

# Female Reproductive Potential and 3x by 2x Breeding Approach for Hybrid Seeds Development in Sukali Ndizi Genotype (Musa genome AAB)

Henry Buregyeya, Naboth Oyesigye, Doreen Amumpiare, Priver B. Namanya, Kephass Nowakunda, Wilberforce K. Tushemereirwe, Eldad Karamura, and Patrick Rubaihayo

## ABSTRACT

*Sukali Ndizi* is one of the most popular local desert banana cultivars in Uganda. The crop is affected by a number of diseases and pests. Genetic improvement of the crop by hybridization may be hindered by very low seed set and poor seed germination. The main characteristics for assessing the female reproductive potential (seed set, seed quality and embryo germination of hybrid seeds) and 3x by 2x breeding approach were assessed in crosses of eleven different male diploid parents either wild or improved diploids with *Sukali Ndizi* landrace as the female parent for a period of 5 consecutive years planted in pollination blocks at National Agricultural Research Laboratories Kawanda (NARL). The month of pollination did not show any pattern throughout the pollination period but the crosses showed considerable variability in seed set, seed quality, and seed germination for different male diploids used. The water gravity test which differentiated the extracted seeds as sunken/viable and floating/nonviable seeds showed that a substantial amount of seed was floating and the sunken externally characterized by black hard integuments was only 39% with a range = 24%–60% which contained embryos, of which 22% (range = 0–37%) germinated. The increase in non-viable seed suggested that ovule abortion in *Sukali Ndizi* which mainly involved embryo and endosperm abortions was the cause of limited seed germination and the paternity of the zygotes was a major factor underlying abortions. Flow cytometric analysis of nuclear DNA content was used to estimate ploidy levels of the progenies with results showing that using 3x by 2x breeding approach differing levels of ploidy were achieved [3x (288), 4x (61), 5x (14) and 2x (2)] signifying the predominance of 3x progenies and the presence of 5x and 4x conforming to the possibility of sexual polyploids. Although pollination of *Sukali Ndizi* can be done all around the year, the seed set both quantity and quality and germination were very poor, necessitating the development of an efficient regeneration protocol for zygotic embryos at varying maturity stages to increase the recovery of hybrids.

**Keywords:** Banana, Endosperm and Embryo abortion, sexual Polyploidisation, viable seeds.

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**H. Buregyeya\***

National Banana Research Program,  
National Agricultural Research  
Organization, Uganda  
Bioversity International, Uganda  
Department of Agricultural Production,  
College of Agricultural and Environmental  
Sciences, Makerere University, Uganda

**N. Oyesigye**

National Banana Research Program,  
National Agricultural Research  
Organization, Uganda

**D. Amumpiare**

National Banana Research Program,  
National Agricultural Research  
Organization, Uganda

**P. B. Namanya**

National Banana Research Program,  
National Agricultural Research  
Organization, Uganda

**K. Nowakunda**

Food Bioscience and Agribusiness,  
National Agricultural Research  
Organization, Uganda

**W. K. Tushemereirwe**

National Banana Research Program,  
National Agricultural Research  
Organization, Uganda

**E. Karamura**

Bioversity International, Uganda

**P. Rubaihayo**

Department of Agricultural Production,  
College of Agricultural and Environmental  
Sciences, Makerere University, Uganda

**\*Corresponding Author**

## I. INTRODUCTION

Bananas (*Musa* spp.) include dessert banana, plantain and cooking banana which form the eighth most important food crop in the world and the fourth most important in least developed countries [1]. A large population (more than 50%) of Uganda depends on bananas as their main source of livelihoods [2] which are produced by rural smallholder farmers, with land holdings of about 2 ha [3]. Household surveys [2] indicate that areas where banana production was a main activity were never hit by famine and had relatively higher household incomes, making the crop one of the most important sources of livelihoods in rural areas. The continuous production of bananas all the year round offers continuous food and income, giving the producers an

