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Polleniferous and fertility assay of USTPx plantain (*Musa* sp.) hybrids

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Conventional plant breeding is the development or improvement of cultivars using conservative tools for manipulating plant genome within the natural genetic boundaries of the species. This is aimed at producing plants that are more superior, in terms of quantity and quality, to the pre-existing ones. However, the success of conventional plant breeding depends completely on the fertility status of the two parent plants involved in the hybridization program. The genetic breeding of plantain and banana through hybridization is limited by the occurrence of sterility in most cultivars. This results in low production or complete absence of seeds. Seed set after pollination is an indication of fertility of both male and female parents which can be used to develop hybrid seeds of commercial varieties and wild relatives. The main objective of the present research was to identify the fertility status of seven (7) plantain hybrids which include USTPx/01/01, USTPx/01/02, USTPx/01/03, USTPx/01/05, USTPx/01/06, USTPx/01/07 and KM5 using *ex-situ* and *in-situ* method of screening for fertility. Staining of pollens from the different species was made using 1% acetocarmine glycerol jelly solution. Different interspecific crosses were made. Result from both *in-situ* and *ex-situ* method indicates that six (6) species were fertile apart from USTPx/01/07 which does not produce seeds and pollen grains. The highest value of seed set, percentage fertility and mean seed set were obtained from USTPx/01/01 and USTPx/01/02 as male and female parent with 1076 seeds, 61.6% seed set and mean of 107.6 and 1346 seeds, 77.0% seed set and mean of 134.6 respectively. The highest value of mean and pollen grain percentage fertility from the *ex-situ* studies were obtained from USTPx/01/01 with a mean of 19.0 and 95% fertility.

Keywords: *Ex-situ*, Plantain, *In-situ*, Hybrid, Fertility

INTRODUCTION

Plantains and bananas (*Musa* sp.) are perennials which grow to produce generations of ratoon crops. They represent the world's second largest fruit crop with an annual production of 129,906,098 metric tons. They rank as the fourth most important global food commodity after rice, wheat and maize in terms of gross value of production. They originated from South East Asia. Plantain and Banana are reliable sources of energy and fibre; they store carbohydrate reserves in the form of starch and sugar respectively. Bananas provide a starchy staple across some of the poorest parts of the world in Africa (with consumption up to 400 kg per person per year) and Asia, while dessert bananas are a major cash crop in many countries (FAO Stat, 2007).

Cultivated bananas and plantains are giant herbaceous plants within the genus *Musa*. Bananas (including plantains) grown today worldwide are cultivars derived from the interspecific hybridization of *Musa acuminate* and *Musa balbisiana*. There are over a thousand *Musa* cultivars embracing a wide genetic diversity, which reveal their multiple origins through hybridization from these two ancestral diploid species (Heslop Harrison and Schwarzacher, 2007). Inter and intra-specific hybridizations have led to parthenocarpic diploid and triploid cultivars. However, the difficulty in generating hybrids and the sterility of many cultivars showing parthenocarpy makes *Musa* cross-breeding challenging. The main objective for the genetic improvement of banana is to breed sterile triploid hybrids through the recombination of fertile cultivars and species that meet farmers' needs and consumers' demand (Ortiz and Vuylsteke, 1996). Although sterility and parthenocarpy are important factors that contribute to the desirability of banana fruits, sterility has impeded progress in breeding programs. Through natural somatic (vegetative) mutation, hybridization and selection over many thousands of years, considerable genetic variability have arisen within the cultivated bananas, giving rise to more than 1000 varieties worldwide.

Musa breeding started about one century ago in Trinidad, aiming to develop pest and disease resistant cultivars for the export trade. Even so, all banana export cultivars grown today are still selections from somatic mutants of the group Cavendish and have a very narrow genetic base (Perrier *et al*., 2011). As noted by Dirk R. Vuylsteke "a broad-based, improved *Musa* germplasm with pest/disease resistance will be a major component to achieve sustainable production of this vegetatively propagated, perennial crop." Such germplasm can be produced through conventional cross-breeding, enhanced by the utilization of innovative methods for the introduction of additional genetic variation. Also, the increased use of molecular markers will accelerate the process of recurrent selection of improved *Musa* germplasm and, hence, facilitate the development of new hybrids. The prospects of banana and plantain breeding are unlimited and increased effort would initiate a new phase of *Musa* evolution" (Ortiz, 2001). This research is an attempt to identify the fertility status of some indigenous plantain hybrids.

