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Original Research Paper

Ploidy analysis among Citrus mutants using leaf meristematic tissue

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ABSTRACT

A promising method for preparing metaphase spread for counting the number of chromosomes from the emerging shoot tissue is described in this report. In the present study, we adopted enzymatic digestion of shoot tips to analyse the chromosome number. The chromosomes in metaphase stage of cell division are highly condensed and easy to count in routine cytological technique. Even the morphological features like position of centromere can be seen in metaphase. In prophase it may not be clear as the chromosomes are getting ready for cell division. In enzymatic digestion even the prophase chromosomes are visible, which can be counted. Hence enzymatic digestion technique is more efficient in citrus as compared to acid digestion method as the citrus crop is a perennial crop with small-sized chromosomes. Furthermore, the sample collection in the field was easy and actively growing vegetative flush was available throughout the year. This technique was attempted in the tissue culture lab of ICAR- CCRI in various *in vitro* and *in vivo* ploidy induction experiments in *Citrus sinensis Osbeck* (Sweet orange cv. mosambi), *C. reticulata Blanco* (Nagpur mandarin) and *C. jambhiri Lush* (Rough lemon), for confirmation of diploidy (2n=2x=18), triploidy (2n=3x=27), tetraploid (2n=4x=36), hexaploid (2n=6x=54).

Keywords: Citrus, chromosome, enzymatic digestion, mutants and ploidy

INTRODUCTION

Citrus is one of the most important fruit crops of the world grown in more than 114 countries. More than 70 per cent of the world total citrus production is from northern hemisphere particularly in China, Brazil, India, USA and countries around the Mediterranean. In India, the area under citrus is 1.05 million ha with a production of 13.97 million tonnes and average productivity of 10.30 tonnes/ ha (NHB 2019-20). India is at 3rd position in the production of citrus (FAO 2018). Previous investigation shows that modern *Citrus* species originated in north eastern India and adjacent northern Burma (Gmitter and Xulan Hu 1990). The *Citrus* genus belongs to the family Rutaceae that includes 162 species (Tanaka 1993) and is grown in tropical and subtropical region of the world. In India, 30 *Citrus* species have been reported (Singh and Chadha 1993) in which, nine species are available throughout India. North-Eastern region is a hot spot for biodiversity of citrus and having germplasm of 23 taxa including 68 varieties reported by Sharma *et al*. (2004). ICAR CCRI, Nagpur is officially holding largest collection of valuable citrus germplasm. There are 614 accessions of citrus including 23 rootstocks from exotic sources (from U.S.A. and Australia), 552 from indigenous sources and 39 scion cultivars (mandarin, sweet orange, grapefruit and pummelo from U.S.A., France, Japan and Niger). Besides, 55 superior clones of Nagpur mandarin, 12 of acid lime, 5 of 'Mosambi' sweet orange and 6 of pummelo have been identified (www.ccri.org.in).

ICAR-CCRI had been identified as National Active Citrus collection sites by NBPGR, New Delhi and cytogenetic study of entire citrus germplasm could help in identification of minute genetic variants, in detection of true hybrid in hybridization propagation. This study has enabled to understand chromosome number in evaluation of citrus group (Guerra *et al.* 1997). Further, there is dire need to understand the genetic variation at ploidy level and

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morphology level, due to the existence of huge genetic biodiversity and economic importance of several Citrus species (Hynniewta *et al.* 2011). The available published literature reports suggested the prevalence of different chromosome number in different species such as 2n=18 or 2n=27 in *C. aurantifolia* (Longley 1925; Krug and Bacchi 2003) and 2n=18, 27, 36 in *C. limonia Osbeck*, (Frost 1925a, b) are some examples. Therefore, there is a need to undertake investigations on cytogenetical approaches to define the existing genetic variation in the *Citrus* genus. The present investigations were an attempt to analyse sample which were developed as a part of polyploidy breeding programs at ICAR-CCRI, and samples were triploids, tetraploids and hexaploids, in the citrus scion and rootstock, cultivars as observed in flow cytometric studies.

Citrus chromosomes are small with 2-4µm length (Krug 1943). The mitotic index is mostly low in root tips. High-grade metaphase preparation requires a proper high-resolution metaphase spread. Cytogenetic studies such as chromosome counting in various *in-vitro* and *in-vivo* ploidy experiments, *in-situ* hybridization of higher ploidy species help in cultivar improvement. Currently available and routinely used squash preparation methodology is unable to obtain good quality chromosome spreads.

