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Study of Androgenesis in (*Musa balbisiana*) cv. Bhimkol Banana and *in vitro* Regeneration of Haploids Using Isolated Microspore Culture

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A B S T R A C T

In-vitro androgenic haploids in *Musa. balbisiana* L cv. bhimkol were produced by anther culture at uninucleate stage of pollen. Young 30 day's old immature male flowers with 5 stamens obtained by cutting out the bracts in between 20th to 30th were used as explants for induction of callus. The best medium for inducing callus from anthers were found to be MS (Murashige and Skoog) basal medium supplemented with 6-BAP (1.0 mgL^{-1}) and IAA (Indole-3- acetic acid 2 mg/l.) with a frequency of 27.33±2.52 while best media for regeneration of shoots from callus was found to be MS medium supplemented with 7mg/l 6-BAP, 0.5mg/l IAA, 350 mg/l CH. Regenerated shoots were isolated and inoculated on MS medium supplemented with four different concentrations (0.25, 0.5, 1.0 and 2.0 mg/l) of IBA to induce root. IBA at a concentration of 1.0 mg/l produced best rooting. In our study out of 350 anthers inoculated only 12 plants were regenerated from anther callus and embryogenesis, all the plants were transferred and established in the potting mixture containing soil, vermicompost and farm yard manure in the ratio of 1:1:2. Using direct method of chromosome counting 58.33 % plants were found to be haploids and 41.66 % plants were diploids. This is the first report on the production of haploid plants in cv. bhimkol However, diploids banana plants were also found.

Introduction

Bananas (*Musa* spp.), including dessert and cooking types, are giant perennial monocotyledonous herbs of the order Zingiberales, a sister group to the well-studied Poales, which include cereals. A draft Musa balbisiana genome sequence for molecular genetics in polyploid, inter- and intra-specific Musa hybrids reported by (Davey *et al.*, 2013) and recently draft genome of *Musa balbisiana* with genome size of 430 Mb (87%) assembled into 11 chromosomes was reported by (Wang *et al.*, 2019). Bananas are vital for food security in many tropical and subtropical countries and the most popular fruit in industrialized countries (Lescot, 2011).

The *Musa* domestication process started some 7,000 years ago in Southeast Asia. It involved hybridizations between diverse species and subspecies, fostered by human migrations (Perrier, X. *et al.,* 2011) (Stover and Simmonds, 1987) and (Devi *et al.,* 2017). The global sales of banana is more than 5 billion ($US) per annum and the crop ranks fourth in terms of production in the world and is second most significant fruit crop in India next to mango (Subramanyam *et al.,* 2011). Banana is cultivated in nearly 140 countries it is the fourth most important food crop in the world and second in India after mango. Fruits are rich in carbohydrate, minerals, phosphorous, calcium, potassium and vitamin-C, and are popular for its year-round availability, good production as well as high consumers preference for its taste and aroma. Cultivated bananas are derived from two species of the genus Musa, *M. acuminata* (AA) and *M. balbasiana* (BB) (Van den houwe *et al.,* 1995). Mixing of A and B genomes have given rise to different genotypes in cultivated edible bananas, including diploid (AA, BB and AB), triploid (AAA, AAB and ABB) and tetraploid (AAAB, AABB, ABBB) variants (Simmonds and Shepherd 1955).

In Assam, India *Musa balbisiana* has two cultivars known as Athiyakol and Bhimkol found both in wild habitat and cultivated form under (BB) genomic group is a diploid with 2n=2x=22, (Simmonds,1962) the various parts of the plant, like fruit peel, pseudostem, flower, leaf, fruit pulp, are widely used in food preparations, rituals and in traditional medicine. Young tender banana pseudo-stem is a very rich source of iron and fibers (Kalita *et al.,* 2004). It is considered as staple baby food due to highly nutritious sweet pulp and various parts of the plant are used either as food, in religious rites and also as medicine for treatment of diseases like jaundice, dysentery etc. (Borborah *et al.,* 2016). The people of Assam and parts of NE India consume the fruits of bhimkol banana as dietary supplement to obtain nutrition as it is rich in carbohydrates, vitamins and proteins. Fresh ripe pulp of the fruit possesses antiperoxidative and antioxidant properties which can prevent oxidative stress related disease (Bhattacharjya *et al.,* 2015).