MATERIALS AND METHODS

The experiment was conducted in the Teaching and Research Farm of the Rivers State University, Port Harcourt. Rivers State has a landmass of 19420 sq.km, and lies within tropical rainforest zone of Nigeria, located in latitude 4°-6°N and longitude 6°-8°E. The project site (University Farm) is located on latitude 4.5°N and longitude 7.01°E with an elevation of 1.8m above sea level. The rainfall pattern is essentially bimodal with peaks in June and September, while in April and August there are periods of lower precipitation. The long rainy season is between April and October. The dry season lasts from November to March with occasional interruption by sporadic down pours. Annual rainfall is an average of 2000mm to 4500mm. The mean monthly temperature ranges between 28℃ and 33℃ while the annual monthly minimum is between 20℃ and 23℃. The highest temperatures are experienced during the months of December through March and coincide with the overhead passage of sun.

The *Musa* accessions used for the experiment were seven (7) plantain hybrids. These putative plantain and banana hybrids of the USTPx series developed by the Department of Crop/Soil Science of Rivers State University, represent Du-novo genetic resources that could be utilized in any breeding and genetic research (Davids, 2001, Orluchukwu and Ogburia, 2014, Ogburia, 2017); and they include USTPx/01/01, USTPx/01/02, USTPx/01/03,USTPx/01/05, USTPx/01/06,USTPx/01/07 and KM5 which were growing *in-situ*.

KM5 served as the control, and the different cultivars as the treatments. They were replicated ten (10) times in a Complete Randomized Design. Soil samples were collected and analysed for Soil pH, Total Nitrogen, Potassium and Available Phosphorus. The aim was to determine the initial nutrient status of the soil.

At maturity of the plant, i.e. 90 days plus after shooting, using a steel ladder to reach the male buds of the tall plants, pollen grains of freshly exposed male flowers were excised on a daily basis during the morning hours of 7.00- 10.00. The pollen grains were manually dislodged from the stamen, spread on a glass slide and stained with 1% acetocarmine glycerol jelly (Marks, 1954). The preparation was covered with cover slide and allowed to stand for 24 hours to allow passive uptake of stain. The slide preparations were then observed under bright-field illumination (x 40 magnification) using Leitz Diaplan Binocular Microscope. Only completely rounded and deeply stained grains were considered viable pollen.

Stainability with acetocarmine glycerol is an established method to determine pollen viability for estimating the level of male fertility for *Musa* clones (Dessauw, 1988). The diameters of fifteen randomly selected deeply stained and round grains were measured with the aid of a graduated eyepiece. Giant pollen grains having diameters >/=140um were classified as 2n pollen, since 2n pollen normally have 1.25 times the diameter of haploid or n pollen (Darlington, 1937), which never exceeds 128um in non-2n pollen producers of *Musa* (Ortiz, 1997). The most frequent (n) pollen size, the number of normal or haploid (n), diploid (2n) and giant (4n) pollen grains was recorded per specie. Percentage pollen fertility was calculated using the following formulas below;

Percentage pollen fertility = No. of deeply stained pollen / total No. of pollen x 100%.

Clones producing at least 25% viable pollen (completely rounded and deeply stained) at moderate production levels are considered sufficiently male-fertile. This assumption is based on the observation that 'Maraw', an edible banana with this level of male fertility

(UIburghs, 1994), was a functional male parent that produce true viable seeds when its pollen was used in crosses with ‘Mbi Egome’, a female-fertile triploid plantain (Vuylsteke *et al*., 1993b).

A total of 200 pollen grains were counted in ten microscopic field views. Only completely rounded and deeply stained grains were considered viable while those with irregular shape and unstained pollen grains were considered as non-viable.

Different interspecific crosses were carried out, at the end of which seeds were produced in some of the pollinated plants.

The first set of seed retrieved from the six out of the seven USTPx plantain hybrid were planted in the month of February, 2018 in the green house inside polybags of dimension 30cm by 30cm by 30cm. The bags were filled with topsoil rich in organic matter to about 3-4cm height. Watering was done early in the morning and in the evening every day.

The seeds of the cross between USTPx/01/05 and USTPx/01/01 emerged first after 3weeks of planting.

