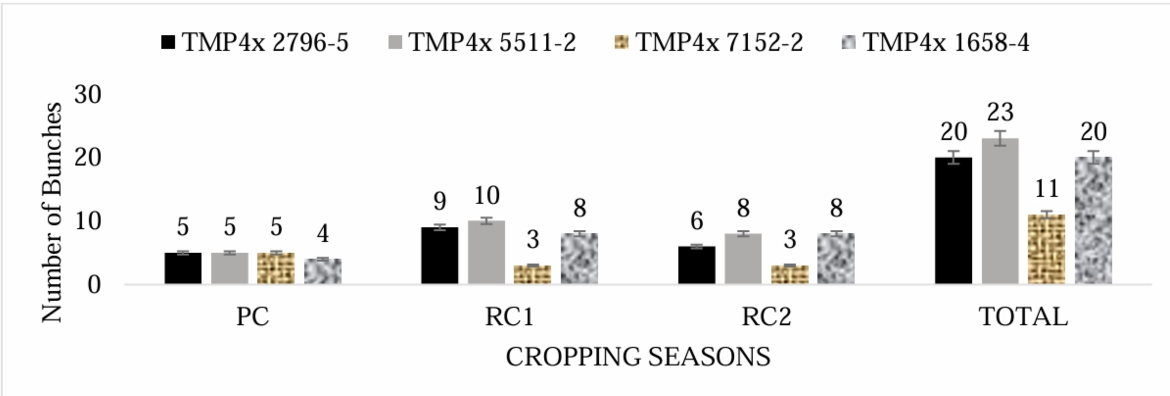


Fig. 1. Bunch yield by the four female (4x) parents in the polycross over three cropping seasons: plant crop (PC), ratoon crop 1 (RC1), ratoon crop 2 (RC2)

**Seed Production**

There were significant differences ($P = 0.05$) in seed production in each crop cycle (Fig. 2). In the plant crop, total number of seeds produced was 1,729. In ratoon crop 1, 3,272 seeds were produced which was significantly higher than those produced in the other crop cycles. The least number of seeds was produced in ratoon crop 2 amounting to only 794 seeds.

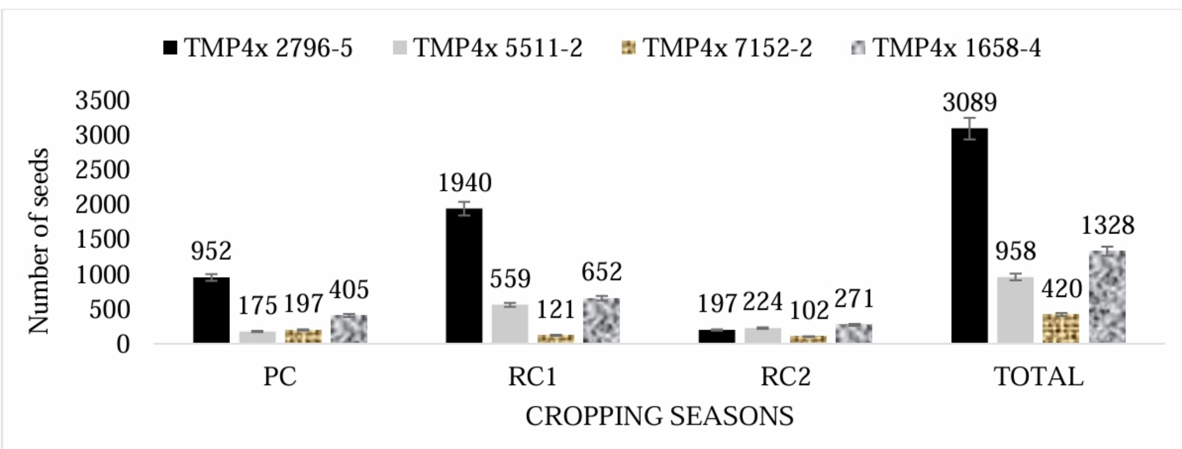


Fig. 2. Seed production by four female (4x) parents in the polycross over three cropping seasons: plant crop (PC), ratoon crop 1 (RC1), ratoon crop 2 (RC2)