Bhimvita and Bhimsakti are two such nutritious baby food products made from fresh fruits of bhimkol available in the market. Tender inner pseudo-stem and male buds are used as vegetable, leaves and pseudo-stem are used in different ways in rituals and ceremonies to serve food and prasad. As such, this plant has an immense commercial value in the region (Borborah *et al.,* 2016). In spite of many qualities bhimkol banana has not gain much popularity due to lack of systematic planting strategies, long crop cycle (740 days) and presence of seeds (150-160) in numbers which are compactely arranged in the fruit this hinders pulp extraction.

Naturally haploids are produced through parthenogenesis (development of haploid plants from unfertilized eggs) or androgenesis (development of haploid from pollen culture). They can be artificially induced through culture of ovaries (Muren, 1989). The conventional breeding methods are tedious and require many years to obtain haploids. The other approach to commercial plant breeding is the use biotechnological means, use of tissue culture techniques and development of *in-vitro* haploid plants through androgenesis. As the haploids are sterile, the fruits produced by them would be seedless. The in vitro production of haploid plant is a tremendious asset in genetics and plant breeding, as with haploids, homozygosity can be achieved in a single step by a doubling of the chromosomes by using colchicine (Guha and Maheshwari, 1964).

This is particularly important for a long-generation banana cultivar bhimkol which bear fruits in 740 days from planting (kotoky 2000). To date there is no report on halpoids in cv. Bhimkol. Therefore our study on androgenesis, highlights the feasibility of *in-vitro* haploid production in bhimkol using anthers at uninucleate stage of pollen. These haploids plants obtained could be used in conventional breeding programs to generate pure homozygous lines and auto-tetraploids in a single generation.

Materials and Methods

Explant collection and sterilization

Banana cv bhimkol (*Musa balbisiana*) inflorescence of 30 days old after branch shooting were collected to be used as explant (Fig. 3 a). from Lichubari, Jorhat, Assam, we also collected the ripe fruits to count the number of seeds arranged in the pulp.The inflorescence and fruits were treated with 70% ethanol for 2 minutes then dipped in fungicide solution 0.2% bavistin for 5 minutes followed by 5 time rinse in sterile distilled water and again treated with 0.2% $Hgcl_2$ for 10 minutes and followed by 5 washings with sterile distilled water. Anthers were dissected out using binocular steriozoom microscope using pre-sterilized Petriplates, forceps, and fine needles. The male flowers possessing five stamens were taken from the 20^{th} to 30^{th} bract of the inflorescence (koldil in Assamese language) (Fig. 3 b and c). Anthers measuring 3.5 cm in length were carefully isolated and transferred into 55×15-mm pre-sterilized Petri plates under laminar airflow cabinet and all operation were carried out under aseptic conditions. Once dissection was over one anther from each bract was set aside for cytological studies. The stage of microspore were ascertained by using 0.8 % aceto-carmine stain, microspores were found to be at uninucleate stage with centrally located nucleus and vacuole (Fig. 3d), at this uninucaleate stage anthers were cultured for inducing callus or indirect embryogenesis in pre-sterilised, disposable Petriplates containing 10 ml of MS (Murashige and Skoog1962) modified medium supplemented with different concentration of 2,4-D, and 6-Benzlyaminopurine (BAP) either alone (Fig. 1) or in combination with Indole acetic acid (Fig. 2) with 3 % sucrose and 0.8 % agar Himedia®, pH was adjusted to 5.8 before autoclaving at 121ºC. The Petriplates were sealed with Parafilm and kept in dark for 8 weeks. All cultures were maintained at 24^{o}C at 50–60% relative humidity under a 16/8-h (light/dark) photoperiod with diffuse light.

