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Studies on the Karyotype of the Genus Musa L.

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ABSTRACT

The development of a simple way to display and study the mitotic chromosomes of *Musa* plant is reported in the present paper. Use of enzyme mixture has been employed to arrive at this less expensive method. Enzyme mixture was used to digest the root-tips. The mixture and the root-tips were incubated at 37^{0} C in water bath for 2 hours. After the digestion, the root-tips became very soft and were handled with care. Just one drop of 3:1 absolute alcohol/acetic acid solution was used to burst the cells. This method involved both economy of material and time.
Derived chromosomes were without breakages. This is an encouraging result that could be used for further research into *Musa* species. Appropriate precautions taken on application of 3:1 alcohol /acetic acid solution in maceration produced safe structures of chromosomes. This work also provides prime record of the production of the karyotype of *Musa* plants. Photo–idiograms and idiograms of *M. acuminata* and *M. balbisiana*, which are the ancestors of the edible bananas and plantains were clearly outlined.

KEYWORDS: *Musa*, Cytology, Mitotic Chromosomes, Karyotype.

INTRODUCTION

The genus *Musa* belongs to the family *Musaceae*. There are two genera in this family – *Ensete* Horan and *Musa* L. The genus *Ensete* consists of monocarpic herbs that bear fruits that are not edible while the genus *Musa* has four sections *Eumusa* Baker, *Australimusa* E.E. Cheesman, *Calimesa* E.E. Cheesman and *Rhodaclamys* Baker (Shepherds, 1968). *Musa* belongs to the section *Eumusa*. This is the largest and geographically most widely ranging section. It is the origin of the great majority of the edible bananas. It is believed that edible bananas originated from *Musa acuminata* Colla and *Musa balbisiana* Colla which are both members of the *Eumusa* section. The two species of *Eumusa* concerned have rather unequal contributions to the origins of edible forms, *M. acuminata* being the more important (Stover and Simmonds, 1987).

The genomic make-up of edible *Musa* depends largely on the genomes acquired from the two wild progenitors, *M. acuminata* (AA) genome and *M. balbisiana* (BB) genome (Simmonds and Shephard, 1955). These two hybridized to give rise to diploid, triploid, as well as tetraploid clones (Cobley and Steel, 1977, Ortiz and Vuylsteke, 1996). Cultivated *Musa* species are virtually seed-sterile but develop fruits by parthenocarpy. These fruits are sweet when ripe. In many parts of Africa as well as in the Caribbean and Central and South Africa, the banana could be eaten raw when riped or boiled. Banana fruits are used for dessert as well as culinary purposes The plantain fruits are high in starch content and are used as staple food in most African countries. Both immature and mature fruits as well as ripe and unripe or even over- riped fruits are used for culinary purposes because they mix with other vegetables in delicious preparations (Shanmugavelu *et al.*, 1992).

Plantain and highland bananas provide 25% of the carbohydrate intake of the people. It is estimated that over two million people derive about 10% of their carbohydrate requirement from plantains and bananas which supply about 7% of the daily caloric intake, 20% of vitamin C and 48% of vitamin A (Jaffe *et al.* 1962). In Columbia, Venezuela, Hawaii, Africa and other South Pacific Islands, plantain is consumed mainly as fried, baked or as preparations made from plantain flour (Shanmugavelu *et al.* 1992). There is a good demand for plantain. Its production has remained static over the years. Usually the first and second harvest of bananas and plantains are high. There is a decline even where adequate nutrient level is available. This decline has been attributed to the behaviour of the species due to build up of nematode and insect pest, adverse changes in soil physical and chemical properties, high mat rate and the rate at which suckers are developed (Rao and Edmonds, 1985).

Cheesman (1932) gave evidence that the cultivated bananas and plantains are polyploid (like many temperate fruit plants) and suggested that the complicating circumstance of parthenocarpy necessitate cytological survey. There are a number of wild, seed-bearing species of *Musa*, which are ancestors of cultivated forms. In fact, many crops currently classified as different species and genera may have evolved from common ancestors (Fatokun, 1999). Lukaszewski (1983) who worked on wild as well as cultivated relatives of wheat arrived at the same conclusion.