The enzymatic digestion method for leaf chromosome preparation is a reliable technique to solve drawbacks in conventional squash preparation methodology (Kesara, 2003). The method was reproducible initially for *Citrus sinensis Osbeck* (Sweet orange cv. mosambi), *C. reticulate Blanco* (Nagpur mandarin), *C. jambhiri Lush* (Rough lemon), in woody tree species. Root-tips were not easily available for analysis from the field because growing roots are small and are fibrous in nature. Roots from seedlings are too small and hence only one slide can be prepared from approximately ten roots. Emerging shoot tissue is reliable and most dependable source of active metaphase chromosomes in any plant species.

In the present investigation, ploidy samples with higher number of chromosomal counts such as triploid (2n=3x=27), tetraploid (2n=4x=36) and hexaploid (2n=6x=54) and smaller size of citrus chromosome were hindrance in getting a proper metaphase spread and subsequent chromosome counting. Hence research efforts were directed to develop a technique, based on enzyme digestion hypotonic protoplast dropping methodology, which enables getting a high-quality metaphase spread and counting of chromosomes by using actively dividing meristematic tissue of shoot tips.

MATERIALS AND METHODS

Enzyme digestion and Protoplast dropping methods were used for studying chromosome numbers of mitotically active leaf meristem cells from regenerated ploidy plants following the protocols of Kesara, (2003) with some changes in the protocol, which helped in resolving the problem of spreading and visualization of chromosome in citrus crop. Citrus chromosomes are small in size 2-4µm (Krug 1943) and mitotic index is mostly low. In HCl digestion and squash preparation methodologies, the frequency of getting good quality chromosome spreads especially in citrus crop was low. For tissue collection, the diploid, triploid and tetraploid plants of different citrus species obtained from various sources *i.e.,* in-vitro, greenhouse and natural open field conditions in the experimental block of ICAR-CCRI were collected. The fresh emerging shoots of approximately 2-3 mm in size, harvested from the plants were used as the source of the mitotic cells for leaf chromosome preparation and were placed in ice water (0-4^{0}c) for 24 h to retain in metaphases. The excess water was drained with the help of filter paper and the samples were placed in 1 ml of cold fixative in 2 to 3 micro tubes that were then kept at room temp for 2 h. Carnoys fixative was changed once during this process of fixation. After completing the fixation procedure, the samples were removed from fixative and were rinsed with double distilled water and kept immersed in water for the next 30 minutes. The digestion period gets extended with the size of bud. The small buds measuring 1 to 2 mm were used. Two to four shoot buds were placed in 100µl of Cellulase / pectinase enzyme mixture incubated at 37-38°C temp for 4hrs in water bath. For Protoplast isolation, post 4-6 hrs of digestion treatment, the bud tissues were crushed into Cellulase /pectinase enzyme mixtures with help of needle and filtered with the help of the tissue filter made of nylon mesh with pore size of 30 µm. The suspension must run down inside the wall of the micro tube 2ml. The filtered suspension was kept at 4° c for about 15 to 30 min.

The tissues were then subjected to hypotonic treatment of protoplasts where, the filter suspensions in vials/ microtubes were added with 1.5 mL of cold 75mM KCL solution. The protoplast suspensions were mixed by gently inversing the tubes for 15 to 20 min and were left in stand still position for 5 to 7 min. Cleaning and fixation of the protoplasts were carried out by centrifuging the protoplast suspensions at 7000 rpm for 5min. The supernatants were discarded and added with 1 mL of ice-cold fixative to the protoplast pellet. This fixative + protoplast mixtures were kept at room temperature for the next 5 min or at 4°C for a longer time. Again, the protoplasts were spinned down at 7000 rpm for another 5 min. The fixative supernatants were discarded and this fixation procedure was repeated 2-3 times as above. A 50µl of fresh cold fixative (A mixture of 1part glacial acetic acid and 3 parts absolute ethanol) was added to each of the protoplast pellets and gently mixed into suspension. Glass slides were kept wet and chilled for further use at 4°C. The protoplast suspension was dropped on the ice-cold and wet slide from 15 cm height. The slide was immersed in the absolute ethanol for few seconds, after drying the protoplast drop followed by air drying again and adding a drop of carmine solution. The coverslip was placed and sealed with the help of transparent nail paint (Kesara, 2003).

