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Ex-situ cytogenetic male fertility characterization of selected cassava clones

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Ex-situ **cytogenetic studies had been used for the determination of male fertility of clones of cultivated crops for conventional breeding and consequent production of ideotypes. The experiment consisting of seven (7) cassava cultivars in which pollen grains were excised from all the cultivars at anthesis, each treatment was replicated six (6) times and a total of two hundred (200) pollen grains were counted per cultivar. Treatments (pollen grains) were subjected to cytogenetic male fertility screening which was conducted in the Microbiology laboratory, Rivers State University. The treatments collected were arranged in Complete Randomization Design (CRD). Some baseline cytogenetic data were taken from all the cultivars such as number of stained pollen grains per cultivar, number of unstained pollen grains per cultivar, number of N-pollen grains per cultivar and number of giant pollen grains per cultivar. The Number of stained pollen grains were highest in Tms98/0505 followed by Tms96/1632 with the means 26.50 and 26.1 respectively while the least was obtained from 016/137. The highest number of unstained pollen grains were observed in cultivar 016/137 followed by 01/1368 with mean 14.83 and 14.33 respectively while the least were obtained from cultivar Tms98/0505 followed by Tms96/1632 with the mean 6.83 and 7.17 respectively. The highest number of N and giant/2N pollen grains were obtained from cultivar Tms419 Tms07/0593 respectively. Stainability/fertility percentages were calculated in which Tms98/0505 had the highest (79.5%) which showed significant difference (P>0.05) from the other cultivars except Tms96/1632 and the least was 55.5% obtained from 016/137 cultivar. The ex-situ findings of this research revealed that all the different species are fertile though their inherent fertility level/percentage varies from specie to specie as paternal parents and therefore, should be used for cassava breeding programs due to their inherent fertility as male parents especially Tms98/0505 and Tms96/1632.**

Keywords: Breeding,*Ex-situ*, Cytogenetics, Fertility, stainability.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), is a dicotyledonous plant native to South America (Allem, 2002), is an important storage root crop worldwide (Ceballos *et al*., 2004). It is a primary component of the diet of over 800 million people across differnt continents. The crop is a high starch producer with levels of up to 90% of its total storage root dry mass (Jansson *et al*., 2009). It is currently the world's fourth most important staple and carbohydrate rich food crop (El-Sharkawy, 2012), with a worldwide production estimated at 257 million tonnes (MT), of which about 146 MT come from Africa.

The crop is grown by most subsistent farmers due to its ability to yield better than other staple food crops under conditions of extended drought and poor soils (Ceballos *et al*., 2011). It is a perennial crop native to South America and was among the first crops to be domesticated.

Cassava, *Manihot esculenta* Crantz (Euphorbiaceae), is one of the leading food and feed plants of the world. It ranks fourth among staple crops, with a global production of about 160 million.

Cassava crop is the third more important source of calories in the tropic, is consumed by more of 700 million of humans of little resources in Africa, Asia and South America. Cassava is a highly heterozygous crop and the production of homozygous parents is difficult, because conventional crossings delay between 8-10 years (Ceballos *et al*., 2007); this makes it difficult for use in breeding programs (Ceballos *et al*., 2007). The cytogenetic study of pollen fertility as a way of characterizing paternal parent in cassava helps in averting time wastage and cross incompatibility during introgression of useful traits The production of homozygous plant using pollen grain isolation is the most efficient method for the production of haploid plants (Dubas *et al*., 2010), reducing to two generations the time required to generate fully homozygous lines. The advantage in using doubled haploid plants is that the traits useful can be fixed without multiple cycles of hybridization, (Ferrie and Möllers, 2011).