All females produced the highest quantity of seeds in the second crop cycle (ratoon crop 1) with the exception of TMP4x 1658-4 which produced its highest quantity of seeds in the first crop cycle (plant crop). Cumulatively, over the 3 crop cycles, TMP4x 2796-5 produced 3,089 seeds; a significantly ($P = 0.05$) higher quantity of seeds than other females, with TMP4x 1658-4 producing the second highest (1,328), whereas TMP4x 7152-2 produced the lowest number of seeds amounting to only 420.

**Ploidy status and types of offspring produced**

**in each crop cycle**

Flow cytometry analysis (FCM) revealed four ploidy levels (2x, 3x, 4x and 5x) among the polycross offspring in each crop cycle. The ploidy status and types of offspring in each crop cycle is presented in Table 1. The frequency of triploids produced increased from the plant crop (44%) to the first ratoon crop (76.3%) to exclusively triploids in the second ratoon crop (100%).

**Table 1**. Relative frequency of p loidy types among offspring from the polycross in each crop cycle

| Cropping Cycle | Relative frequency (%) of ploidy types among offspring from polycross | | | |
|---|---|---|---|---|
| | 2x | 3x | 4x | 5x |
| Plant crop | 28.0±0.32 * | 44.0±1.31 | 28.0±0.70 | 0.0±0.00 |
| Ratoon crop 1 | 22.7±0.46 | 76.3±1.01 | 0.0±0.00 | 1.0±0.01 |
| Ratoon crop 2 | 0.0±0.00 | 100±1.25 | 0.0±0.00 | 0.0±0.00 |
| LSD $_{0.05}$ | 20.3 | 22.9 | 18.6 | ns |

*± = S.E; ns =not significant at P=0.05*

**Ploidy Status / types of offspring from female parents across the three crop cycles**

The cumulative ploidy status/ types among the offspring across the crop cycles is presented in Table 2, the frequency of which differed with each female. Offspring from the female tetraploid TMP4x 7152-2 were 100% triploids. Offspring from the female tetraploid TMP4x 2796-5 were 93.5% triploids with 6.5% as diploids. Offspring from the female tetraploid TMP4x 5511-2 were 61.5% triploids with 38.5% as diploids. Finally, offspring from the female tetraploid TMP4x 1658-4 had all four ploidy levels 82.8% triploids, 8.6% diploids, 5.7% tetraploids and 2.9% pentaploids. The average number of triploids produced made up 84.5% of the various ploidy types in the polycross, while diploids made up 13.4%. Tetraploid and pentaploid offspring were only produced by TMP4x 1658-4 and constituted only an insignificant 1.4% and 0.7% respectively of the ploidy types.

**Table 2.** Relative frequency of ploidy status / types of offspring in each of the four tetraploid females in the 4x - 2x polycross mating scheme averaged over the 3 cropping cycles

| Tetraploid Female parents | Relative frequency (%) of ploidy types of offspring from polycross | | | |
|---|---|---|---|---|
| | 2x | 3x | 4x | 5x |
| TMP4x 2796-5 | 6.5±0.87 | 93.5±1.53 | 0.0±0.00 | 0.0±0.00 |
| TMP4x 5511-2 | 38.5±1.96 | 61.5±1.41 | 0.0±0.00 | 0.0±0.00 |
| TMP4x 7152-2 | 0.0±0.00 | 100±1.93 | 0.0±0.00 | 0.0±0.00 |
| TMP4x 1658-4 | 8.6±0.69 | 82.8±2.32 | 5.7±0.16 | 2.9±0.09 |
| LSD $_{0.05}$ | 19.27 | 16.86 | ns | ns |