Regeneration of planlets

The MS media contained both macro and micro- nutrients augmented with 30 g/l sucrose, 8 g/l agar, 160 mg/l adenine sulfate, 100 mg/l tyrosine and the growth regulator 2, 4-D tested at the range of 0.3 to 1.5 mg/l. The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min. The cultures were kept at 25±2°C in darkness and maintained on the same medium until callus emerged. Subculture before callus development turns the anthers into brownish black in colour might be due to polyphenol secretions therefore culture were kept untill calli appeared. Calli were observed on MS basal medium supplemented with BAP (1.0 mgL^{-1}) and IAA (2.0 mg L^{-1}) (Table 1).

Calli thus obtained were cut into small suitable pieces which ranged 3-4 for each callus depending on size and were transferred to regeneration medium composed of MS medium frequency was evaluated after 45 days of incubation. However blackening of calli was observed due to release of polyphenol from the cut surface after 30 days of inoculation. Following two round of subculture at 15 days interval in MS Media

with 100mg/l of adenine sulphate and tyrosin regeneration was observed. Regenerated androgenic embryos like structures developed from callus were transferred to regeneration medium containing 6-BAP 7mg/l + IAA 0.5mg/l and CH 350 mg/l Initially the calli first turned brownish black in the regeneration media but as we kept the cultures for 6 weeks without subculture green nodular structures appeared all over the calli. The media was fortified with 100mg/l of adenine sulphate and tyrosin. After another 14 days distinct green shoot buds appeared on the nodular portions of calli. In 8- week-old cultures twenty five percent of callus had turned green and produced shoots (Fig. 3e).

Induction of roots in regenerated plants and transfer

The well grown elongated shoots were aseptically transferred to the medium containing 0.25, 0.5, 1.0 and 2.0 mg/ l IBA and without hormones fortified with 0.2% activated charcoal for the proper development of root system. The roots were induced at 1.0mg /l of IBA, the plantlets with 2-3cm in length were removed from culture jars and transferred to soil in the net house for primary hardening where the root of the plantlets are first gently washed free of agar and sucrose present in the medium adhered to roots.

All rooted plants were washed with 0.2% of bavistin solution then followed by primary hardening in cocopeats, maintained in polythene house tunnel for a period of two weeks.

During this period, 80% humidity is maintained for the initial 20 days under diffused light, later plants were kept at 70 %, humidity and 26 °C then transferred to green house for secondary hardening in a polythene bags containing soil, vermicompost and farm yard manure in the ratio of 1:1:2 respectively and later transferred into pots (Fig. 4 f).

Cytogenetics study for determination of ploidy level

There are two main methods first one is Cytogenetics studies which is performed by direct chromosome counting from root meritem tips and second methods is Flow cytometric analysis which is performed to see and record the leaf DNA intensity from plants growing under in-vitro regenerated plants where a prominent peak of nuclei in PI fluorescent intensity in the interphase stage is observed. However in our study, we performed direct chromosome counting as per the protocol described by (Sharma and Sarkar 1955) on prestaining treatment and squashing for somatic chromosomes in coconut palm. In our study, the root meristem tips were collected in the morning hours between 9.0 to 10.00 am from the anther derived plants grown in vitro, a diploid parental plant was used as an internal standard. Root meristem tips were cut into 1 cm size inoculated in a tube containing saturated para-dicholobenzine and aesculine and treated for 2.5 to 2 hour at 18^0C. Then the meristem tips were fixed onto slide using 1: 3, acetic acid: ethanol solution for 12 hours (overnight) next day after pretreatment at room temperature root tip were treated with 5N HCL solution and kept for 5 minutes in deep freezer and thereafter root tips were taken out from the deep freeze, these were washed properly using 1% aceto-orchine (Sigma®) and kept it for 2 days. After 2 days the root meristem were cut and on a top of a glass slide, concentrated acetic acid was added and proper pressure was applied using a cover slip to smear it uniformly then number of chromosomes were counted using compound microscope at 100x magnification. On counting we found n=11 number of chromosome from anther derived plant, however, in the standard diploid plants the number was found to 2n=2x=22. As shown in the Fig (5 a and b). The percentage of haploid are concerned 58.33 % plants were found to be haploids 41.66 % plants were diploids.

