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Estimation of chromosome numbers (Ploidy) and AMF associations in few *Musa* spp.

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Abstract: Banana (*Musa* spp.) are giant perennial grasses, commonly known as 'Apple of paradise'.The chromosome counting of Elachi, Poovan and Nendran Banana traits were recorded. In average, a number of chromosomes recorded in Poovan (13.2), followed by Elachi with 11.1 chromosomes and Nendran with 10.9 chromosomes. There are various methods of determining banana ploidy level, one among is estimating stomatal density and size. The present investigation Elachi, Nendran and Poovan stomatal density range from 50 to 55/mm^2 which are diploid, while Robusta ranges 34/mm^2 which is triploid. Highest stomatal size recorded in Poovan traits (48 µm^2) followed by Robusta (45 µm^2), Nendran (28 µm^2) and Elachi (21 µm^2). Another method of ploidy determination is by chloroplast count in a guard cell of stomata. Elachi, Nendran, and Poovan contain 8 chloroplasts in the guard cell thus belongs to diploid. In the analysis of floral characters of all the four traits reveals that there is no significant difference in stigma length and width. Similarly, no significant variation recorded in anther length. Pollen size is often used as a biological parameter to estimate the ploidy and viability of mature pollen grains. Broad variation in pollen viability exists among clones with *Musa* species. Mycorrhizal association with the higher plants has also shown an increase in agricultural

productivity. The percent colonization of AMF with the root system was calculated by two methods. Compare to Method – I and Method – II reveals that in Elachi 2.5%, Poovan 13.3% and 17.5% of variations recorded. Overall in the average highest percent of colonization were recorded in Poovan (76.6%).

Key words: Banana, Ploidy, Pollen, AMF, Chloroplast

INTRODUCTION

Banana (*Musa* spp.) are giant perennial grasses that are cultivated in the tropical and subtropical regions of the world, belongs to the family Musaceae. Commonly known as 'Apple of paradise'. These are one of the important staple foods in many countries. The genus *Musa* has 22 chromosomes in wild species and 22, 33 and 44 in cultivated forms with a basic haploid number of 11 chromosomes. Most cultivated clones are parthenocarpic triploids with a very low seed set which makes their breeding very difficult[1]. Banana cultivated varieties are predominantly triploids (2n = 3x = 33) and develop the fruit by vegetative parthenocarpy. Few species are diploids and few are tetraploids[2]. Ploidy is the number of complete sets of chromosomes in a cell. Somatic cells, tissues, and individuals can be described according to the number of sets of chromosomes present per cell (i.e. the ploidy level). The number of chromosomes sets in a cell is one of the defining characters of a species or cultivar. Wild species and subspecies of banana are all diploid, whereas cultivars can be diploid, triploid or tetraploid. Ploidy has conventionally been determined by counting chromosomes in dividing root tip cells, a labor intensive procedure that is made difficult in *Musa* by the fact that the chromosomes are small and numerous. The identification of the VAM fungi directly from the roots has been difficult. One of the striking features of VAM fungi is their very wide host range which includes angiosperm species belonging to almost all the families even the roots of some aquatic plants are colonized by VAM fungi. Mycorrhizal association with the higher plants has also shown an increase in agricultural productivity. Hence now a day it can be used as a tool for increasing the yield of the crop plants by the commercial application of the mycorrhiza to the crop plants of commercial importance[3].Broad variation in pollen viability exists among clones with *Musa* species. Generally diploid species produce more viable pollen than tetraploids, which in turn produce more viable pollen than triploids. A mathematical approach to the evaluation of pollen diameter was used in which the diameter of pollen of tetraploids is comparable to 2n pollen diameter of diploids and bigger than pollen of diploids, thus the evaluation of pollen diameter of different genotypes helps estimation of pollen variety in different *Musa* genotypes[4-5]. The present work decided to select four traits of Banana i.e., Robusta, Elachi, Poovan, and Nendran. Poovan, Cavendish cultivars (Robusta and Dwarf Cavendish), Silk (Rasthali), Karpooravalli (Pisang awak) and Virupakshi, 'Nendran' and Ney Poovan are the important cultivars grown commercially in India. All the four traits were evaluated for the stomatal density, size, chloroplast density in guard cells and determination of the pollen size for the analysis of ploidy.