advantage over those who produce annual crops. The bananas are potentially a high yielder and one of the least labor-demanding crops, *Sukali Ndizi* is one of the two most popular local desert banana cultivars in Uganda, the other one being ‘Gros Michel’ (AAA group). In terms of distribution, *Sukali Ndizi* is the most abundant cultivar, grown by about 85% of all banana farmers [4]. This cultivar is known for its small fingers with thin peels and a slightly acidic apple like flavor, meeting consumers most preferred taste in dessert banana group. Studies have shown that the demand for *Sukali Ndizi* in East Africa and Middle East markets was big, but the supply was limited [5], [6]. The production of *Sukali Ndizi* has declined over the past three decades due to pests like the banana weevil (*Cosmopolites sordidus*) [7] and burrowing

nematode (*Radopholus similis*) [8]; pathogens, of which the most important are *Fusarium oxysporum f. sp. cubense* causing fusarium wilt [9], *Xanthomonas campestris pv. musacearum* producing banana bacterial wilt [10] and reduced soil fertility and drought [11]. Host plant resistance through conventional cross breeding has been identified as a sustainable method for addressing the banana production challenges [12]. Resistance against these diseases and pests have not been found in the cultivated bananas but have been identified in wild species [13] for example the wild diploid species Calcutta-4 (*Musa acuminata* subspecies burmannicoides) [14], which is resistant to black Sigatoka. Similar to East African highland bananas, *Sukali Ndizi* is triploid ( $2n = 3x = 33$ ) with low male and female fertility that results in very low seed yield and germination rates [15].

Inter- and intra-specific hybrids have been successfully produced through conventional crossing methods thus minimizing infertility barriers. Improvement of the East African highland bananas have been achieved through crossing 3x(triploids) landraces with 2x (diploids) wild or improved to produce 4x tetraploids that generally display greater male and female fertility [13] and selected tetraploids crossed with improved diploids to produce sterile secondary triploids [16]. Breeders require appropriate progenitors that can be crossed to produce progenies with the desirable characteristics and choice of parents to be used in crossing is a prerequisite for an efficient breeding program and a necessity to sustain genetic progress. The success of any breeding program is measured by its ability to produce good quantities of quality seeds which greatly depends on the identification of female and male fertile parents that combine best.

Use of climate smart crops in African countries is an urgent concern by the governments and the private sectors, for example Zambia where the weather trends show an increasing low rainfall quantity and length of season, with increased temperatures across the seasons. Breeders have resorted to use of mutation breeding to create variation among cowpea genotypes for climate smart traits (improved yields, resistance to abiotic and biotic stresses) and promising lines have been identified[17]. Most research on bananas, polyploidization is being achieved through somatic doubling, whereas, in nature, sexual events involving 2n gametes represent the main route to polyploid formation. In light of this, the possibility of sexual polyploidization in Sukali Ndizi would be useful step in the crop's improvement.

The Uganda's National Agricultural Research Organization (NARO) under National Banana research program in 2010 embarked on Sukali Ndizi improvement program and this study was carried out under that program with the aims of assessing the female fertility potential (seed production and seed germination and the ability of sexual polyploidization of Sukali Ndizi and implication in the improvement of the crop.

## II. MATERIALS AND METHODS

### A. Plant Materials

Eleven different male diploid parents either wild or improved diploids were crossed with *Sukali Ndizi* in landrace as the female parent (Table 1) with each male diploid crossing with female *Sukali Ndizi* equal numbers of bunches. The diploids used had breeding attributes that were superior to the Sukali Ndizi like host plant resistance or tolerance to black