Results and Discussion

Anthers with uninucleate microspore stage was ascertained by 0.8 % acetocarmine stain at this stage anthers were inoculated into modified MS media for development of callus and kept in the dark. Callus development and shoot regeneration were investigated on MS basal medium supplemented with different concentration of 2,4-Dichlorophenoxyacetic acid, and 6-Benzlyaminopurine (BAP) either alone or in combination with Indole acetic acid (IAA). with increasing 2,4-D concentration callus formation increased in (Figure 1) however maximum callus formation was observed on MS medium supplemented with BAP (1.0 mgL^{-1}) and IAA (2.0mg L^{-1}). On transferring to regeneration media anthers began to swell and the size increases to 3 folds as the vegetative nucleus divides. After 20 days of incubation, callogenic mass was noticed while the anther wall cells turns black (Fig. 3e). After 56 days of culture organization of embyronic nodules were appeared from the sprouted callus mass. The androgenic embryroids differentiated into shoots and our result was similar to the findings of Assani *et al.*, 2003 and Chaturvedi *et al.,* 2003. In our study the interaction of BAP at higher levels (1.0 -7.0 mg/l) and IAA at lower levels (0.1 - 0.6 mg/l) induced the growth of the embryonic mass in to shoot buds. The development of shoots organized at each combination of BAP and IAA is shown in the (Fig. 4 e) obtain after 6 months. The shoot multiplication was optimized at the concentration of 7.0 mg/l BAP and 0.4 mg/l IAA with a mean of 6.10±0.48 shoots per callus. The shoots buds were formed which were maintained on the media up to 4 subculture at an interval of 25 days, and then they were transferred to the root induction media. The emergence of roots depends on the nutrients composition and growth regulators. Shoot buds were transferred to MS medium with 1.0 mg/l IBA, 0.2% activated charcoal initiated roots from the base of microshoots after 30 days of culture. The well rooted plantlets were hardened primarily in coco peats containing equal proportion of soil, sand, and farm yard manure in poly bags and kept in polythene tunnel for a period of 14 days and then they were subjected to secondary hardening in transparent polythene bags in the net house (Fig. 4 f). The diploid plants was also observed among the regenerants the first reason could be when similar looking vegetative nuclei get fuse together producing homozygous diploid plant or callus and the second reason might be due to regeneration of diploid anther tissues such as anther wall or connective tissue, third possibility is the spontaneous chromosome doubling in haploid cells. The possible factors leading to spontaneous doubled-haploid plants could be nuclear fusion in the early divisions of the microspores, endomitosis, endoreduplication or multipolar mitosis during the callus phase (Chen *et al.,* 1982; De Buyser and Henry1986). The polar transport of auxin is essential for the establishment of bilateral symmetry during embryogenesis in dicotyledonous and monocotyledonous species (Fisher *et al.,* 1996) (Table 2).

The process of obtaining haploid plants in *Musa balbisiana* is very difficult through conventional method because of long crop cycle of (740 days) in cv bimkol. On the other hand, homozygous plants can be obtained in a single generation by diploidisation of haploids. However, *Musa balbisiana* species plays an important role in banana breeding because it contains resistant genes against banana diseases (Foure, 1993). Here we showed highest percentage of callus formation was obtained on MS medium supplemented with BAP (1.0 mgL^{-1}) and IAA 2.0mg L^{-1} (Table 3) if the cultured anthers contained microspores at the uninucleate stage and regeneration of haploid plant by inducing axillary buds.