MATERIALS AND METHODS

Chromosome counting in *Musa* spp. by traditional technique: The traditional method of chromosome counting in *Musa* spp. is being done by manually counting the number of chromosomes in the actively dividing metaphase cells under higher magnification of about 1000 X.

Collection of the samples: The collection of plant material (*Musa* spp.) has been done initially by conducting field visits. The collection is done by visiting the local regions in Mysore and Wayanad, Kerala. The material collection details such as variety, date, time and place of the collection are noted done. The information about the variety is obtained with the help of farmers. The material such as leaf and pollen of Banana traits are carefully placed in polythene bags and directly brought to the laboratory for conducting further analysis.

The roots were collected from the plants by removing underground roots and small roots having the small root tips of about 1 cm are excised and brought to the laboratory in the zip-lock polythene bags. For the present work three local traits of *Musa* sp. were selected they are, Elachi, Poovan, and Nendran Banana traits

Storage of collected materials: The collected root tips are directly treated with 0.03% 8-hydroxyquinoline for up to 4-9 hours and then the roots are transferred to the maceration fixation solution/storing solution for a minimum about 12 hours before the slide preparation. The roots may be stored in the same solution until further use.

Sample preparation:

Root sampling and treatment: Collect root tips (1 cm long) after the sunrise. Immerse the root tips directly in 1ml of 0.03% 8-hydroxyquinoline solution at 20-22 ^{0}C for 4 to 9 hours. Transfer the root tips into the maceration fixation solution in sealed tubes (5-10 ml) for about 12 hours (overnight)

Slide preparation: Pull out a root tip from the maceration fixation solution and briefly blot dry on filter paper with the forceps. Cut off the apical root portion of about 1.5 mm length. Place the apical portion in the center of a microscope slide in a drop of the staining solution. Put the cover glass on and firmly squash the preparation with the finger without horizontal moving. Wipe the excess stain off the slide with filter paper. Flatten the preparation with radial vertical pressures from the center to the exterior of the slide with the eraser of the pencil. Pressure must be exercised to remove air bubbles from the preparation with enough force to spread chromosomes within the cells but not to burst cell walls. Wipe the excess stain off the slide again with filter paper. Wait for 6 to 8 hours before starting the observations. The stain should take about two days to intensify and provide good contrast. The preparations should also be sealed with nail polish for later microscopic observation.

Observation of useful cells: Make a mark on the top right of the slide. Put the slide on the platform of the microscope with the mark on the right. Scan the slide in forwarding and backward directions. Locate the position of all good cells with well-identified chromosomes on the slide. Complete the scan of the slide and position the previously identified good cells by the noted coordinates. Pass the microscope to the 100 X objective and observe the cells to count the chromosomes.

Chromosome counts on useful cells: Select only the cells with the chromosomes well separated and little overlap, in which chromosomes can be unequivocally defined and counted. Make a drawing and a photograph or an electronic image of the cell showing all the chromosomes. Count chromosomes from five or six independent cells from the same plant material.

Study of stomatal density and size: From the collected leaf find a suitable leaf and identify the upper and lower surfaces of the leaf. Spread a thin layer of clear nail polish on each surface, the upper side and

lower side of leaf surface leave it to dry. Place a strip of clear sticky tape over the nail polish press the tape down to make a good connection with nail polish. Peels off the sticky tape, the layer of nail polish should come off with tape. Place the tape impression on a microscopic slide. Use a razor blade to trim the excess sticky tape from the edge of the slide. Place the impression from the other side of the leaf on the other part of the slide. Label the slide with the plant name and the upper and lower surface. View under 100 X or 400 X magnification in the microscope. Calculate the stomata in at least five FOVs on each leaf surface. Record the results in a table and calculate stomatal density by using the formula.

FOV (field of view) at 400 X magnification = 0.45mm.

Area of FOV = $3.14xr^2$

Stomatal density = No. of stomata in entire FOV/Area of mm^2

Calculation of Chloroplast density: The sections of 3-5 true leaves at emergence stage were taken. The lower epidermis is removed by piercing the leaf with hand and place on a glass slide after addition of a drop of distilled water. The number of the chloroplast is recorded under observation by a microscope at the number of chloroplast in each side of guard cells of stomata have to be noted. Five stomata per leaf were observed on the basis of evaluation.