Cassava bears separate male and female flowers on the same plant, and is thus referred to as a monoecious crop (Kawano, 1980). The time interval between planting to flowering depends on the specific genotype and environmental conditions, and may vary from one to more than 24 months (Jennings and Iglesias, 2002). Male and female flowers are borne on the same branched panicle, with female flowers at the base and male flowers toward the tip. Male flowers are more numerous than female flowers (Fukuda *et al*., 2002). Flowers often begin to open around midday, remain open for about one day. Female and male flowers in an inflorescence open at different times. Female flowers open first and the male flowers follow from one to a few weeks later, a condition called protogyny. By the time male flowers open, the female flowers on the same branch will have been fertilised or have aborted. However, since flowering on a single plant may last for more than two months, pollen from one flower may fertilise other flowers on the same plant (Kawano, 1980). Thus, both self-pollination and cross-pollination occur naturally. Cassava is an outcrossing and highly heterozygous species due to the protogynous nature of the flower anthesis (Fukuda *et al*., 2002). However, there is no genetic or physiological barrier that prevents self-pollination (Kawano *et al*., 1982). A number of factors influence flowering in cassava and they include: genotype, soil moisture, soil fertility, photoperiod and temperatures (Kawano, 1980).

Cassava pollen grains are relatively large in size and sticky, and therefore wind-pollination appears to be of little consequence (Kawano, 1980). Several species of honeybees andwasps are main pollinators of cassava flowers. Pollen grains show size differences within the same genotype. The larger grains are about 130 to 150 µm in diameter, whereas the smaller grains range from 90 to 110 µm (Kawano, 1980). In some genotypes, the larger grains are more abundant, whereas in others the smaller grains are more common. There are differences in pollen size indicating trimorphism with larger, normal and smaller diameters; however pollen dimorphism via abnormal meiosis or mitosis is still to be investigated and the percentage of viability was higher in the normal and large sized pollen grains as compared with the smaller ones (Wang *et al*., 2010); the formation and different amounts of micronuclei with distinct sizes in each microspore at tetrad stage, irregular wall development producing abnormal pollen spores, conducing to unbalanced gametes and low sterile in pollen grain (Vasquez and Nassar, 1995; Ogburia *et al*., 2002).

Reports of diploid pollen resulting from abnormalities during microsporogenesis (Ogburia *et al*., 2002). The larger pollen grains have been observed to have better in vitro germination (60% after 2 h at 40°C) than the smaller ones, which may have less than 20% germination (Chavarriaga-Aguirre and Halsey, 2005). Cassava pollen rapidly loses viability after it is shed. Ninety-seven per cent of seed set occurs when the pollen is used immediately after its collection, 56% when pollen is stored for 24 h at 25°C, and 0.9% after keeping pollen for 48 h. In practice, however, cassava breeders take care to perform pollinations within one 1 h after collection of pollen to ensure successful fertilisation (Kawano, 1980; Chavarriaga-Aguirre and Halsey, 2005).

After pollination, the flower ovary develops into a fruit within 70 to 90 days. The fertility of genotypes is variable and may be very low. An average of one seed per fruit is commonly achieved through controlled pollination from a potential three seeds from a tri-locular ovary (Jennings and Iglesias, 2002). The genotype of the female parent is more important in determining success of fertility than that of the pollen parent (Jennings, 1963).

Fruit maturation generally occurs 75 to 90 days after pollination. The newly harvested seeds are dormant and require three to six months' storage at ambient temperatures before germination. Seeds take about 16 days to germinate. Germination can be hastened by carefully filing the sides of seed coats at the radicle end and by temperature management. Ellis *et al*. (1982) found that few seeds germinated unless the temperature exceeded 24^0C, and that the best rates occurred at 30 to 35^oC.

Cassava hybridization process is affected by the flowering ability and/or low rate of flower production by some genotypes, lack of synchrony in the flowering period of the genotypes and male sterility (Kawano *et al*., 1978). Different levels of male sterility have been reported in cassava genotypes (Bai, 1985). Cours (1951) studied the morphological variation of many cassava varieties and reported that 20% of the varieties presented deformed anthers and were sterile males. When Magoon *et al*. (1968) assessed a number of cassava genotypes

they identified different levels of male sterility. Male sterility in cassava is attributed to several factors, including the non-disjunction of the microspore; abnormal behaviour; cytological abnormalities and functional male sterility which is reflected by absence of anther dehiscence.

MATERIALS AND METHODS

This experiment was done in the Microbiology Laboratory, Department of Microbiology, Rivers State University, Port-Harcourt.

All plant materials used were field established in the Teaching and Research Farm of the University and all clones developed by the IITA2010. Some morphological features and traits of selected clones one as presented here under.