The cytogenetic technique for plant breeding requires constant reference to the chromosome that plays an indispensable role in plant systematics. Chromosome features like chromosome number, size, structure, behaviour and karyotype are useful in classifying genetic materials. The study of chromosomes of genera serves

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as an important source of data for agronomists to provide answers to various systematic and evolutionary problems (Larmer, 1988). The genetics of *Musa* has not yet gone into advanced stage. Cytogenetic analysis of *Musa* germplasm including its artificial hybrids adds to our knowledge of the genome and its evolution (Osuji *et al.* 1997). Although Dantas *et al.* (1998) reported a preliminary karyotype of *Musa*, yet there is need for more detailed studies of *Musa* chromosomes. The individual chromosomes in *Musa* have not been identified and numbered partly because of the small size of the chromosomes and the absence of suitable cytological markers. The objective of the report here is to identify a cheap and easy way for displaying the mitotic chromosomes of *Musa* as well as provide a karyotype of the displayed chromosomes.

MATERIALS AND METHODS

The materials for this investigation into the cytology of bananas and plantains were collected from the field gene bank of International Institute for Tropical Agriculture (IITA) stationed at Onne, a high rainfall area of Rivers State of Nigeria. The plant materials used were taken from two genomic groups that were thought to be the ancestors of the bananas and plantains. Thus *M. acuminata* ssp. burmanicoides 'Calcutta 4' represented AA genome group while *M. balbisiana* 'Eti Kehel' represented the BB genome group.

Meristematic tips used in this work were collected from the sword suckers and matured plants in the field. The banana plant has a reputation for needing plentiful supply of water (Champion 1963, Simmonds 1966). To stimulate these plants to produce young roots they were mulched and watered in dry season. Fresh solution of 50mM-phosphate buffer was used. Three parts phosphate buffer solution A and 2 parts of phosphate buffer solution B were mixed and adjusted to pH 7. About 400 μl of 0.2% β-mercaptoethanol was added into the solution. Phosphate buffer enabled the cell division to continue why β-mercaptoethanol prevented oxidation that may lead to the root-tips turning black. This was dispensed into vials (collection tubes) and taken to the field. Fresh and actively growing secondary roots were selected. Using a pair of forceps about 10 to 15 of these roots, (about 1 to 2mm thick) were clipped at a point about 1cm long. These were quickly inserted into the vial and taken to the laboratory. In the laboratory, the root tips were placed on filter paper in a petri-dish and saturated with aqueous solution of 0.002m 8-hydroxyquinoline for 45 minutes to 1 hour to arrest cell division at pro-metaphase stage. The material was kept in dark cupboard because of the photosensitive nature of 8-hydroxyquinoline.

Root-tips were fixed in 3:1 absolute ethanol/acetic acid (Clark's fluid) according to the method of Okoli (1983) for about 20 – 24 hours. The root tips were preserved in 70% ethanol after fixation and stored in the refrigerator till required. The root-tips were rinsed in water for 15 minutes and hydrolyzed with 0.1N. HCL for 5 minutes. The root tips were again rinsed in water for 10 minutes and washed in cold 0.10M citrate buffer solution (same as citric acid butter solution CASC) and kept at 4^{0}C for about 5 minutes.

PREPARATION OF SLIDES

Prepared root tips were preserved in 70% alcohol. They were washed in distilled water for 5 minutes and immersed in fresh distilled water for 15 minutes. They were washed in citric acid buffer solution (CASC) for 2 minutes at interval of 5 minutes for 3 times at room temperature. The root tips were placed in Petri dish. This dish was put over some crushed ice. With a surgical blade, the terminal meristematic region of the root-tip (cream colour at the tip) was cut out and transferred into a centrifuge tube (Appendoff tube) containing 0.10M citric acid buffer (CASC) solution to remove fixative before incubation.