Floral characteristics of *Musa* spp.: Female and male flowers of *Musa* genotypes were carefully detached from the inflorescence and taken to the laboratory. The stamen and the pistil were carefully cut out with a razor blade. The stigma length was measured by carefully placing the measuring rule on the stigma and reading out the value in centimeters. Stigma width was measured horizontally while the style length was measured by placing the rule straight from the lower to the upper part of the style. Anther length was measured carefully by placing the rule vertically on the anther.

Analysis of Pollen size in four traits of Banana: Pollen samples were collected from different traits of Banana between 7:30 am and 10:30 am. Pollen grains were manually dislodged from the stamens. Spread the pollen on a glass slide and stained with aceto-carmine with glycerol jelly. The preparation was covered with a coverslip and allowed to stand for 24 hrs to allow passive uptake of stain. The slide preparation was then observed under higher magnification (40 X). The diameters of ten randomly selected deeply stained grains were measured with the aid of a graduated eyepiece.

Study of Vesicular-arbuscular mycorrhizal (VAM) association in *Musa* spp.: The mycorrhizal association with the banana in their roots can be observed by clearing and staining of the roots collected from the underground root system. Hence the clearing and staining of the roots can be done by the following procedure.

Fine roots from plants of the same species were randomly collected and mixed properly and a composite root sample was obtained. Modified trypan blue method was followed for the determination of the intensity of root colonization as described by Vierheiling[6] in1998.

Sample preparation: Wash the VAM infected roots thoroughly and they were cut into 1cm bits and fix them overnight or store in FAA. Remove FAA from the roots by washing with several changes of tap water. Transfer samples into 10% KOH solution taken in autoclave resistant jars. Clear the samples by autoclaving those at 15 lb pressure for 15 minutes (Delicate root samples require shorter clearing times).

Rinse with several changes of tap water and wash with deionized water. Stain roots into staining solution [Made of equal volumes of 80% lactic acid, glycerine and distilled water with 0.1% (w/v) chlorazol black-E or even the 5% ink (blue ink) is diluted in Vinegar (5% acetic acid) can also be used for the staining]. Keep stained roots into glycerine overnight for storage. Mount roots on a clean glass slide using mounting fluid. 4-5 slides per accession should be made. Observe slides under the microscope for VAM fungi (vesicles and arbuscules).

In the present investigation, there are two methods to follow for the calculation of percentage association of AMF with Banana root system.

The method I: Assessment of VAM fungal colonization: Percent root colonization of VAM fungi in the representative root samples was evaluated by using the formula as follows:

$$\text{Percentage of root colonization} = \frac{\text{Total no. of root bits shows colonization}}{\text{Total no. of root bits observed}} \text{ X } 100$$

Method II: Assessment of VAM fungal colonization: Percent root colonization of VAM fungi in the representative root samples was evaluated by using the formula as follows:

$$\text{Percentage of root colonization} = \frac{\text{Total no. of microscopic fields show colonization}}{\text{Total no. of microscopic fields observed}} \text{ X } 100$$

RESULTS AND DISCUSSION

The chromosome counting of Elachi, Poovan and Nendran Banana traits, the following results were recorded. In the traditional technique of the chromosome counting, 10 microscopic fields of well-spread cells were taken into consideration. In the Elachi trait, 11-12 chromosomes per cell are being observed, whereas in the Poovan, 13-14 chromosomes per cell were seen. In Nendran, about 10-11 chromosomes per cell were counted. On average, a number of chromosomes recorded in Poovan (13.2), followed by Elachi with 11.1 chromosomes and Nendran with 10.9 chromosomes.

In the genus Musa, accurate determination of the ploidy by chromosome counting is laborious[7]. Agrawal[8] revealed cytological abnormalities of 17 South Indian banana varieties. Chromosome counts of 20 Bihar banana cultivars were reported by Roy and Sharma[9]. Valsala and Nair[10] in 1990 reported chromosome number of 98 cultivars. Somatic chromosomes of 53 *Musa* landraces and hybrids were reported by Osuji *et al.*,[11] and 16 *Musa* species and landraces by Dolezel *et al.*,[12]. Variations were observed within groups may have resulted in the evolution of new cultivars. In the present investigation, the Bakry and Shepherd[13] protocol was followed for the counting of chromosome numbers. Chromosome counting methods are a basic tool for the determination of ploidy in Banana.