TABLE I: PARENTS USED IN CROSSES

| Genotype      | Source and type                                      | Disease characteristics                                                                       | Other traits of interest                                                                 | References |
|---------------|------------------------------------------------------|-----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|------------|
| 131k-15       | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka<br>resistance and FOC resistant,<br>burrowing nematode resistance              | Every long laxed bunch and fertile                                                       | [18]       |
| 131k-25       | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka<br>resistance and FOC resistant,<br>burrowing nematode resistance              | Every long laxed bunch and fertile                                                       | [18]       |
| 131k-57       | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka resistance and FOC<br>resistant, burrowing nematode<br>resistance              | Every long laxed bunch and fertile                                                       | [18]       |
| 131k-3        | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka resistance and FOC<br>resistant, burrowing nematode<br>resistance              | Every long laxed bunch and fertile                                                       | [18]       |
| 131k-63       | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka resistance and FOC<br>resistant, burrowing nematode<br>resistance large fruits | Every long laxed bunch and fertility                                                     | [18]       |
| 131k-8        | Diploid- derived<br>EAHB hybrid from<br>Uganda       | Black sigatoka resistance and FOC<br>resistant, burrowing nematode<br>resistance              | Every long laxed bunch and fertility                                                     | [18]       |
| TMB2x8075-7   | Diploid hybrid<br>banana from ITC                    | Sigatok leaf disease and burrowing<br>nematode resistance FOC resistant                       | High %Brix, big, long and compact bunch,<br>good combining ability, normal<br>pseudostem | [19]       |
| Cultivar Rose | Wild diploid from<br>ITC                             | Resistant to <i>F.oxysporum f.sp</i><br>cumbens, burrowing nematode<br>resistant              | High %Brix,Excellent fruit pulp texture,<br>drought tolerant, short cycle, short stature | [21]       |
| Kokopo        | Cultivated diploid<br>Papua New Guinea<br>accession. | Resistant to Sigatoka leaf disease &<br>FOC, drought tolerant, short cycle                    | High carotene (pVACs) contents1,276-<br>3,428 ug/100 g short cycle, drought<br>tolerant, | [21]       |
| Pitu          | Cultivated diploid<br>Papua New Guinea<br>accession  | Resistant to Sigatoka leaf disease &<br>FOC                                                   | carotene(pVACs) contents1,276-3,428<br>ug/100 g short cycle, drought tolerant,           | [21]       |
| Sukuli Ndizi  | Cultivated triploid<br>Landrace in uganda            | Susceptible Sigatoka leaf disease and<br>FOC                                                  | Best landrace with consumer (market)<br>preferred traits                                 | [22]       |
| Pisanga Lilin | Cultivated diploid<br>from ITC                       | Sigatoka leaf disease & <i>F.oxysporum</i><br><i>f.sp</i> cumbens                             | Good bunch size and positioned                                                           | [19]       |

sigatoka, Fusarium wilt, weevils and nematodes, parthenocarpic fruits and big bunch size.

### B. Study Location and Crossing Procedure

The parents were planted in pollination blocks at National Agricultural Research Laboratories Kawanda (NARL) in September 2010. NARL is located in Central Uganda at 32°36'E and 0°25'N, 1210 m above sea level. The spacing of plants was 3×3 m. The procedure of crossing described by [13], (2006) was adopted where mature male flowers from the male parents were covered with cotton bags to avoid pollen contamination from other pollen sources. Female inflorescences of *Sukali Ndizi* were also bagged immediately at emergency but this time with transparent plastic bags [23]. The crossing exercise started in 2012 up to 2016 using Top-cross mating design [24]. Hand pollination was carried out by picking a cluster of male flowers from the selected male parent containing pollen and rubbed onto freshly exposed flowers of selected female *Sukali Ndizi* after which the flowers were re-bagged. The exercise was repeatedly carried out daily between 6.00 h and 8.30 h [13].

### C. Seed Extraction and Embryo Germination

The bunches were harvested when the first fruit started yellowing and placed in a room at 20-25°C with no other treatment until all fruits ripened to yellow with brown spots. Seed extractions were carried out and seeds from each cross were taken to the tissue culture laboratory for the process of invitro seed germination and embryo culture as per [25] protocol. The water gravity test was used to differentiate the extracted seeds as sunken/viable and floating/nonviable.

### D. Determining the Nuclear DNA Content of Seedlings

The nuclear DNA content of each seedling was analyzed by flow cytometry to determine the ploidy levels using procedures described by [26] with some modifications at NARL Biotechnology lab at Kawanda. The cell nuclei were isolated by chopping the midrib tissue from young leaves with a sharp razor blade in cold OTTO I buffer (0.1 M citric acid monohydrate, 0.5% Tween 20) to release cell nuclei [27] and the nuclei suspension was filtered through a 50 µm nylon mesh to remove large cellular material and then the nuclei were stained in OTTO II buffer (0.4 M Na<sub>2</sub>HPO<sub>4</sub>, supplemented with 4 µg/ml DAPI, 4',6-diamidino-2-phenylindole). Fluorescence detection was carried out with a Partec Ploidy Analyzer PA—II (Partec GmbH, Munster, Germany) whereby relative fluorescence intensities were translated into histograms corresponding to the relative DNA content which indicated the ploidy status of tasted sample [27]. Two reference accessions of known ploidy level, calcatta 4 (diploid) and *Sukali Ndizi* (triploid), were used as internal standards and the analytical instrument was calibrated so that the G1 peak of nuclei isolated from the control diploid plant was on channel 50, while that of the triploid was on channel 75 [26]. The setting was kept constant during analysis of sample prepared from the breeding population to compare their peak to that of the reference plants. Thus peaks appearing on channel 50, 75, and 100 corresponded to diploid, triploid and tetraploid plants respectively.