Table.1 Callus formation from anthers

Formation of Callus per 50 explants (Mean ± Standard Deviation)			
Conc. 6-BAP(mg L^{-1})↓		Conc. 2,4-D(mg L^{-1}) ↓	
0	8.33±0.58	0	11.67±1.15
0.3	12.33±1.15	0.3	13.67±1.21
0.5	13.67±0.60	0.5	15.70±1.53
0.7	15.63±0.55	0.7	16.35±1.47
1	15.35±1.53	1	19.33±1.70
1.3	10.67±0.58	1.3	16.67±1.36
1.5	11.67±1.53	1.5	15.33±1.15
P Value(one sample t-test): P(BAP)=0.000; P(2,4-D)=0.001;			
LSD: LSD(BAP)=0.835; LSD(2,4-D)=1.195			

Table.2 Interaction of 2,4-D and IAA on frequency of callus per 50 explants

Formation of callus per 50 explants (Mean ± Standard Deviation)				
Concentratiuon. 2,4-D (mg L^{-1})↓	Concentration of IAA(mg L^{-1})			
	0.5	1	1.5	2
0	9.67±0.58	12.0±1.73	14.67±1.15	14.67±1.53
0.3	15.67±1.15	11.33±1.15	16.33±1.20	10.67±0.72
0.5	8.65±0.55	7.40±0.60	7.70±0.63	10.00±0.90
0.7	7.67±0.58	8.70±0.63	10.67±1.15	10.33±0.58
P Value: P(2,4-D)=0.000; P(IAA)=0.000; P(2,4-D*IAA)=0.000				
LSD: LSD(BAP)=0.403; LSD(2,4-D)=0.403				

Table.3 Interaction of 6-BAP and IAA on frequency of callus per 50 explants

Formation of callus per 50 explants (Mean ± Standard Deviation)				
Conc. 6-BAP(mg L^{-1})↓	Concentration of IAA(mg L^{-1})			
	0.5	1	2	3
0	7.67±0.58	10.33±0.58	14.33±1.15	11.67±0.58
0.3	10.67±0.58	12.33±1.15	16.33±1.53	11.00±1.00
0.5	15.67±1.15	11.33±0.58	16.67±1.53	12.67±1.15
0.7	18.33±1.53	21.67±2.08	23.00±2.00	17.33±1.53
1	15.67±1.53	22.0±2.0	27.33±2.52	18.67±1.53
1.3	15.33±0.58	15.67±1.15	17.00±1.63	13.33±0.58
1.5	12.67±1.53	16.33±1.53	17.53±0.58	12.00±1.00
P Value: P(BAP)=0.000; P(IAA)=0.000; P(BAP*IAA)=0.000				
LSD : LSD(BAP)=0.554; LSD(2,4-D)=0.419				

Fig.1(a) Graph showing relation between increase in 2,4-D conc. and no. of callus formed out of 50 inoculated explants. 1(b): Graph showing the relation between increase in 6-BAP conc. and no. of callus formed out of 50 inoculated explants