There are various methods of determining banana ploidy level, one among is estimating stomatal density and size [14]. Among the four *Musa* species studies where done on stomatal density and size (Table – 1), the diploid stomatal density varies from 50 to 80/mm^2 while in triploid, it varies from 32 to 47/mm^2 in tetraploids, it ranges from 13 to 17/mm^2. The stomatal density decreased with the increase in ploidy level. In the present investigation Elachi, Nendran and Poovan stomatal density range from 50 to 55/mm^2 which are diploid, while Robusta ranges 34/mm^2 which is triploid. In Table – 2 illustrate the size of the stomata

in μm^2, highest stomatal size recorded in Poovan traits (48 μm^2) followed by Robusta (45 μm^2), Nendran (28 μm^2) and Elachi (21 μm^2). The leaf stomata frequency of the genotypes can be related to the process of adaptation of the trees. Verification of ploidy levels in progenies from triploid x diploid crosses is required due to the production of diploid, triploid, tetraploid, hyperploid and aneuploid hybrids. Ploidy levels are estimated by phenotypic appearance and confirmed either by root-tip chromosome counting or stomatal size and density[15].

The aims of this work were to document how stomata are distributed on the leaf surface and to determine if there is any significant variation in stomatal characteristics among *Musa* species, and finally to study the relationship between the stomatal density of the four traits. Stomatal density (SD) can vary within leaves, plants, and individuals of a single species[16].

Table – 1: Stomatal density of four Banana traits

Sl. No.	Name of Traits	Magnification (ocular × objective)	Area of FOV (Field of view)	No. of stomata in entire FOV	Stomatal density Stomata/mm^2
1	ROBUSTA	400 X	0.45mm	22	34.59
2	ELACHI	400 X	0.45mm	34	53.47
3	NENDRAN	400 X	0.45mm	33	51.89
4	POOVAN	400 X	0.45mm	35	55.04

Table – 2: Stomatal length, breadth and size of four Banana traits

Sl. No.	Name of Traits	Stomatal Length (µm)	Stomatal Breadth (µm)	Stomatal Size (μm^2)
1	ROBUSTA	9	5	45
2	ELACHI	7	3	21
3	NENDRAN	7	4	28
4	POOVAN	8	6	48

Table – 3: Total number of chloroplasts in guard cells of the traits**.**

Sample Name	Number of chloroplast in guard cell
ROBUSTA	14
ELACHI	8
NENDRAN	8
POOVAN	8

Table – 4: Floral phenotypes of four traits of Banana

Sl. No.	Name of Traits	Stigma length (cm)	Stigma width (cm)	Style length (cm)	Anther length (cm)
1	ROBUSTA	0.4	0.2	3.4	2.4
2	ELACHI	0.5	0.3	4.5	2.3
3	NENDRAN	0.4	0.3	4.2	2.2
4	POOVAN	0.4	0.3	4.3	2.5

Another method of ploidy determination is by chloroplast count in a guard cell of stomata. The chloroplast count of diploid ranges from 5 to 7 chloroplasts, while for tetraploids 10-12 chloroplasts. Here Elachi, Nendran, and Poovan contain 8 chloroplasts in the guard cell thus belongs to diploid, while Robusta posses 14 chloroplasts (Table – 3). In the analysis of floral characters of all the four traits reveals that there is no significant difference in stigma length and width (0.1 cm variation between each other)(Table - 4) but variation found in style length, it is high in Elachi (4.5 cm) compare to Robusta (3.4 cm). Similarly, no significant variation recorded in anther length (0.1 cm between the traits).

The polyploidy has been an important factor in the development of improved commercial varieties and hybrids of the *Musa* spp. A correlation between the mean number of chloroplasts and the number of chromosomes was observed by Frandsen[17] and estimated the number of chromosomes in potato using the mean number of chloroplasts per pair of guard cells. The chloroplast number in epidermal guard cells as an indirect ploidy indicator was evaluated on seed-grown and tissue culture-derived maize plants[18]. The suitability of stomata length as a criterion in the distinction between diploid and tetraploid rye-grass plants was tested[19]. The differences observed from the length, breadth, and nature of the style and anther was significant among the various genotypes[20].