### E. Data Collection

The data were recorded from January 2012 to December 2016 on the following traits; date of pollination, harvest date, total number of good seeds, number of extracted embryos, number of germinated embryos after two months, total number of seeds when pollinated by particular diploid male, total number of bunches pollinated which produced no seed, highest seed per pollinated bunch per diploid male and mean number of seeds per bunch was calculated.

### F. Statistical Analysis

Data per cross over the 5 years was analyzed using MS Excel, and frequencies generated. Trait means was generated using SAS version 8.2 for windows (2001) and Fisher's protected least significant test at  $\alpha = 5\%$  was performed.

## III. RESULTS AND DISCUSSION

### A. Seed Separation

The seed separation results of some of seeds generated by the crosses using the water gravity test are presented in Fig. 1.

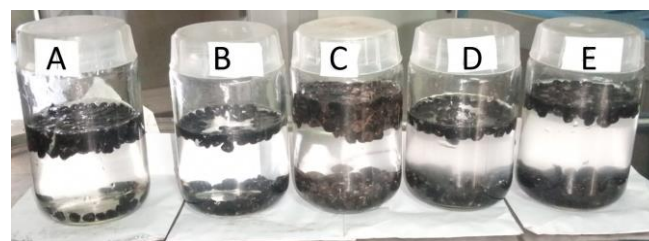


Fig. 1. Number of seeds recovered in the floated and sunken fractions for crosses: A = Pitu, B = 131k-57, C = Pisanga Lillin, D = C.Rose, E = TmB2x-8075-7.

Results of the seed extraction showed that cross C had the highest number of seeds and cross B had the least. The water gravity test revealed that the highest number of seeds floated in all the crosses suggesting that the large proportions of the collected seeds were nonviable (empty, shriveled, and anatomically underdeveloped seeds) indicating that the large quantity of empty and dead seeds was due to pollination failures, underdevelopment of female gametophytes, degeneration of male gametophytes and/or post-zygotic degeneration during the various stages of embryogenesis [28], [29].

### B. Variation in Seed Set

The results of average seed production for each month of pollination for a period of five consecutive years are presented in Fig. 2.

The results of the study showed monthly variation for total seed production in the cross combinations in the 5-year period which suggested that seed set did not follow any monthly pattern indicating no seasonal influence on seed production implying that pollination in *Sukali Ndizi* can be done all the year around irrespective of the month. [30] reported that seed set in Plantains was very high in the early part of the year (peak in February), then declined to a very low level in May and reached a second peak in Aug-September but [31] working with EAH bananas reported that Pollination success was similar for each month at a 95% confidence level.