Rivers State has a landmass of 19420 sq.km. The rainfall pattern is essentially bimodal with peaks in June and September, while in April and August there are periods of lower precipitation (Ukpong, 1992). 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 average of 2000mm to 4500mm (Anderson, 1967).

Microscopic sample of male flowers of male parental lines were sampled and fixed in fixation (95% ethanol: glacial acetic acid in a 3:1 ratio) in the field and taken to the laboratory for microscopy.

ANOVA method was used to analyzedthe treatments and means were tested using Tukey means method of grouping at 5% level of probability (Minitab, 2010).

The experiment consists of a total of seven (7) *Manihot*clones 01/1368, Tms96/1633, 016/137, Tms419, Tms98/0505, Tms07/0593 and unknown from which pollen grains were obtained. The experimental materials were made up of seven (7) different *Manihot* species which serve as the treatments and each treatment was observed six times. The treatments were arranged in a Complete Randomization Design (CRD).

The data collected includes Number of viable (stained) pollen, Number of non-viable (unstained) pollen, Number of pollen grains per microscopic field, Number of pollen grains observed per cultivar, Number of n pollen per cultivar, and Number of 2n pollen per cultivar.

Pollen samples were collected from the genotypes between 7:30 and 10:30 am and were taken to the laboratory. Pollen viability was determined using acetocarmine stains (Nassar 1978d). The preparation was covered with cover slide and allowed to stand for 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 will be considered as viable pollen. Stainability with acetocarmine is an established method to determine pollen viability for estimating the level of male fertility (Dessauw, 1988). The diameters of ten randomly selected deeply stained grains were measured with the aid of a graduated eyepiece. Pollen grains show size differences within the same genotype. The larger grains 2n Pollen) are about 130 to 150 µm in diameter, whereas the smaller grains (n pollen) range from 90 to 110 µm (Kawano, 1980). Percentage Pollen grain fertility were calculated using the formula below;

$$\% \text{ Pollen Grain Fertility} = \frac{\text{No.of deeply stained pollen grains}}{\text{Total No. of Pollen grains observed}} \times \frac{100}{1}$$

Percentage N and 2N Pollen grain frequency were calculated using the formulas below;

$$\% \text{ N Pollen Grain Frequency} = \frac{\text{No. of N pollen grains}}{\text{Total No. of Pollen grains observed}} \times \frac{100}{1}$$

$$\% \text{ 2N Pollen Grain Frequency} = \frac{\text{No. of 2N pollen grains}}{\text{Total No. of Pollen grains observed}} \times \frac{100}{1}$$

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 (Ulburghs, 1994), A total of 200 pollen grains per cultivar were counted in ten microscopic field views. Only completely rounded and deeply stained grains were considered viable.

RESULTS AND DISCUSSION

Cytogenetic Fertility Characterization of Paternal Parents of Cultivars

The cytogenetic fertility characterization and evaluation of male parent parameters are shown in Table 1.The highest numbers of stained pollen grains were observed in Tms98/0505 followed by Tms96/1632 while the least were obtained from cultivar 016/137. Therefore, Tms98/0505 had the highest stainability and fertility percentage (79.5%) amongst the different cassava cultivars. The more the number of stained pollen grains, the higher the stainability and fertility percentage of the cultivar and the better the cultivar for introgression of desirable trait during genetic breeding of cassava species. The highest number of unstained pollen grains were observed in cultivar 016/137 which invariably indicates its inherent low stainability and fertility percentage, and its less chances of bearing viable hybrid seeds for development of better and superior hybrids. The highest number of N and giant/2N pollen grains were obtained from cultivar Tms419 Tms07/0593 respectively. The more the frequency/percentage 2N pollen grains, the

better the chances of producing fertile hybrid seeds when hybridized and the lesser the frequency/percentage 2N pollen grains, the lesser chances of bearing hybrid seeds after hybridization. In general, the higher the number of 2N pollen grains, the better the chances of bearing viable hybrid seeds than that of more N-pollen grains. Clones producing at least 25% viable pollen (completely rounded and deeply stained) at moderate production levels are considered sufficiently male-fertile, therefore, findings indicate that all the cultivars are fertile.