Ploidy analysis was carried out using a flow cytometer (Partec Gmbh, Munster, Germany). Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy was presented in the form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample. The protocol includes a series of steps starting with excision of a 0.3-cm^2 piece of emerging leaf tissue and placing in a Petri dish. The sample was prepared for analysis using a High-Resolution Staining Kit (Partec GmbH). The samples were chopped with a sharp blade in the presence of 500-800µl of Nuclei Extraction Buffer and the nuclei were filtered through a nylon screen 30-µm filter into a 3.5-mL tube and stained with 0.5- 1ml of Nuclei staining buffer (4^2, 6-diamidino-2-phenylindole dihydrochloride). After that, samples were run in the flow cytometer. When the cells with labelled with fluorescent colouring due to the staining buffer passed through the measuring area one after the other, the individual cells or particles got illuminated by the excitation light and the fluorescent light intensity was proportional to DNA content. The samples were analysed in a UV-LED Partec flow cytometer with light emission at 365 nm, adjusted to fluorescence optical detection to gain as per the control sample of cultivar or as per species. More than 5000 nuclei were assessed in each sample. Nuclear DNA histograms were constructed using CyView software (Partec Gmbh, Germany), which determines peak position and relative ploidy level of the tested samples.

RESULTS AND DISCUSSION

Research studies on polyploidy breeding program initiated at ICAR-CCRI, since last few years. Standardized the protocols for induction of triploidy, tetraploidy in commercial citrus rootstocks and scions via endosperm rescue, micro budding coupled with colchicine treatment, and also by colchicine treatment of meristimatically active seeds. (Vijayakumari and Pooja, 2013), and generated various polyploids which were tested by flow Cytometry method but the results of flow Cytometry analysis were found varying due to the shifting of histogram inconsistent peaks. At this juncture to reconfirm the results, alternate chromosome counting technique was employed to ascertain the chromosome number. The flow cytometry methods sometimes yield fluctuating results depending on genome size, age of the sample and also with parents and progeny due to prevalence of introgressive hybridization in Citrus species. The estimated counts of seed derived plants differ with that of parent trees.

Citrus is mostly propagated by vegetative/asexual means, but in this study, we generated polyploidy plants by innovative *in vitro* and *in vivo* propagation techniques. Seed is the product of natural hybridization or sexual reproduction which leads to variation in the genetic makeup of both in parent and progeny trees. Chance of getting the obvious/clear convincing results /chromosome numbers of control and test plants through flow cytometry was observed to be tough. Polyploidy occurs naturally in citrus, mostly through spontaneous mutations, and polyploids are generally slow in growth less vigorous than the diploid counterparts (Gmitter *et al.,* 1991). In our experiments we have successfully regenerated large no of polyploidy plantlets via somatic embryogenesis from hybrid endosperm rescue and also by colchicine treatment of meristematically active seeds, but while assessing the ploidy level of all polyploidy plants with conventional method of using the root tips by squash spread techniques is quite difficult due to small size

of chromosomes in citrus, mitotic index is mostly low in root tips (Hynniewta, 2011). Further the slow growth rate of polyploids creates a difference in age group of test plant sample and control plant sample. The chromosome preparation method described in this paper is technically possible and simple for implementation, for cytogenetic confirmation of ploidy of large population of citrus both in the field and at nursery level and also plants obtained by different propagation methods. In Citrus cultivars chromosomes are small in size so sometimes accurate chromosome counting is generally difficult to achieve in mutants where ploidy is high. This improved methodology of chromosome counting helped in revalidation of flow cytometry analysed samples. In this investigation, polyploids generated in various experiments were confirmed by chromosome counts and also flow cytometry (Table 1, Fig. 1-3).