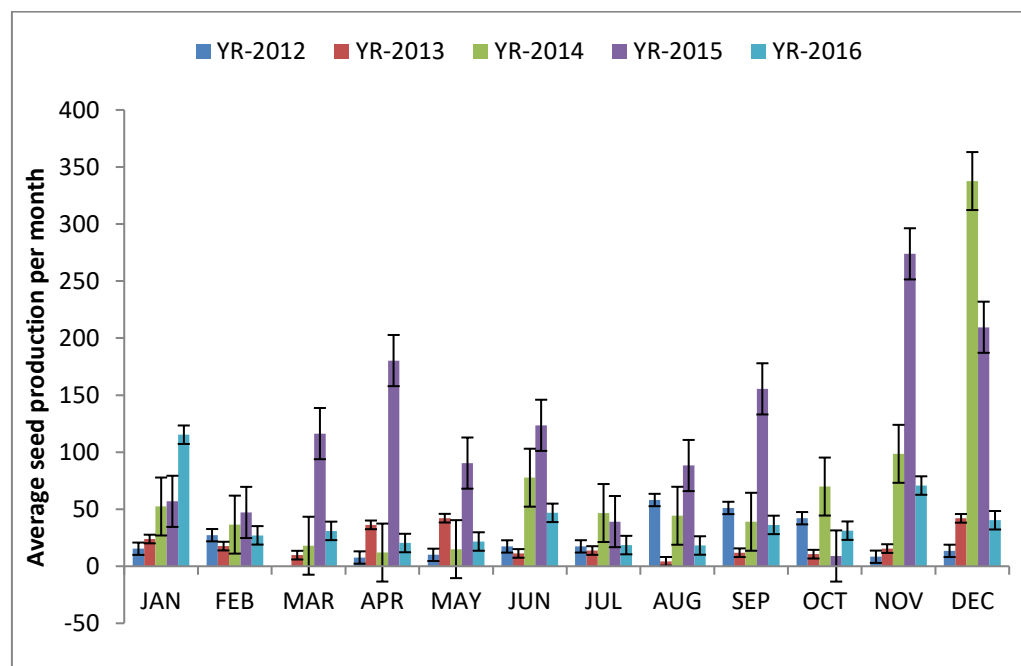


Fig. 2. Monthly variation in seed set in Sukali Ndizi pollinated with various diploids for five consecutive years (2012–2016).

The results of the mean seed generated by Sukali Ndizi using 11 different males as source of pollen in five years are presented in Fig. 3.

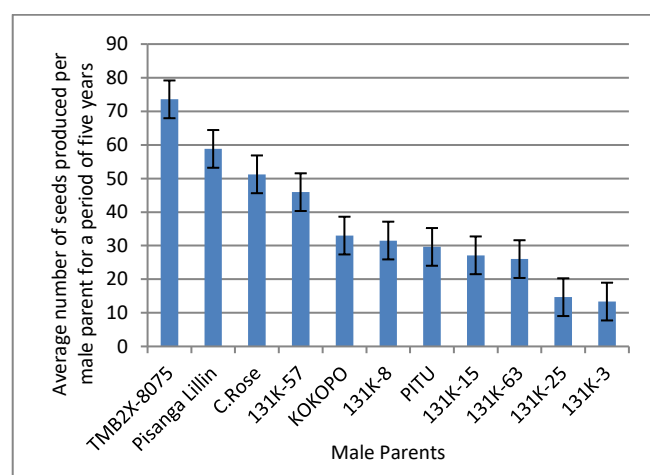


Fig. 3. Average number of seeds produced per male parent crosses with Sukali Ndizi.

Results of the pollination success and seed set in Sukali Ndizi revealed that the highest number of seeds per bunch was Sukali Ndizi X TMB2X-8075-7 with 73.6 seeds per bunch followed by Sukali Ndizi X Pisanga Lillin with 58.8 seeds per bunch. Pisanga lillin, 131k-57 and C.rose were not significantly different in their seed production, Kokopo, 131k-8, Pitu, 131k-15 and 131k-63 were not significantly different in seed production and finally 131k-25 and 131k-3 not significantly different. The results suggested that Seed production efficiency was dependent on male diploid parents' compatibility with female Sukali Ndizi thus, crosses using diploid TMB2X-8075-7, Pisanga lillin, 131k-57 and Cultivar rose as male parents were more likely to be successful than the rest of the male parents implying that the three would be the best vehicle to transfer the preferred traits. Triploid plants usually have high abortion rates owing to unbalanced meiotic chromosome segregation, but the limited viable gametes could participate in the transition to different ploidy levels

[32]. The relatively high seed set obtained in the cross Sukali Ndizi and TMB2X-8075-7 was probably due to the high fertility residuals of the wild diploid male parent Km5 in the background of TMB2X8075-7 which is a pedigree of SH3362 × Calcutta 4/Km5 where Km5 has high Seed production [33].

The results of percentage mean seed with embryos for each male parent are presented in Fig. 4.