*± = S.E; ns =not significant at P=0.05*

**DISCUSSION**

Cumulatively, TMP4x 7152-2 produced significantly the lowest number of seeds even when it produced the same number of bunches as other females in the plant crop; confirming a similar earlier report by Ortiz and Crouch (1997). Ironically its entire offspring though few were triploids, the ploidy status most desired (Tushemereirwe *et al.*. 2015). Interestingly although TMP4x 5511-2 produced the highest number of bunches cumulatively, its seed production was significantly lower than those of TMP4x 2796-5 and TMP4x 1658-4. These results would indicate that there is a genotypic influence and considerable cycle to cycle variation in flowering phenology and seed production of *Musa* spp. that needs to be further explored in order to maximize any positive benefits arising from this. With respect to flowering, when flowering among different clones is synchronized, it helps to accomplish random mating also known as panmixis thereby ensuring first-rate output of seeds (Wilson *et al.,* 2020), but attention must be paid to synchronizing flowering with the periods when pollinators are foraging as an important strategy for maximizing seed production (Elzinga *et al.,* 2007; Rafferty and Ives, 2012). The variation in the bunch and ultimately seed production by the females across the crop cycles may be related not only to genotypic influence but also to the variation in the number of female and male plants that exhibited floral synchrony and overlap in each crop cycle with the activity of pollinators. The number of seeds produced provides a measure of reproductive success (Wilson *et al.,* 2020). A paramount constraint to the genetic improvement of *Musa* spp. is the limited production of viable seeds (Brown *et al.,* 2017). Ortiz and Crouch (1997) had reported long ago that open-pollinated plants produced a greater quantity of seeds having healthier embryos than hand-pollinated plants. Others have stated, that open and hand pollination methods did not produce significant differences, nor adverse effects or serious decrease in performance of yield and yield attributes of resulting 4x - 2x progeny of similar genotype (Wilson and Tenkouano, 2019b). Evidently, therefore use of a polycross mating technique using 4x females and 2x males for seed production will decrease labour, cost, time and stress and other costs associated in *Musa* breeding and development. With respect to the ploidy status of offspring, although offspring from TMP4x 7152-2 were (100%) triploid, it produced only 14% of the total number of seeds obtained from TMP4x 2796-5 the highest producer of seeds. Overall, approximately 5,000 triploid seeds were produced from this polycross mating technique. Triploid seeds produced can be easily distributed by national agricultural stations in developing economies to researchers to conduct on-station and on-farm demonstration trials; and to farmers directly enabling farmers also to make their own selections in their own environment (Nakagonge *et al.*, 2007), thereby accelerating and decentralizing *Musa* breeding. This study showed the valuable contribution in the use of the 4x – 2x polycross mating technique for the production of viable 3x seeds in *Musa.* Such seeds can be widely distributed for planting *ex-vitro* in the soil directly to benefit farmers and researchers thereby reducing dependency on *in-vitro* germination and embryo rescue by tissue culture. This study will help researchers to uncover other critical areas of use of polycross mating technique in *Musa* breeding that many researchers were not able to explore in order to further accelerate and decentralize *Musa* breeding.

## CONCLUSION

The polycross mating technique in *Musa* showed that the offspring of the four female tetraploid parents were predominantly triploids. Although lower frequencies of 2x, 4x and 5x progenies were observed in the plant and first ratoon crops, the second ratoon crop had only triploids. The production of a high frequency of triploid offspring from three out of the four females across the three crop cycles in this study suggests that the use of the polycross mating technique is a viable option for seed production to accelerate and decentralize *Musa* breeding. There are no formal

*Musa* seed production schemes in most *Musa* producing countries and this study indicates that such prospects could be initiated.

**Author Contributions**: This work was carried out in collaboration between both authors. Author AT designed the study. Author VW carried out the field work, collected and analyzed data and developed the manuscript.

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**Conflicts of Interest**: Authors have declared that no competing interests exist.

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