Table 1: Measurement of calculation of somatic chromosomes of *M. acuminata* ssp burmanicoides 'Calcutta 4'

Chromosome pair	Total length	Long arm (μm)	Short arm (μm)	Position of centromere
1	3.26	2.46	0.80	t
2	2.84	1.60	1.24	m
3	2.22	1.67	0.55	t
4	2.16	1.35	0.81	m
5	2.14	1.17	0.97	m
6	2.10	1.10	0.90	m
7	2.08	1.24	0.84	m
8	1.83	1.28	0.55	sm
9	1.76	1.29	0.82	m
10	1.75	1.10	0.65	m
11	1.68	1.00	0.68	m
Total	23.82			
Average	2.16			
Total form length F%	36.98 %			

Table 2: measurement and calculation of somatic chromosomes of *M.balbisiana* "Eti Kehel"

Chromosomes pair	Total length	Long arm (µm)	Short arm (µm)	Position of centromere
1	3.11	1.63	1.48	m
2	2.80	2.13	0.77	sm
3	2.75	2.01	0.74	sm
4	2.60	1.52	1.08	m
5	2.38	2.00	0.60	st
6	2.19	1.68	0.51	t
7	2.28	1.77	0.51	sm
8	2.07	1.11	0.96	m
9	2.00	1.60	0.44	st
10	1.83	1.42	1.02	m
11	1.67	1.34	0.33	t
Total	25.68			
Average	2.33			
Total form length F%	32.86%			

The 0.10M citric acid buffer (CASC) solution was then decanted and replaced with 0.2ml of enzyme mixture [The enzyme solution was made by dissolving 0.152g of pectolyase from *Aspergillius japonicus* Biochemika Fluka, 0.625g of cellulase from *Tricoderma viride* Karlan. And 0.125g of pectinase from *Rhizopus* sp (crude powder) Sigma. All these were dissolved in 12.5ml of 0.10M citric acid buffer solution, (CASC)]. The content of the tube was incubated at 37°C in water bath for 2 hours. After digestion, the root-tips became very soft and had to be handled with great care. The enzyme solution was sucked away with a micropipette and the tube content washed 3 times in 0.10M citric buffer (CASC) solution. It was left to stand for 35 minutes over some ice block. With the micropipette, one root tip was sucked up at a time and placed on distilled water in petri-dish. It was then picked with a fine forceps and placed on a clean slide, which had been soaked in 70% alcohol (to keep the slides clean and ready for the next treatment). Just one drop of 3:1 absolute alcohol/acetic acid solution was placed on the root-tip. This root-tip was macerated by tapping a pair of forceps on top and smearing it on the slide. Following maceration of the root tip on the slide in a drop of 3:1 alcohol/acetic acid, the cell walls that were now tender were broken when tapped with a pair of forceps. As the mixture evaporated, the surface tension made the protoplasts wider from side to side and eventually caused the cells to burst (Dolozel *et al.*, 1999). The above treatment released the metaphase plate from the protoplast by dispersing the cytoplasm to release the chromosomes. This preparation was viewed on a phase contrast microscope. Good slides with clear chromosomes were selected, air dried and preserved for further processing.

STAINING OF THE SLIDES

Prepared slides were left on the laboratory bench to dry. The slides were stained using Leishman's stain. About 15ml of Leishman's solution was mixed in 150ml of Sorenson's phosphate. This was poured over the slides and left to stay for between 10-30 minutes.

At intervals, the slides were removed from the solution, dipped into distilled water twice, and viewed under the low power of a light microscope. Promising slides were selected and air-dried overnight. They were then immersed in xylene solution in staining jar for a few minutes, picked up, placed on laboratory paper towel and cover-slipped with DEPEX solution [DEPEX or DPX, is a mountant like Canada balsam, is miscible with xylene and has other advantages such as being neutral. It is colourless and quick-drying]. These slides were left to dry overnight on the laboratory table at room temperature.

The prepared slides were viewed in bright field under a Leitz Diaplan binocular light microscope. The cells that showed the chromosomes clearly were focused using the oil immersion objective at 100x. Micrographs were taken with Leica Wild MPS 52 microscope camera using colour filters on the microscope.