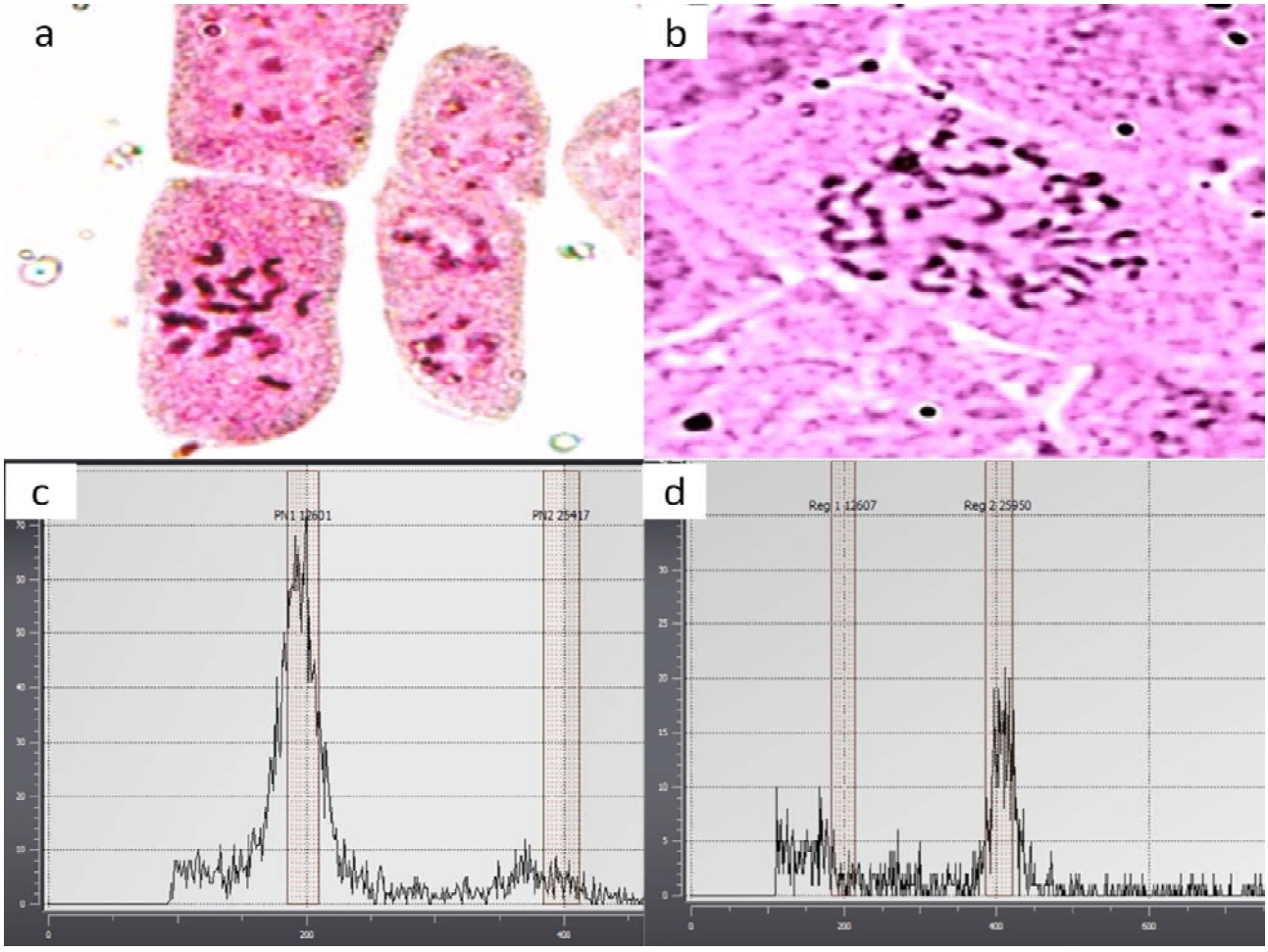

Fig. 1. Processed metaphase cells of *Citrus jambhiri Lush*. (Rough lemon) sample

(a) diploid (2n=18), (b) tetraploid(2n=4x=36).Result of Chromosomes count is correlated with flow cytometry analysis (histogram peaks) in control sample of Citrus jambhiri Lush. (Rough lemon). (d) with tetraploid sample (2n=4x = 36)

Table 1. Chromosome count of diploid mother plant and developed Polyploidy plants

Sl.No.	Species name	Chromosome count diploid mother plant	Developed Polyploidy plants chromosome counts
1	*Citrus jambhiri Lush.*	18	4x-36
2	*Citrus sinensis Osbeck*	18	(3x-27), (4x-36),(6x-54)
3	*Citrus reticulata Blanco*	18	(3x-27), (4x-36)