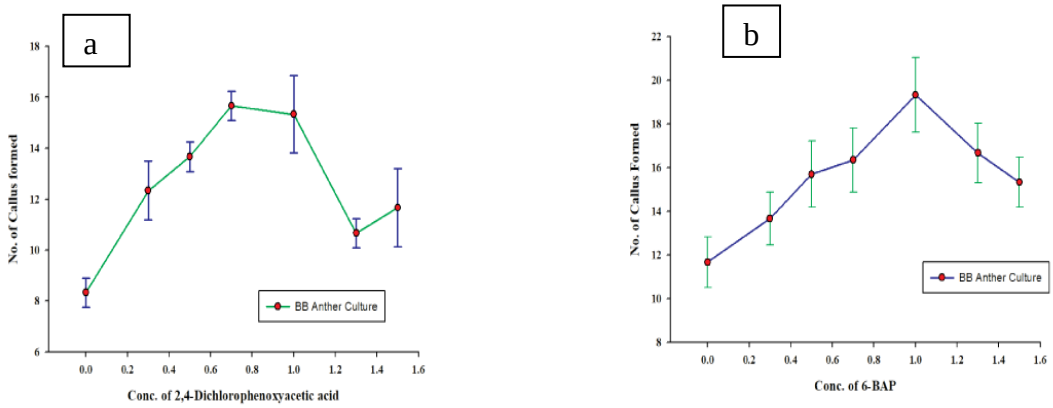


Fig.2 (a) Graphs showing interaction of Interaction of IAA and 6 –Benzylaminopurine 2,4-D on callus formation (b) Interaction of IAA and 2,4 –Dichlorophenoxyacetic acid

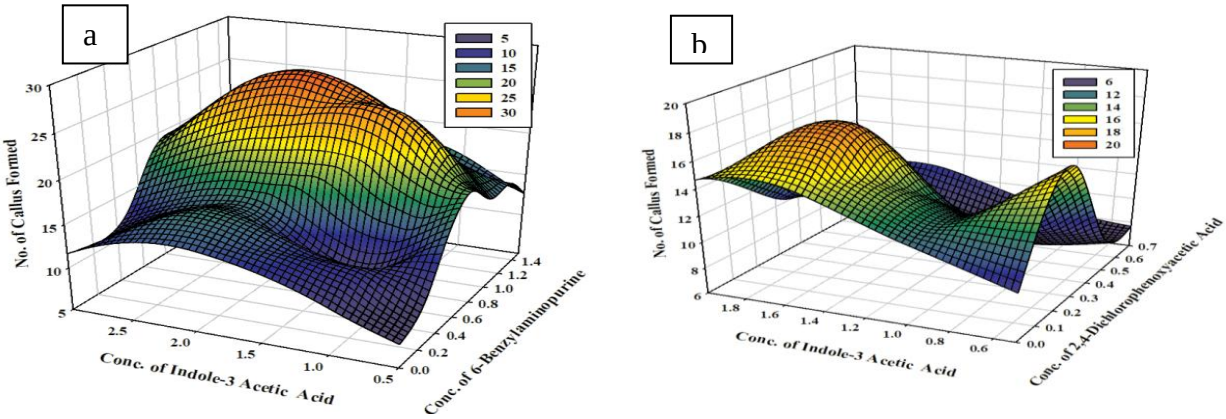


Fig.3 (a) Male inflorescence of cv. Bhimkol banana b) Male flower extracted from 20th to 30th bract c) dissected flower with exposed anthers d) Highly vacuolated uninucleate microspores and nucleus in the periphery observed under steriozoom microscope with 0.8% aceto-carmine staning under 100x magnification