Table 1: Cytogenetic Fertility Characterization of Paternal Parents of Cultivars

Cultivars							
Parameters	**01/1368**	**Tms96/1632**	**016/137**	**Tms419**	**Tms98/0505**	**Tms07/0593**	**Unknown**
No. of Microscopic Slides	6	6	6	6	6	6	6
No. of Pollen Grains Counted	200	200	200	200	200	200	200
No. of Stained/Viable Pollen Grains	114	157	111	145	159	136	140
No. of Unstained Pollen Grains	86	43	89	55	41	64	60
No. of Giant Pollen Grains	82	82	83	66	73	85	70
No. of N Pollen Grains	118	118	117	134	127	115	130
%Fertility/Viability of Pollen	57	78.5	55.5	72.5	79.5	68	70
%2N Pollen Grains	41	42	41.5	33	36.5	42.5	35
%n pollen grains	59	59	58.5	67	63.5	57.5	65

Mean ex-situ cytogenetic male fertility characterization of cultivars

The mean numbers of viable/stained pollen grains of cultivars are shown Table 2. The highest number stained pollen grains were obtained from Tms98/0505 followed by Tms96/1632 with the means 26.50 and 26.17 respectively which are declared not significantly different from one another but significantly different from the rest cultivars. Tms07/0593 and Unknown are not significantly different from one another but are declared significantly from the rest cultivars. 01/137 and 01/1368 were also declared significant from the other cultivars, although having the least in terms of the number of stained pollen grains respectively.

The more the number of stained pollen grains, the higher the stainability and fertility percentage of the cultivar and better the chances of bearing viable hybrid seeds for the development ideotypes. The lesser the number of stained pollen grains, the lesser the stainability and fertility percentage of the cultivar the lesser the chances of bearing hybrid seeds.

The mean numbers of unstained pollen grains are shown in Table 2. More number of unstained pollen grains were obtained from 016/137 followed by 01/1368 with mean 14.83 and 14.33 which are declared not significant from one another but significantly different from the rest cultivars while the least number of unstained pollen grains were obtained from cultivar Tms98/0505 followed by Tms96/1632 with the mean 6.83 and 7.17 respectively and they not declared significant from one another but significantly different from the other cultivars.

The more the number of unstained pollen grains, the lesser the stainability and fertility percentage of the cultivar and lessen the chances of bearing viable hybrid seeds for the development ideotypes. The lesser the number of unstained pollen grains, the higher the stainability and fertility percentage of the cultivar the better the chances of bearing hybrid seeds.

The mean number N-pollen grains of the different cultivars are shown in Table 2. The highest numbers of N-pollen grains were obtained from Tms419 followed by the Unknown cultivar with the mean 22.33 and 21.67 respectively. Tms419 is declared significantly different from the other cultivars while the Unknown cultivar and Tms98/0505 are declared not significant from one another but significantly different other cultivars. 01/1368, Tms96/1632, 016/137 and Tms07/0593 are not significantly different from one another while 01/1368, Tms96/1632 and 016/137 are declared significantly different from the rest cultivars except Tms07/0593 with the least mean of 19.17.

The mean number 2N-pollen grains of the different cultivars are shown in Table 2. The highest numbers of 2N-pollen grains were obtained from Tms07/0593 followed by 016/137 cultivar with the mean 14.17 and 13.83 respectively. Tms419 is declared significantly

different from the other cultivars except from Unknown cultivar while Tms98/0505 is declared significant from the other cultivars. 01/1368, Tms96/1632, and 016/137 are not significantly different from one another but different from the rest.

Table 2: Mean Characterization of cytogenetic Paternal Parent Fertility of Cultivars

CULTIVARS							
Parameters	**01/1368**	**Tms96/1632**	**016/137**	**Tms419**	**Tms98/0505**	**Tms07/0593**	**Unknown**
No. of Stained/Viable pollen grains	19.00±9.06^{d}	26.17±9.64^{a}	18.50±6.60^{d}	24.17±9.60^{b}	26.50±6.28^{a}	22.67±5.47^{c}	23.33±9.89^{c}
No. of Unstained pollen grains	14.33±4.41^{a}	7.17±4.17^{d}	14.83±5.88^{a}	9.17±7.25^{c}	6.83±2.79^{de}	10.67±8.02^{b}	10.0±5.48^{b}
No. of 2N/ giant pollen grains	13.67±5.35^{b}	13.67±5.35^{b}	13.83±7.25^{b}	11.00±8.85^{de}	12.17±3.92^{c}	14.17±6.77^{a}	11.67±6.86^{d}
No. of n pollen grains	19.67±5.92^{c}	19.67±5.92^{c}	19.50±7.64^{c}	22.33±8.76^{a}	21.17±4.88^{b}	19.17±6.40^{cd}	21.67±5.89^{b}