RESULTS AND DISCUSSION

Pollination

Pollination is the transfer of pollen grains from the anthers of one flower to the stigma of the same or different flower. Pollination was done at anthesis on the female flowers. Pollen from fertile flowers were brushed against the stigma of the female flowers. This is an essential process for the fertilization of the flowers and for the development of seeds. The most effective time of pollination according Simmonds (1952) is between 7.00 a.m and 10.30 a.m. Pollen grains are structures that house the male gametophyte generation of seed plants and are the vehicles through which the microsporangium is carried to the female gamete. The pollen grain has a very complex structure that may be reflective of specific species’ functional adaptation. The ability of fertilization is considered to indicate the ability of the pollen grain to perform its function of delivering the sperm nuclei to the embryo sac and final fertilization after compatible pollination. Hence the seed set is dependent on pollen viability (Oselebe *et al*., 2014).

Large inflorescence size, visual conspicuousness, floral odor and copious nectar production in Musaceae have been identified as classic examples of adaptations for cross-pollination. In conformity with Simmonds (1952), both male and female flowers of the different USTPx plantain hybrid series are nectiferous and male flowers produce sticky pollens which are dispersed by a variety of natural pollinators. In the female flowers of plants, nectaries are limited to the upper part of the ovary above the locules. In male flowers with aborted ovaries, the nectaries entirely occupy the aborted ovaries.

It was discovered that in diploids such as USTPx/01/01, the pollen grains deposited on the stigma developed pollen tubes faster than in triploids such as USTPx/01/02. Four hours after pollination, pollen grains were not visible on the stigma anymore, which indicates that germination of the pollen tube was complete. In triploids, pollen germinated on the stigma, but apparently it didn’t penetrate into the style, which suggests the lack of some sort of stimulus to direct the pollen tube, or the presence of a physical barrier.

According to Simmonds (1952), both *M. Balbisiana* and *M. Acuminate* are considerably differentiated. In these two species, pollination among closely related clones can produce significantly higher number of seeds than self-pollinated plants. Pureline crosses were also made and few seeds were obtained from such crosses, while significantly higher numbers of seeds were obtained from compatible interspecific crosses.

Stigma receptivity

Specific cell (intercellular) interactive events (interactions) are important components in pollination and fertilization processes. In a successful pollination, shortly after pollen capture, the interaction between a pollen grain and the stigma surface leads to hydration and germination of the pollen grain (Heslop-Harrison, 1975). As a result, pollen grains were collected and possible crosses were made in the morning hour which is the most receptive period of the stigma and to circumvent the eventual dehydration and death of pollen grains due to high environmental temperature.

Seed Set

During ripening and consequent squashing of the fingers, it was discovered that all hand pollinated stands produced seeds expect for USTPx/01/07, though some were slightly fertile such as USTPx/01/05, USTPx/01/06 and KM5. Among other factors, seed set in *Musa* was highly influenced by the genetic composition of the cultivar. Thus some cultivars are sterile while others are slightly fertile and, they set seeds less frequently (Stover and Simmonds, 1987).

Seed set percentages were calculated based on each cultivar using the formula: No. of seeds produced per cultivar /Total No. of seeds x 100.

Tetraploids are prone to produce viable seeds due to even chromosome number. The seeds obtained were counted and subjected to floatation test to sort out the viable seeds from the non-viable ones.

In-situ and Ex-situ **Palynological Fertility Studies**

From the pollen grain studies, it was discovered that, using acetocarmine solution as a stain on pollen grains gives more distinguished and clearer view of the pollen grains for easy identification of viable pollen grains from non-viable pollen grains than using 1% acetocarmine glycerol jelly as a stain. This clarity is more evident within few minutes after staining; though, the view may change when slides are kept for many days due to the of the reagent (acetocarmine) scorching effect.

Table 1: *In-situ* and *ex-situ* male fertility by the different cultivars

Cultivars Parameters	USTPx/01/01	USTPx/01/02	USTPx/01/03	USTPx/01/05	USTPx/01/06	USTPx/01/07	KM5	TOTAL
No. of stands pollinated	10	10	10	10	6	10	10	66
No. of Ripe Bunches	10	10	10	10	2	10	10	62
No. of Seeds Produced (Seed set)	1076	412	35	209	2	0	14	1748
% Seed Set	61.6	23.6	2	11.9	0.1	0	0.8	100
No. of Mats Producing Seeds	10	5	1	2	2	0	2	22
No. of stands/microscopic field views	10	10	10	10	10	10	10	70
No. of Pollen Grains Counted	200	200	200	200	200	0	200	1200
No. of stained/viable pollen grains	190	189	80	100	99	0	90	748
No. of giant pollen grains	160	140	55	40	42	0	60	497
No. of n pollen grains	40	60	145	160	158	0	140	703
%Fertility/viability of pollen	95	94.5	40	50	49.5	0	45	374
%2n pollen grains	80	70	27.5	20	21	0	30	248.5
%n pollen grains	20	30	72.5	80	79	0	70	351.5