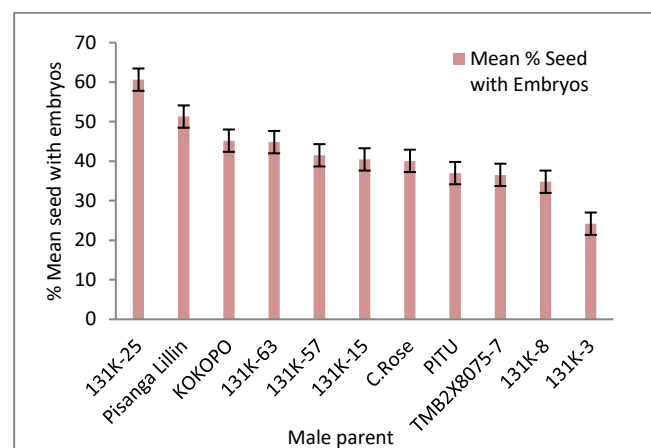


Fig. 4. Variation in % of Seeds with embryos per male parent crossed with Sukali Ndizi.

The results showed that in individual crosses the percentage of seeds with embryos ranged from 24% (Sukali Ndizi × 131k-3) to 61% (Sukali Ndizi × 131k-25) which was 2.5 fold. The percent of seeds with developed embryos per bunch in the crosses varied with the male parent with some of the crosses producing few good seeds with majority having embryos for example of the few seeds produce in Sukali Ndizi × 131k-25 cross, 61% seeds hand embryos. The mean number of seeds with embryos was low in the crosses suggesting the inherent Seed abortion in Sukali Ndizi due to endosperm degeneration during early embryogenesis [34]. The ovule abortion was characterized by the presence of floating seeds which were devoid of either embryo or endosperm or both and the sunken seeds which hand the endosperm and the

embryo or endosperm alone which were the major cause of low female fertility.

### C. Embryo Germination in Sukali Ndizi hybrids

The results of mean percentage seed with viable embryos are shown in Fig. 5.

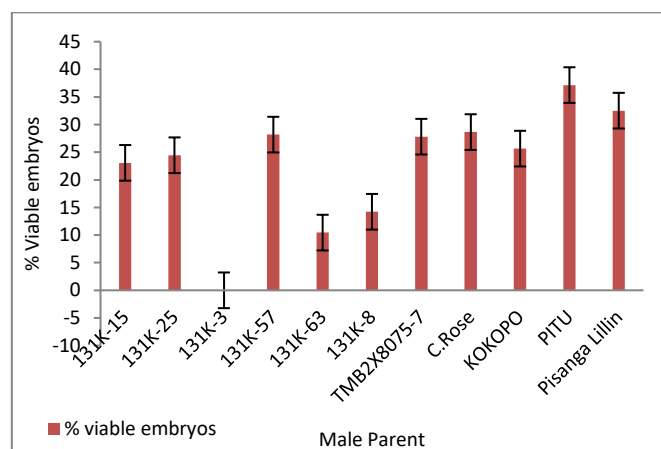


Fig. 5. Seed viability in Ndizi hybrids from different male parents.

The viable embryos obtained varied with the crosses between *Sukali Ndizi* and the male diploids. No embryo germinated from the cross *Sukali Ndizi* X 131k-3 and seed germination percentage in the remaining 10 crosses ranged from 10% in *Sukali Ndizi* X 131k-63 to 37% *Sukali Ndizi* X Pitu which was 3.7 folds signifying wide variation in seed germination from different male parents due to the large number of aborted zygotic embryos which emphasized the need to rescue *Sukali ndizi* hybrid embryos [35]. The embryos from *Sukali Ndizi* x Pisanga lillin resulted into 10 hybrid seedlings, *Sukali Ndizi* x TMB2X8075-7 resulted into 8 hybrid seedlings and *Sukali Ndizi* x C.Rose resulted in 7 hybrid seedlings indicating the limited and variable seed germination exhibited by *Sukali Ndizi* hybrids [36]. When the embryos were excised and germinated in vitro according to [25] the seed dormancy was eliminated suggesting that ovule abortion in *Sukali Ndizi* mainly involved embryo and endosperm as the cause of limited seed germination confirming the need to carry out embryo rescue to avoid most progenies being lost at abortive stages [37].

### D. Ploidy Distribution in Hybrids

The results of ploidy analysis of the 365 progenies are shown in Fig. 6.