*Means that do not share same letter are significantly different (Turkey method at 95% confidence level)

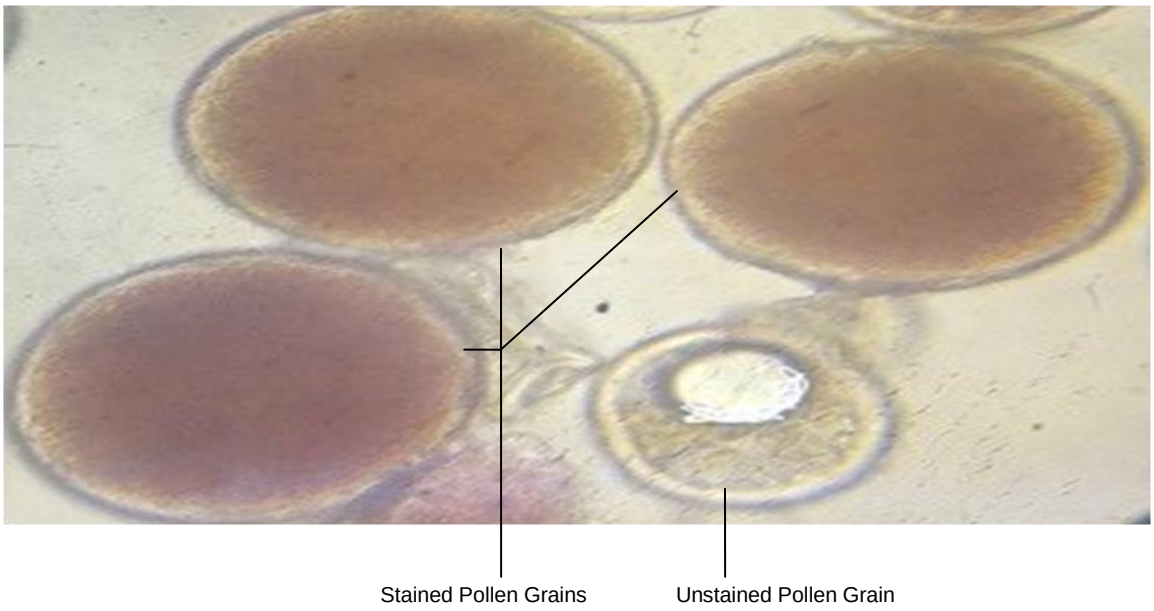

Figure 1: Microscopic View of Stained and Unstained Pollen Grain

Mean Number of Viable/Stained Pollen Grains of Cultivars

The mean numbers of viable/stained pollen grains of cultivars are shown Table 2 and Figure 2. The highest number stained pollen grains were obtained from Tms98/0505 followed by Tms96/1632 with the means 26.50 and 26.17 respectively which are declared not significantly different from one another but significantly different from the rest cultivars. Tms07/0593 and Unknown are not significantly different from one another but are declared significantly from the rest cultivars. 01/137 and 01/1368 were also declared significant from the other cultivars, although having the least in terms of the number of stained pollen grains respectively.

The more the number of stained pollen grains, the higher the stainability and fertility percentage of the cultivar and better the chances of bearing viable hybrid seeds for the development ideotypes. The lesser the number of stained pollen grains, the lesser the stainability and fertility percentage of the cultivar the lesser the chances of bearing hybrid seeds.