The male seed set using pollen grains of the various cultivars are shown in Table 1. The highest number of seed set was obtained from using pollen grains from USTPx/01/01 with a mean of 107.6 which is completely significant from any other cultivar. It is followed by USTPx/01/02 with a mean of 41.2 and it is declared significant. There was no seed set obtained from USTPx/01/07 which evident its inherent male sterility. USTPx/01/03, KM5 and USTPx/01/06 are declared insignificant from one another, though; they are significant from the other cultivars. The minimum of seeds were retrieved from USTPx/01/06.

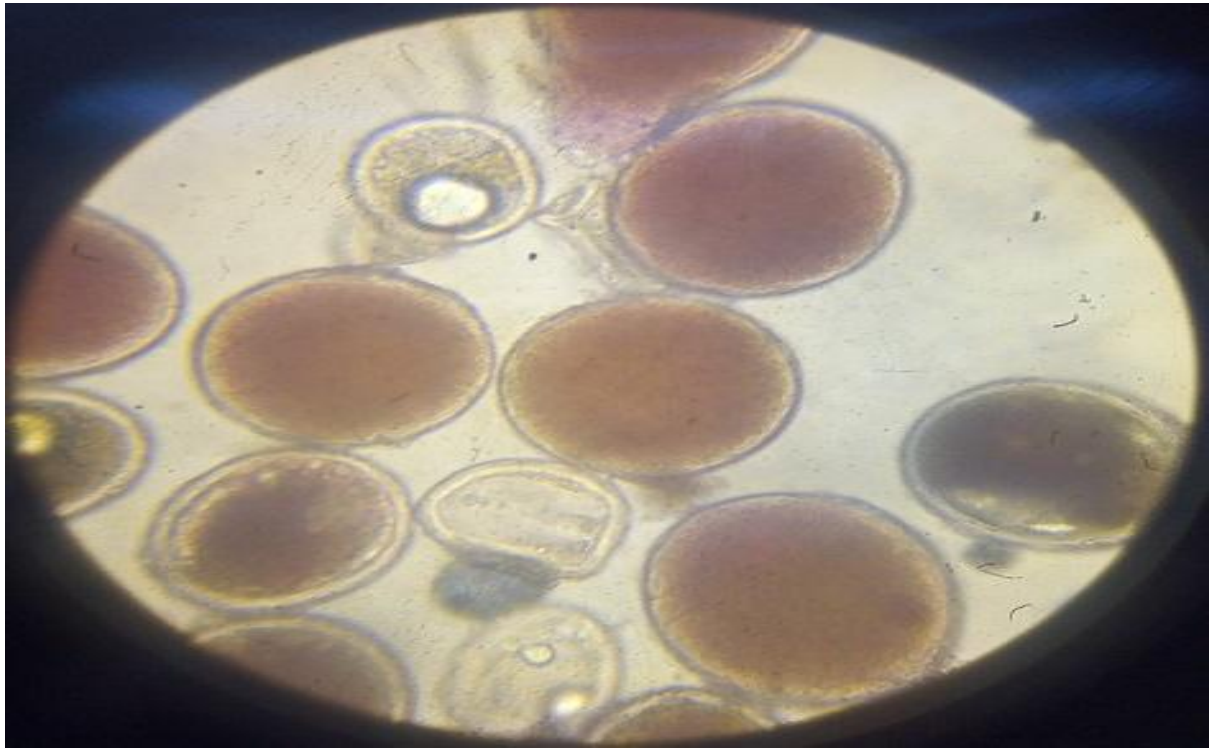
From Table 1 above, the highest percentage fertility as a male parent was obtained from USTPx/01/01 followed by USTPx/01/02. The higher the percentage fertility/viability as a male parent, the higher the seed set when crossed with fertile female parent under optimal genetic and environmental conditions and vice-versa.

Lower percentage fertility was observed in USTPx/01/03, USTPx/01/05, USTPx/01/06, and KM5.