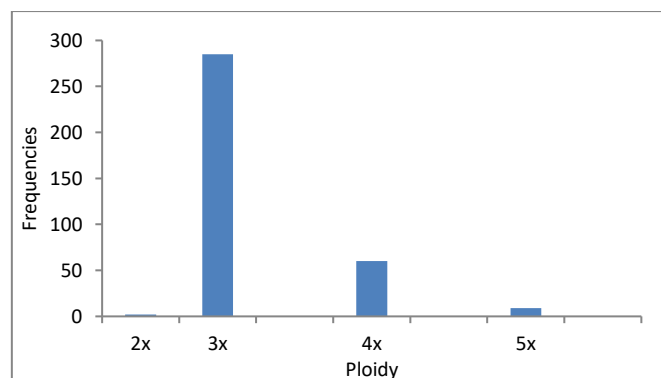


Fig. 6. Ploidy distribution in Ndizi hybrids.

The results of ploidy analysis of the 365 progenies which resulted from crosses of *Sukali Ndizi* and the various diploids male parents showed that 3x ploidy class was the highest (288), the 4x ploidy class had (61) the 5x had (14) and lowest class was the 2x (2). The ploidy distribution had a 142.5 fold dominated by the 3x progenies signifying a very wide variation in progenies with different ploidy levels. The 3x progenies arose from an egg containing either AB/AA genome from *Sukali Ndizi* and one 'A'/B' half chromosomal set from male parent and the tetraploids arising from unreduced gametes of AAB combining with one 'A'/B half chromosomal set from the male parent indicating the ability of *Sukali Ndizi* to produce unreduced egg cell during megasporogenesis as reported by [38]. The presence of 4x progenies was an indication of the unreduced 2n gametes from the male diploid parents whereas the 5x progenies was an indication of the unreduced gametes in the two parents, indicating great potential for sexual polyploidization in the breeding program. In *Sukali Ndizi* breeding scheme, results showed presence of Polyploidization which refers to the multiplication of a complete chromosome set of a certain species to give birth to a new species. These multiple sets of chromosomes coexist in one nucleus and are inherited to progenies. Polyploidisation induces several modifications in plant cells, resulting into plant anatomical structure changes usually manifests with the most noticeable polyploidisation effect referred to as the so-called "gigas" effect, resulting into change in the phenotype of plants [39]. Polyploidisation generally results in better plant adaptation to changing environmental conditions and increased resistance to biotic and abiotic factors. [40] reported that Tetraploids of *Z. officinale* had significantly more soluble proteins, sugars, and proline (PRO), substances associated with plant stress resistance. [39] has reported that tetraploids are currently being used to increase the yield of industrial hemp biomass as a raw material for the production of second-generation biofuels. [41] reported that Polyploidisation is an important phenomenon in plants and animals which promotes speciation, biodiversity, and adaptation to changing environmental conditions. Therefore, the occurrence of sexual Polyploidisation phenomenon in the *Sukali Ndizi* 3x by 2x breeding scheme can result in climate smart genotypes with potential use in climate change mitigation. The Despite the multiple ploidy nature of the progenies produced in the crossing scheme irrespective of the male parents, this study showed that the 3x by 2x breeding scheme constituted a reliable means of generating triploid Ndizi hybrids. [31] observed that seeds from 3x × 2x crosses had a higher percentage of germination (11%) than seeds from 4x × 2x crosses (7%) while working with a group of 27 EAHB hybrids further justifying the 3x × 2x scheme.

## IV. CONCLUSION

Seed production in *Sukali Ndizi* was possible all year-round as long as flowers were available but seed quality, seed quantity and embryo germination was diploid male dependent. The gradual loss of viability of embryo along the developmental path emphasized the need to rescue hybrid embryos. Unreduced gametes were produced in the process of hybrids formation thus sexual polypolization was possible

in Sukali Ndizi improvement. Diploids Pisanga Lillin, TMB2x8075-7 and Cultivar Rose were more promising male diploid parents to use in Sukali Ndizi improvement program. Unlike in the East African Highland Bananas where 3X by 2x to form primary tetraploids followed by 4x by 2x approach produce new hybrid combinations, in Sukali Ndizi Triploid x diploid approach to breeding is a viable method to produce new hybrid combinations.

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#### DISCLOSURE OF CONFLICT OF INTEREST

The authors have no conflicts/competing of interest to declare that are relevant to the content of this article. All the authors have consented to the publication.

#### DECLARATIONS

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