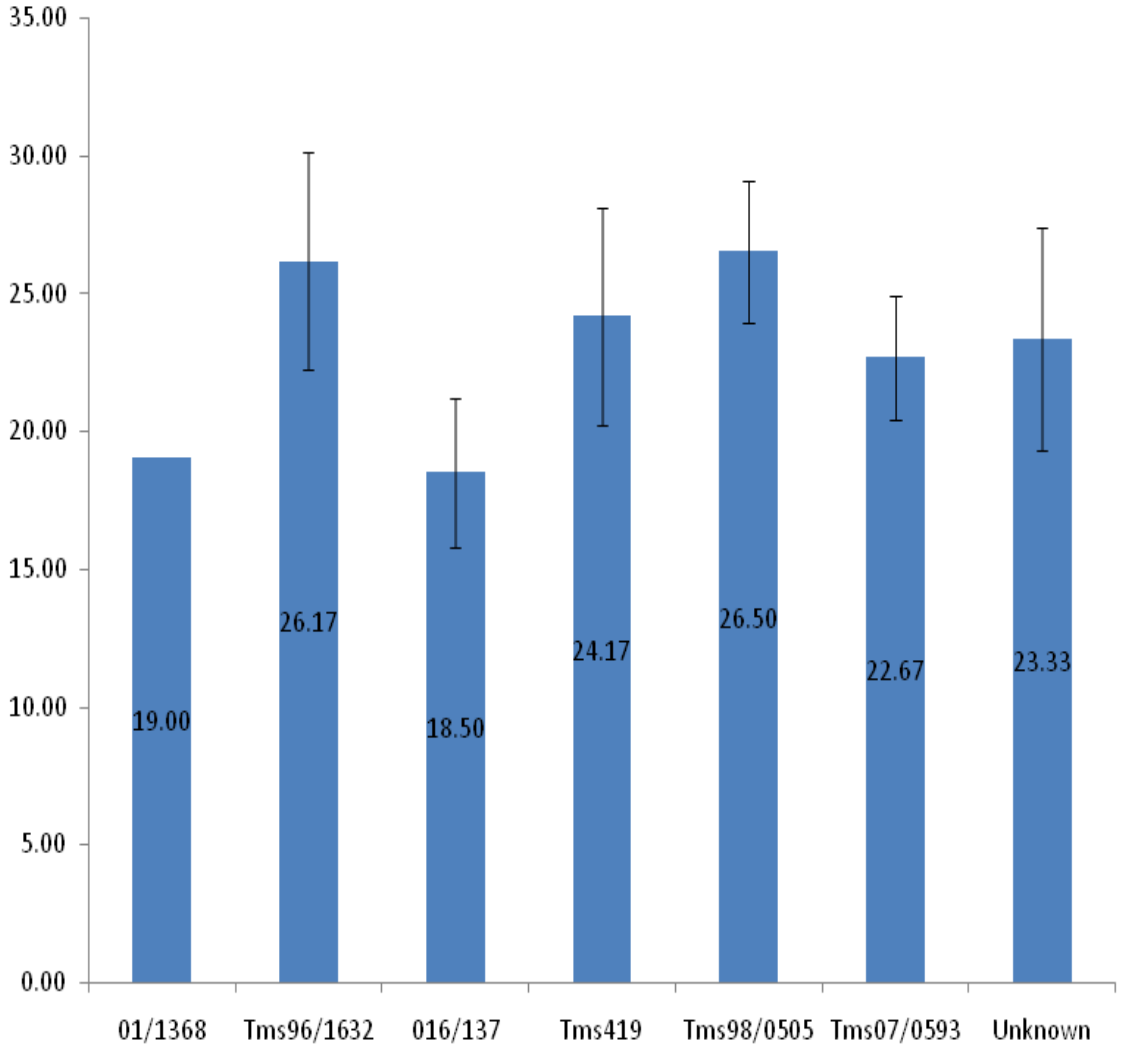

Figure 2: Mean Number of Viable/Stained Pollen Grains of Cultivars

Mean Number of Non-viable/Unstained Pollen Grains of Cultivars

The mean numbers of unstained pollen grains are shown in Table 2 and Figure 3. More number of unstained pollen grains were obtained from 016/137 followed by 01/1368 with mean 14.83 and 14.33 which are declared not significant from one another but significantly different from the rest cultivars while the least number of unstained pollen grains were obtained from cultivar Tms98/0505 followed by Tms96/1632 with the mean 6.83 and 7.17 respectively and they not declared significant from one another but significantly different from the other cultivars.

The more the number of unstained pollen grains, the lesser the stainability and fertility percentage of the cultivar and lessen the chances of bearing viable hybrid seeds for the development ideotypes. The lesser the number of unstained pollen grains, the higher the stainability and fertility percentage of the cultivar the better the chances of bearing hybrid seeds.