The pollen grain fertility of the various cultivars are shown in table 1. The highest value of pollen grain fertility was obtained from USTPx/01/01 with a mean of 19.0 which is declared significant from the other cultivars except from USTPx/01/02. Amongst USTPx/01/05,USTPx/01/06, KM5 and USTPx/01/03 were declared not significant from one another, though; they are declared significant from the other cultivars. The least mean pollen grain fertility was produced by USTPx/01/03 since there is not pollen production in USTPx/01/07.

In *Musa*spp L., 2n eggs opened the path for the genetic improvement of bananas (Rowe, 1984) and plantains (Vuylsteke *et al*., 1997). Furthermore, the higher the frequency of 2n pollen, the higher the seed set when crossed with female fertile parent and vice-versa.

Figure 1: Microscopic view of the stained and unstained pollen grains of USTPx/01/01



Unstained Pollen **Stained Pollen**

Table 2: *In-situ* Female Fertility by the Different Cultivars

Cultivars / Parameters	USTPx/01/01	USTPx/01/02	USTPx/01/03	USTPx/01/05	USTPx/01/06	USTPx/01/07	KM5	TOTAL
No. of stands pollinated	10	10	10	6	6	10	10	62
No. of Ripe Bunches	10	10	10	10	2	10	10	62
No. of Seeds Produced (Seed set)	379	1346	18	1	1	0	3	1748
% Seed Set	21.6	77	1.0	0.1	0.1	0	0.2	100
No. of Mats Producing Seeds	7	7	8	1	1	0	3	27
No. of stands per cultivar	10	10	10	10	10	10	10	70

From the table 2 above, female fertility percentage (percentage seed set) differs from variety to variety using fertile male parents such as USTPx/01/01. Swennen and Vuylsteke work at IITA identified 12 female fertile French plantains, although they concluded that fertility varies from variety to variety in unpredicted manner. From Table 1 above, USTPx/01/01 and USTPx/01/02 are fertile, USTPx/01/03, USTPx/01/05, USTPx/01/06 and KM5 are slightly fertile and USTPx/01/07 is sterile. Low seed set in triploids are attributed to embryo mortality, deranged embryo endosperm relations, irregular growth of pollen tube in styles of female flower and great variations in potency existing between pollens of different cultivars. Pollens of the different cultivars were collected and used for interspecific crosses to ascertain fertility and cross compatible between and among the different species. Cultivated edible *Musa* species produce fruits through vegetative parthenocarpy in diploids like USTPx/01/01. The highest percentage seed set was obtained from USTPx/01/02 and therefore it's the most fertile female parent among the different cultivars. The higher the percentage seed set, the higher the chances of selecting such specie as a potential female parent in conventional breeding program. Plantain and Banana are monoecious plants in which the male and female reproductive organs, pollen grain and ovule respectively are found on the same plant. Female fertility is known by the ability of a given species of Plantain and Banana to produce seed after successful intra/interspecific crosses with a fertile male parent which is shown above in Table 2.

The number of seed retrieved from the various cultivars as female parent are shown in table 2. The highest seed set was obtained from USTPx/01/02 which evident its high female fertility and it is declared significant from any of the cultivars with a mean seed set of 134.6. It is followed by USTPx/01/01, though; it is not declared significant from the rest cultivars excluding USTPx/01/02. There was no seed set in USTPx/01/07 which evident its inherent female sterility.

Figure 2: Seedlings of USTPx Plantain hybrids

CONCLUSION

Conventional plant breeding approach to *Musa* is time consuming; nevertheless it is an essential part of the breeding process. The success of breeding plantain and banana should be measured by the extent to which new *Musa* cultivars are used profitably and sustainably by farmers. Access to bred germplasm of plantain and banana is therefore exceedingly vital, especially in a crop with a few active breeding programs but a great demand from farmers worldwide.

The transgenes of a desired gene (trait) in conventional breeding is made possible by selection of potentially fertile parents for the eventual introgression of the gene. The primitive wild species of *Musa* usually carry seeds and do not need pollination for hybrid seed set, but for the formation of the fruit pulp that makes its edible. While the cultivated species require pollination for hybrid seed set, and not for fruit parthenocarpy. Therefore pollination is a vital step towards production, improvement and introgression of desirable marker gene.

Both male and female sterility prevail predominantly in *Musa* clones, it is essential to screen the cultivars for male and female fertility before selecting them as potential seed parents in any hybridization programme. This is done by crossing female flowers of the selected cultivars with pollen grains of a fertile diploid male. The relatively high female fertility of plantains is a key component of the rapid breeding advances at IITA (Vuylsteke *et al*., 1997).

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