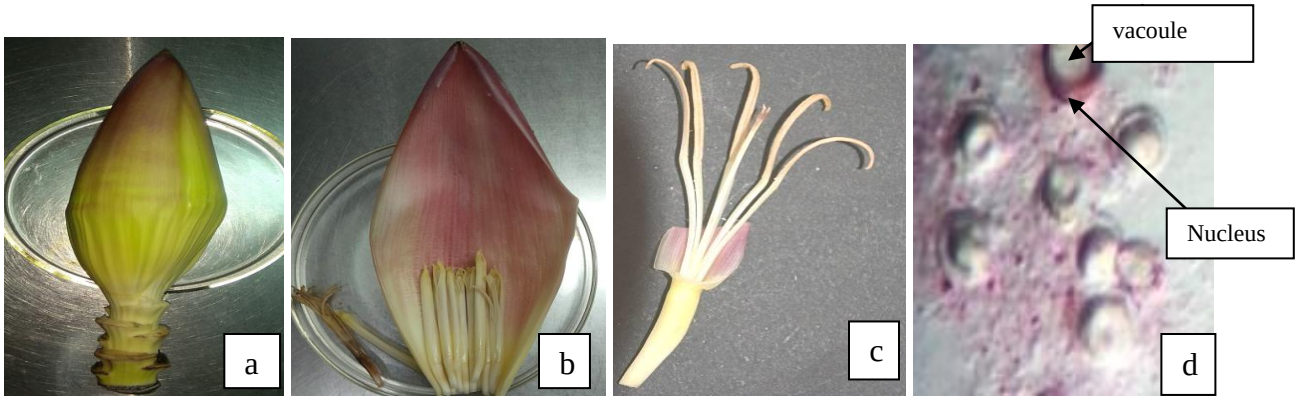


Fig.4 a) Anther culture b) Induction of callus from anthers with uninucleate microspore after 56 days of culture c) Embryogenic calli d) Planlets regenerated from embryonic calli e) Shoot and root induction f) Plants transferred in 1: 1: 1 Soil, FYM and vermicompost in poly bags inside the net house

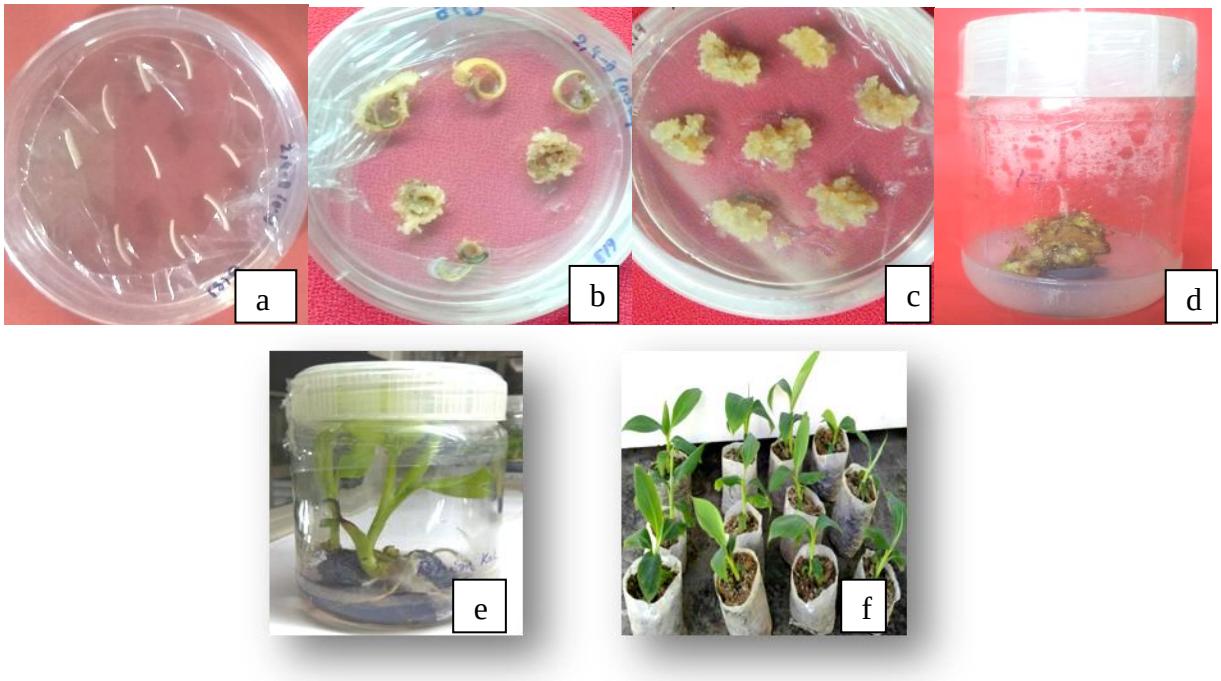
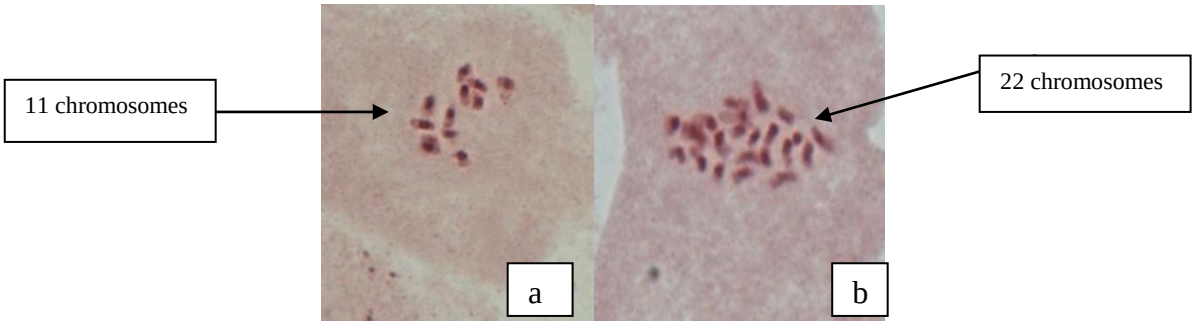