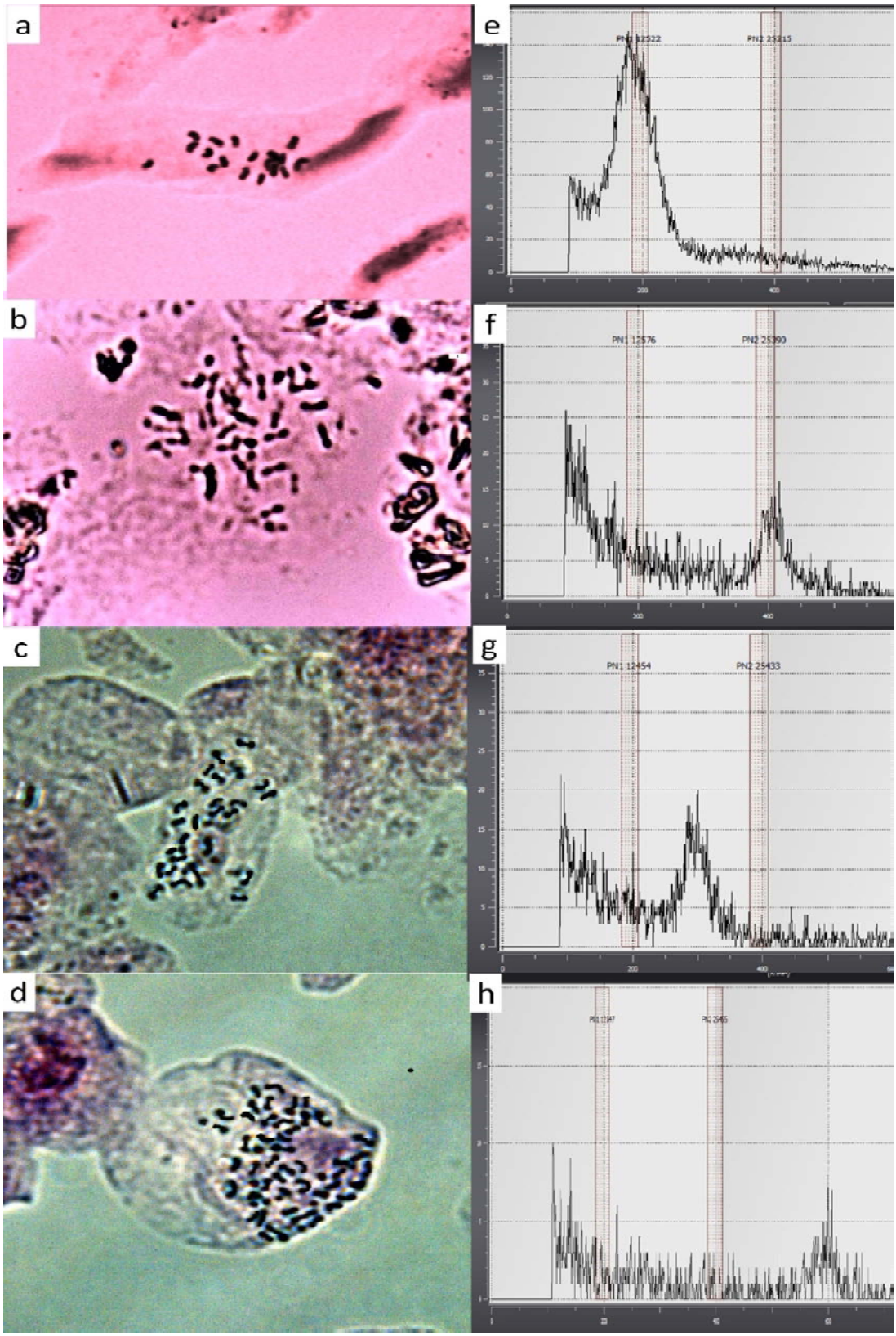

Fig. 2. Processed metaphase cells of *Citrus sinensis Osbeck* (Sweet orange cv. mosambi)

(a) Diploid (2n = 18), (b) tetraploid (2n = 4x = 27), (c) triploid (3n = 27), (d) Hexaploid (6n =54) in comparison with the flow Cytometry analysis (by histogram peaks - e to h).