MEASUREMENT OF THE CHROMOSOMES

The oil immersion objective of the microscope was calibrated to find what one unit on the disc eye piece micrometer that is evenly calibrated will equal in known units of linear measurement. Comparing this against the stage micrometer standardized the available ocular micrometer. The figures derived here showed that each space on the eyepiece equals 0.0035mm, when converted to milli-micron (Faluyi 1992). This calculation was done for each of the chromosomes and measurements recorded. The length of each chromosome arm was measured from its extremity to the centromere and the toal length of the chromosomes were measured independently.

Figure 1: Chromosome preparations from diploid cultivar of *Musa* L with AA genome constitution - Late metaphase of *M. acuminata* ssp. Burmanicoides 'calcutta 4'

Figure 2: Chromosome preparations from diploid cultivar of *Musa* L with BB genome constitution - Early metaphase of chromosome of *M. balbisiana*. 'Etikehel'.

KARYOTYPE PREPARATION

For each set of chromosomes, the attached photograph was enlarged by 200%. From these enlarged copies, chromosomes were carved out. These carved pieces were matched in pairs according to their morphological features (such as chromosome length and the position of the centromere). The chromosomes of each cell were arbitrarily arranged to reflect the haploid origin. These were arranged in decreasing order of their lengths to form the karyotype of each cultivar. Drawings of the idiogram of the karyotype were also made.

RESULT

Leishman's stain was used and it stained the chromosomes bright red. The chromosomes were distinct from the background. Staining time of 30 minutes was optimal. Longer period of staining led to over staining of the chromosomes. The pH value of the phosphate solution used also affected the staining. At pH 6.8 the chromosomes were well stained. A lower pH gave bluish colouration that did not allow the distinct features of the chromosomes to be evident. When observed, under oil immersion high power, the stained chromosomes were evident. From the microscopic slides, photographs were taken and displayed (Figs 1& 2). All the 22 chromosomes of 'Calcutta 4' and 'Eti Kehel' were seen under the phase contrast microscope. The lengths of the chromosomes did not differ sharply rather there was a gradual decrease in length that could easily be noticed. Each haploid unit had one chromosome with a satellite. Thus the diploid unit had a pair of satellites. The information for the production of the photo-idiograms (Figs. 3&4) were also obtained from the photographs. For the idiograms, the karyological data on Tables 1-2 were used.

Based on estimated arm ratio rate and centromere position, the metaphase chromosomes of *M. acuminata* 'Calcutta 4' (in the AA genome group) fell into three groups (Table 1 line 4), 8 pairs of the chromosomes were metacentric, 2 pairs were telocentric while only one pair was sub-metacentric. The longest chromosome measured 3.26μm and the shortest was 1.68μm. The longest arm was 2.46μm while the shortest arm was 0.55μm . The average length of the chromosomes was 2.16 μm. The total form length was 36.98% [Percentage of total form is directly proportional to the total length of all short arms and inversely proportional to total length of chromosome complement]. The BB genome group, *M. balbisiana* 'Eti-Kehel' in Table 2. The longest chromosome measured 3.11µm and the shortest measured 1.67µm in length. Four chromosomes were metacentric, 3 were sub-metacentric while 2 were sub-telocentric and 2 were telocentric. The average length of the chromosomes was 2.33µm but the total form length was 32.86%.

DISCUSSION

Bananas and plantains are of enormous importance but advanced cytological studies on them had not been achieved. Recent studies had been concentrated on the display of mitotic chromosomes. An area yet to be studied is the chromosome constitution in the wild, cultivated and artificial *Musa* hybrids.

The difficulty in display of *Musa* chromosomes, their small size, morphological resemblance, sticky nature and varying contraction have been given as reasons for delay in full investigation in *Musa* species. Osuji *et al.*, (1996) and Dolozel *et al.*, (1999) had reported clear pictures of *Musa* chromosomes. However, the method adopted in this report was shorter and involved the use of less chemicals. It was quite simple and easily achieved and still gave clear results. Using enzyme digestion had an advantage because it produced clearer chromosome spreads. It also produced chromosomes that were free from cytoplasmic debris, which were usually found in acid digestion protocols (Osuji *et al.*, 1997). Just one drop of the fixative was enough. When excess fixative was applied on the slide to burst the cells, the chromosomal features were affected. The chromosomes appeared as broken pieces. Excess acid attacks the walls of the chromosomes.