The pollen size of all the traits of Banana shown in Table – 5 and the data interpretive that highest small, moderate and large pollen size record in Poovan with 47 µm, 55 µm, and 61 µm respectively. It is followed by Elachi with moderate and large pollen size record as 45 µm and 55 µm respectively. Nendran trait show very less small, moderate and large pollen size (28 µm, 31 µm, and 40 µm). In an average, Poovan stands first with pollen size 54.33 µm followed by Elachi with 44 µm and 40.33 µm in Robusta. Very less pollen size record in Nendran with 33 µm.

Table – 5: Pollen size measurement of different traits of Banana

Sl. No.	Name of Traits	Small Pollen (µm)	Moderate Pollen (µm)	Large Pollen (µm)	Average
1	ROBUSTA	34	40	47	40.33
2	ELACHI	32	45	55	44.00
3	NENDRAN	28	31	40	33.00
4	POOVAN	47	55	61	54.33

Table – 6: Percentage colonization of VAM in three *Musa* traits.

Name of *Musa* traits	VAM fungal colonization Method - I in %	VAM fungal colonization Method - II in %	Average of Method - I and II in %
Robusta	65	68	66.5
Elachi	60	62.5	61.2
Poovan	83.3	70	76.6
Nendran	40	57.5	48.7

Pollen size is often used as a biological parameter to estimate the ploidy and viability of mature pollen grains. Pollen size distribution within a genotype can be explored as a means to efficiently separate triploid from diploid and tetraploid roses and the study was to determine the usefulness of pollen diameter and guard cell length to predict sporophytic and gametophytic ploidy levels in a diverse collection of roses [21]. Pollen size is often used as a biological parameter to estimate the ploidy and viability of mature pollen grains in *Arabidopsis* plants[22]. Pollen size in some plant groups is positively correlated with genome size and/or ploidy; in this study we set out to quantify the relationship between pollen grain size

and ploidy level in species of Rhexia, potentially providing a means to determine ploidy for future studies without the labor-intensive processes and specialized equipment required for chromosome counting[23].

In Elachi cultivar the vesicular endomycorrhiza has been observed as the bladder-like structure is observed along with the hyphae, but the hyphae do not show any arbuscules only the vesicles are being observed. In Poovan the arbuscular endomycorrhiza with the vesicles has been observed and the hyphae produce a branched arbuscules penetrating into it. In some of the cells, the hyphae are present intermingled in a single cell densely with arbuscules. In Nendran the ectomycorrhiza is appeared dense along the cell wall but very little penetration was observed inside the cells. The vesicles and arbuscules were not-a-tall appearing in this trait.

The percent colonization of AMF with the root system was calculated by two methods and it is illustrated in Table – 6. In method – I Poovan trait shows more VAM colonization (83.3%) followed by Robusta (65%), Elachi (60%) and Nendran (40%). In method – II Poovan trait also show more VAM colonization (70%) followed by Robusta (68%), Elachi (62.5%) and Nendran (57.5%). Compare to Method – I and Method – II reveals that in Elachi 2.5%, Robusta 3%, Poovan 13.3% and 17.5% of variations recorded. Overall in the average highest percent of colonization was recorded in Poovan (76.6%).

In banana varieties, an association of VAM fungi has been reported by Girija and Nair[24] and the beneficial effects of VAM association in a micro propagated banana are well documented by Lin and Chang[25]. *In-vitro* derived banana plants inoculated with VAM and with phosphate solubilizing bacteria were significantly taller and produced greater dry matter compared to the untreated plants[26-27]. Vidhya *et al.*[28]also reported maximum bunch weight, no. of hands/bunch, no. of fingers/bunch and yield of Robusta banana in the treatment of 100 % RDF with Azospirillum + Phosphobacteria + VAM.

Phirke and Mahorkar[29] concluded that fortification of soil with organic manures like nitrogen fixers, phosphate solubilizing microbes, Vesicular Arbuscular Mycorrhizae (VAM) and bio-fertilizers not only increased soil porosity but also infiltration of water in banana fields. The above literature reveals that VAM is one of the major required fertilizers for enhancement of yield in Banana. Sukhada[30] also reported a significant yield increase in banana by the use of Azospirillum and VAM association in Poovan and Ney Poovan cultivars of banana. In the present investigation, the determination/presence/absence of VAM in three traits of Banana shows the ability of nutrient uptake from soil.

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