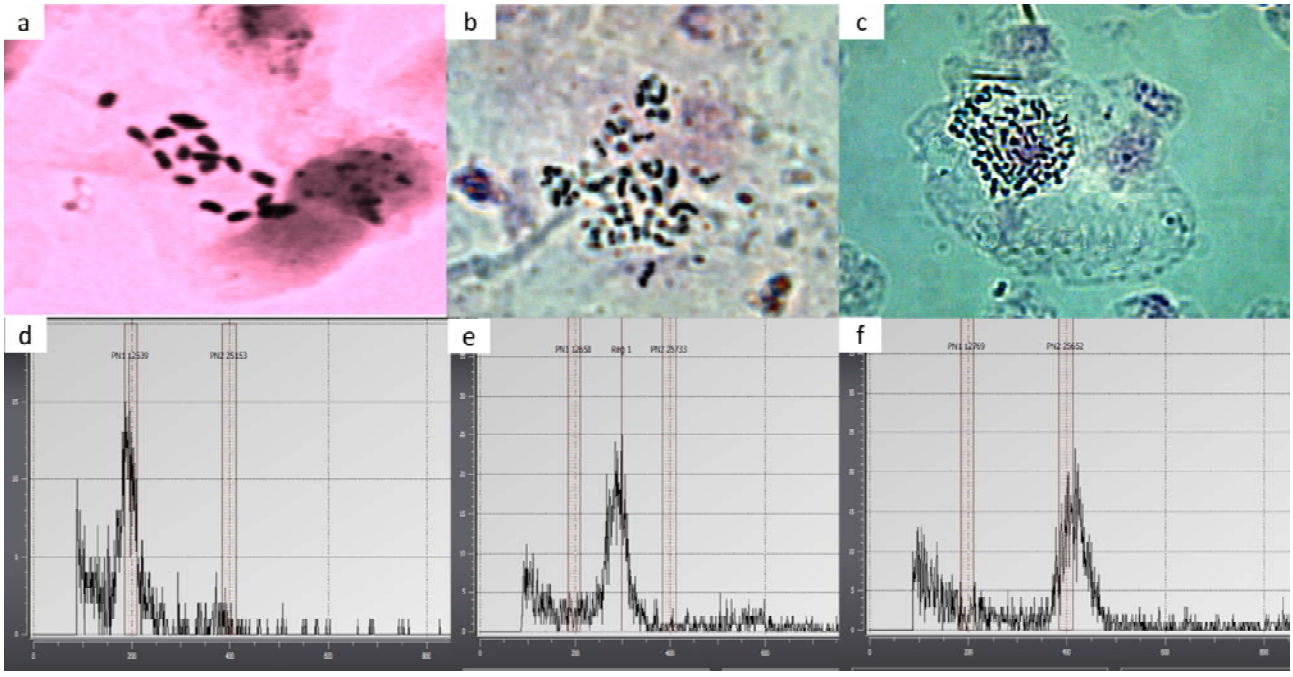

Fig. 3. Processed metaphase cells of *Citrus reticulata Blanco* (Nagpur mandarin) sample

(a) diploid (2x=18), (b) triploid (3x= 27), (c) tetraploid (4x=36), in comparison with the flow Cytometry analysis (by histogram peaks – d to f).

Shoot tip is a very convenient source of sample for chromosome preparations. Collection of shoot sample is the most simple and reliable, and it is mitotically active material from established plants. Approximately 2 to 3 mm of a healthy, vigorous growing shoot collected during active growth stage. The mitotic active phase is observed highest when the bud size is 2-3mm.Ideal time to collect such sample is in the morning (9 AM) where condition of light should be approximately 2000 lux and temperature around 25-27^{0}C. Before attaining above mentioned stage, the plant should undergo for a break of 12 hrs dark phase. After collection, samples were kept in ice cold water in the intermediate duration of carrying it from field to Lab. Collected samples of shoot tips were carefully defoliated and transferred into the ice-chilled water(0-4^{0}C) for 24hr. and treated with0.1% colchicine for 45min in the dark condition to arrest metaphase. This treatment enhances chromosome condensation and more importantly, improved the spreading of chromosome within a cell. The fixation preserves the tissue morphology and minimizes endogenous nuclease activity and other degradation processes. Fixation time affects the tissue sample quality. Higher the duration of fixation approximately up to 10hrs, results into tissue hardening. But here we have observed during research methodology optimum time for this technique in citrus 2 hrs duration is sufficient to generate a quality sample. The Enzyme digestion treatment at 37^{0}C for 6 hr treatment gave best result in citrus crop. Cellulase and pectinase enzyme with 2.5% concentration improves chromosome spreading and better sighting of chromosome. With this methodology the chromosomes are well spread at metaphase enabling well distributed chromosomes for getting clear countable chromosomes, these results are in confirmation of results obtained by (Kesara, 2003). It helps to reconfirm result of flow cytometry. Root chromosomes preparation by traditional squashed technique makes poor quality spreads, either chromosome fused together or some are lost between cells during tapping and squashings, as it was observed in preliminary experiments.