Figure 3: Photo-idiogram of eleven pairs of Chromosomes of *M.acuminata* ssp. burmanicoides -"Calcuta 4".

Figure 4: Photo-idiogram of Chromosomes of *M.balbisiana* "Eti kehel".

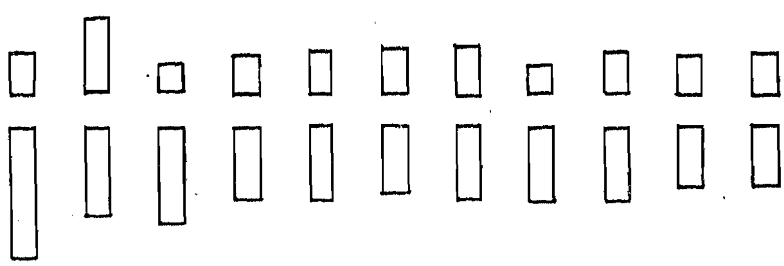

Figure 5: Idiogram of the Karyotype of chromosomes of *M.acuminata* ssp. burmanicoides "Calcut

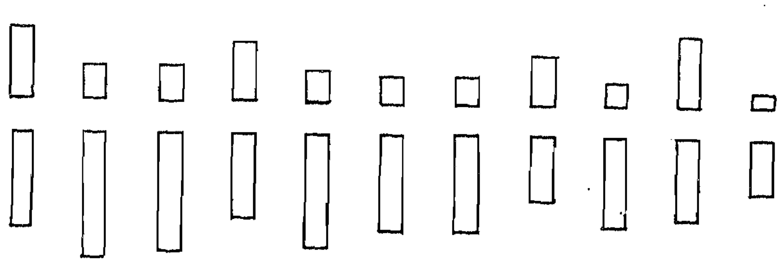

Figure 6: Idiogram of the Karyotype of Chromosomes of *M.balbisiana* "Etikehel"

To design the karyotype of *Musa*, the chromosomes were paired to the haploid number based on structures that had similar morphology. Some of these structures were closely alike and could easily be paired. Most of the satellite chromosomes were detached from the parent chromosomes but in one chromosome it was still intact (Figure 3 No 6). Despite the smallness of the chromosomes of *Musa* particular chromosomes were seen in the set. These were recognized and identified. It would be important from the viewpoint of taxonomy, cytology and genetics and even breeding of *Musa* to identify marker chromosomes as illustrated in the genus *Glycine* (Yanagisawa *et al.*, 1991). The presence of satellite chromosomes at each haploid level in *Musa* could serve as a marker.

As a result of the importance of bananas and plantains in tropical and subtropical countries, as local foodstuff, there is need to protect these crops. Current focus on research in these crops is directed towards development of disease resistant varieties and improved method of production. The standardization of a simple technique for display of the chromosomes of *Musa* was achieved here. This opened the way for further research into the structure of the *Musa* chromosomes. The study of this root-tip chromosome display method outlined here could also serve as a guide to the method to elucidate *Musa* study. A major point in preparation of these chromosomes is in the application of 3:1 alcohol / acetic acid used in maceration of the plant tissue. When there were water droplets the 3:1 alcohol / acetic acid got diluted and the cell wall did not burst completely.

Most of the satellite chromosomes were detached from the parent chromosomes. Nevertheless, all chromosomes were successfully matched to produce the desired karyotype of *Musa*. Many researchers had noticed differential condensation especially among the chromosomes at mitotic pro-metaphase and attempted to utilise it for chromosome identification. The technique adopted here was quite efficient in identifying the chromosomes especially where the problem of the nature of the small size of the chromosomes was evident.

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