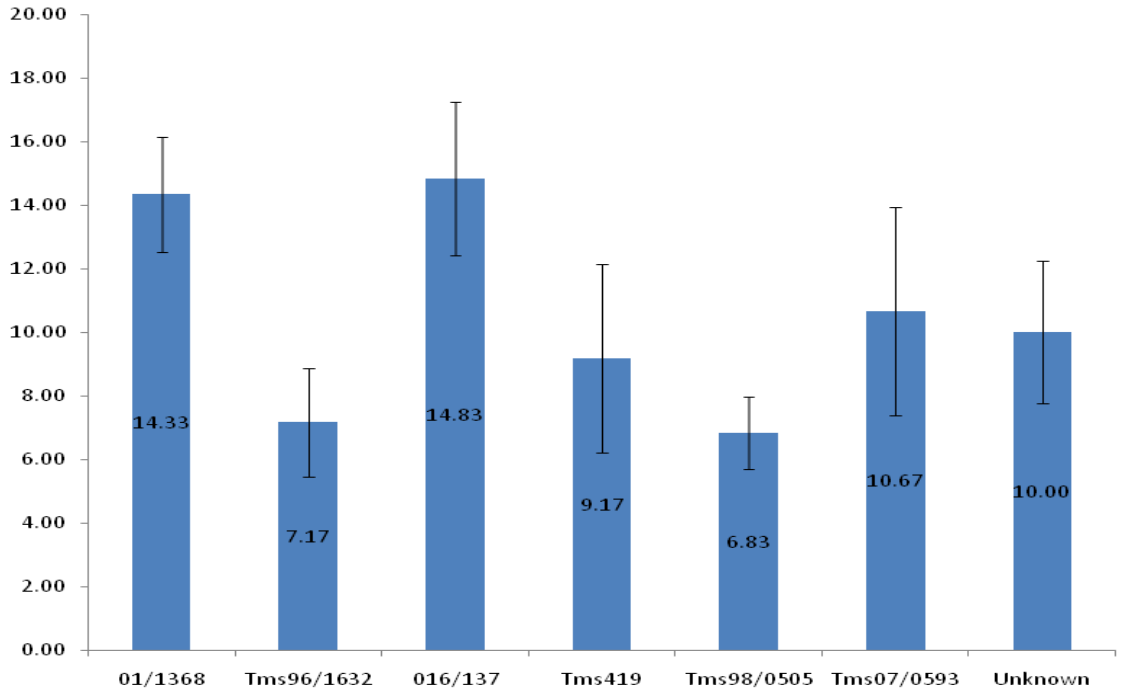

Figure 3: Mean Number of Non-viable/Unstained Pollen Grains of Cultivars

CONCLUSION

Cassava is considered to be one of the most important sources of energy for poor people living in the rural areas where cassava is grown and consumed as a staple food. it is a monoecious crop that is breeded for high yield (>35 t/ha), high starch (> 25%), high harvest index, low HCN content, good cooking and eating quality, early harvest-ability, better root storage quality, shade tolerance for use as an intercrop, wide adaptation, resistance to major diseases and tolerance to adverse soil and climatic conditions which aimed at boasting food security and reduction of food shortage for ever increasing population. However, the breeding of these different cassava species depends completely on their individual reproductive fertility as maternal or paternal parents. highest stainability and fertility percentage(79.5%)which makes it outstanding as a male amongst the different cassava cultivars. 016/137 cultivar had the least fertility percentage of 55.5%.

The more the number of stained pollen grains, the higher the stainability and fertility percentage of the cultivar and the better the cultivar for introgression of desirable traits during genetic breeding of cassava species.

Findings indicate that all the different species are fertile though their inherent fertility level/percentage varies from specie to specie as paternal parents. Tms98/0505 had the highest stainability and fertility percentage (79.5%) which makes it outstanding as a male amongst the different cassava cultivars. 016/137 cultivar had the least fertility percentage of 55.5%.

The more the number of stained pollen grains, the higher the stainability and fertility percentage of the cultivar and the better the cultivar for introgression of desirable traits during genetic breeding of cassava species.

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