CONCLUSION

In this research paper we have used technique based on enzyme digestion treatment, protoplast dropping method and metaphase spread count which enabled better display of accurate chromosomal count. This study facilitated in double check or repetitive validation of ploidy level of samples generated in various *in-vitro* and *in-vivo* ploidy experiments, which were already observed in flow Cytometry. The results which we achieved during research is highly helpful for further karyotype analysis and *in-situ* hybridization application, as chromosomal count can be very much accurate because, of clear well spread, elongated, chromosome morphology on metaphase plates.

REFERENCES

FAO Statistics FAOSTAT Ranking countries by commodity 2018, http://www.fao.org (2018)

Frost, H.B. 1925a. Tetraploidy in *Citrus*. Proceedings of National Academy Science of USA., **11**: 535–537.

Frost, H.B. 1925b. The chromosomes of *Citrus*. *Journal Washington Academy of Science*., **15**:1–3.

Gmittar, F.G. and Xulan, H.U. 1990. The possible role of Yunnan, China, in the origin of contemporary Citrus species (Rutaceae). *Econ.Bot.,* **44**: 2, 267-277.

Gmitter, F.G., Ling, X., Cai, C. and Grosser, J.W. 1991. Colchicine-induced polyploidy in Citrus embryogenic cultures, somatic embryos and regenerated plantlets. *Plant Sci*., **74**: 135-141.

Guerra, M., Pedrosa, A., Silva, A.E.B., Cornelio, M.T.M., Santos, K.G.B. and Soares Filho, W.S. 1997. Chromosome number and secondary constriction variation in 51 accessions of a *Citrus* germplasm bank. *Brazilian Journal of Genetics*., **20**: 489–496.

Hynniewta, M., Malik, S.K. and Rao, S.R. 2011. Karyological studies in ten species of *Citrus* (Linnaeus, 1753) (Rutaceae) of North-East India. *Comparative Cytogenetics*., **5**(4): 277–287,

ICAR Central citrus research institute, Nagpur, official website http://www.ccringp.org.in/ccringp/.

Kesara, A.J. 2003. Preparation of chromosome from plant leaf meristems for karyotype analysis and in situ hybridization. *Methods in cell Science*., **25**: 91-95.

Krug, C.A. and Bacchi, O. 2003.Triploid varieties of *Citrus*. *Journal of Heredity*., **34:** 277–283.

Krug, C.A.1943. Chromosome numbers in the subfamily Aurantioideae with special reference to the genus citrus. *Botanical Gazette*., **104**: 602–611.

Longley, A.E. 1925. Polycarpy, polyspory and polyploidy in *Citrus* and *Citrus* relatives. *Journal Washington Academy of Science*., **15**: 347–351.

NHB. National Horticulture Board Ministry of Agriculture, Governement of India, Gurgaon, Haryana. www.nhb.gov.in, 2019-20.

Sharma, B.D., Hore, D.K. and Gupta, S.G. 2004. Genetic resources of citrus of north-eastern India and their potential use. *Genetic resources and crop Evolution*., **51:** 411-418.

Singh, H.P. and Chadha, K.L. 1993. Genetic Resources of *Citrus*: In: Advances in Horticulture, (Eds) Malhotra Publishing House, New Delhi., **1**:164.

Tanaka, T. 1993. Taxonomie de la Citriculture tropicale. *Revue de Botanique Applique*., **10**:114–119.

Vijayakumari, N. and Pooja, K. 2013. Grow triploid cultivars of Nagpur man darin for better harvest. *Indian Horticulture*., 18-19.

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