Fig.5 Cytogenetic study : counting number of chromosomes (a) Haploid plant with n=11 chromosomes (b) Control diploid plant with 2n= 22 chromosomes



Development of haploids has been reported in *Musa paradisica* cv Puttabale (Girish *et al.*, 2017) in *Musa acuminata* (Kerbellec 1996) and in cereals like barley (Foroughi-Wehr *et al.*, 1982), wheat (Hu and Kasha 1997), maize (Wan *et al.*, 1991) rice (Alemanno and Guiderdoni 1994) neem *Azadirachta indica* A. Juss (Chaturvedi *et al.*, 2003). The average frequency of calli formed was 16.33±1.20 with the interaction 6, BAP and 2,4-d. However, we got 27.33±2.52 % callus formation when 1.0 mg/l 6, BAP and 2mg/l IAA was used under our culture conditions, which is more than that obtained for rice (20%) (Sathish *et al.*, 1995). The androgenic calli developed into embryos, in our study the embryos produced were similar to those reported earlier on embryogenesis in banana

(Assani *et al.*, 2003). After counting the chromosome numbers from the root tips of all 12 plants growing in the pots. Diploid was also observed in the plants regenerated from anthers callus similar result were found in *Musa Paradisica* by (Girish *et al.*, 2017) while the reason could be when similar looking vegetative nuclei get fuse together producing homozygous diploid plant or this might be due to regeneration of diploid anther tissues such as anther wall or connective tissue, in monocots the regeneration of somatic anther tissue has not been reported. The other reason could be spontaneous chromosome doubling in haploid cells. The possible factors leading to spontaneous doubled-haploid plants could be nuclear fusion in the early divisions of the microspores, endomitosis, endoreduplication or multipolar mitosis during the callus phase (Chen *et al.*, 1982; De Buyser, Henry, 1986) and (Chaturvedi *et al.*, 2003) Karyotypic study and flow cytometric analysis was performed to determine the ploidy level of the anther-derived plants. notably, In anther derived plant number of chromosome was found to be n=11 however, in the standard diploid plants the number was found to 2n=2x=22 (Fig. 5 a and b). This validated our work that haploid in cv bhimkol could be regenerated using anthers with uninucleate pollen followed by shoot regeneration from callus. In the present study we obtained 12 plants out of which 7 plants (58.33 %) plants were haploids and 5 plants (41.66 %) were diploids. These haploid plants of bhimkol are being grown in the field under observation and further research would be required to increase the regeneration percentage from callus cells.

In conclusion, anther culture is the most widely used method for haploids production in plants but in conventional methods of production it takes longer duration. The result reported here showed that haploid plants can be produced in cv. bhimkol banana *Musa balbisiana* using anther culture with uninucleate microspore and these haploid banana plants could be used in banana improvement programme through breeding.

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