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Studies on Ploidy Analysis and Chromosome Doubling in Androgenic Plants of Chilli Pepper (*Capsicum annuum L.*)

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***Abstract* – After successful production of chilli pepper (*Capsicum annuum L.*) androgenic plants through direct embryogenesis, the ploidy levels of the plants were checked by chromosome counting, chloroplast counting and flow cytometry methods. These results revealed that all the techniques were successful in assessing the ploidy levels. Besides chromosome count and chloroplast count techniques could be employed effectively as flow cytometry is expensive. The data obtained was that 12 chromosomes and 8-10 chloroplasts were observed in haploid plants, where as 24 chromosomes and 16-20 chloroplasts in double haploid plants. Plants which were haploids and those which did not undergo spontaneous doubling were treated with 0.5% colchicine and were tested for doubling.**

***Keywords* – Chilli Pepper, Chromosome Doubling, Colchicine, Double Haploid, Flow Cytometry, Ploidy Analysis.**

I. Introduction

Increasing demand of food, varied taste for cuisine spices has given scientists a scope to develop *Capsicum annum L.* species. There are many techniques for improving crops, were double haploid production has been considered as the most time reducing and cost effective process.

As the production of in vitro chilli pepper (*Capsicum annum L)* plants from anther culture was successful, there was a need to check ploidy levels of each plant in a cost effective way. Different scientists worked on different methods and concluded flow cytometry as the best accurate technique. Since, hundreds of plants are grown.

But as we grow hundreds of plants, flow cytometry for all of them is expensive and labour intensive. There are other methods performed to check the ploidy levels like chromosome counting techniques from the root tip squash method and chloroplast count in stomatal guard cells. In 1986 [1] studied to show stomatal length measurements could be used as an alternative method for chromosomal scoring in Brussels sprouts. In haploid plants of Brussels sprouts stomatal length was 14mm, 20mm and 24mm in diploid plants and triploid plants. Stomatal length was measured in carrot by [2] for ploidy determination, whereas chloroplast number was counted in haploid plants by [3] in sugar beet, in which diploid plants (2n=2x=18) showed 16 chloroplasts` and 9 in haploid plants (n=x=9). Morphological features, stomatal size, chloroplast count and chromosome count can also be used to distinguish haploid and diploid watermelon plants were shown by [4].

In the present study the ploidy levels of another culture derived Chilli pepper plants was determined by chromosome counting, flow cytometry, chloroplast numbers and by observing plant morphology.

II. Materials and Methods

In this study plants produced in NSL Biotech laboratory from in-vitro anther culture of Chilli pepper (*Capsicum annuum L.*) genotypes PH 8, PH 11, PH 17, PH 43, PH 50, PH51, PH53, PH57, PH58 and PH59 were analyzed. Ploidy levels were checked using plant morphology, chromosome count, flow cytometry and chloroplast number. Plants which were considered to be haploid were treated with 0.5% Colchicine for chromosome doubling and were tested again through the same methods.

A. Chromosome Counting In Root Tips

Root tip squash method using acetocarmine stain was followed for counting chromosomes in root tips. The stain was prepared by mixing distilled water (55 ml) and acetic acid (45 ml)in a conical flask and heating upto its boiling point. Then 1 g (to make 1%) or 2 g (to make 2%) of the carmine dye is added slowly to the boiling solution. Boiling is continued till the dye is dissolved completely. Then the solution is cooled, filtered and if needed ferric chloride or ferric hydroxide is added to intensify the stain.

A 5-6mm long root tip was cut from the plant at 3.20 PM(IST) (this is the time the cells enter the metaphase stage, during which chromosomes can be counted) and placed in a labeled tube containing 2ml of acetocarmine solution, left undisturbed for 24 hours. On a clean glass slide a drop of acetocarmine stain was taken along with the root tip from the tube. Using a scalpel the heavily stained root tip was cut. The glass slide was left undisturbed for some time. Later a cover slip was placed over the root tip, pressed thoroughly with the thumb for even distribution and tapped gently and straight down until the stained tip was spread out to a faint purple monolayer and was viewed under a microscope using 100X oil immersion objective. Chromosomes were counted in 10 plants from each genotype that were raised from anther culture technique.

B. Chloroplast Count Technique

In chloroplast counting method, the lower epidermis of the fully expanded leaf was peeled out and it was stained using 1% silver nitrate solution and then it was placed on glass slide, the chloroplasts were counted under light microscope under 100x magnification.

C. Flow Cytometry

Flow cytometry protocol by [5] was followed, which involved directly staining the DNA content for cell cycle analysis in a solution of propidium iodide made in

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hypotonic sodium citrate, which would lyse the cells and stain the DNA. Hypotonic propidium iodide, 50 µg/ml in 3 g/L trisodium citrate dihydride containing 0.05% (v/v) of Nonidet P-40 or a non-ionic detergent equivalent (Igepal CA-630) containing 2 mg/mL RNase A should be stored in a dark amber bottle in a refrigerator (4-8 °C) and may be stored for years.

In a petri plate 2ml of NIB (Nuclear Isolation Buffer) and two to three ½ inch length leaf tips were taken. With the help of surgical scissor the leaves were chopped finely and the entire liquid was transferred to 5ml glass tube. The liquid was stirred vigorously with the help of a dropper (the most important step). The tubes were capped and incubated at 37°C for 30 minutes and shaken two to three times with the dropper. Later the liquid was filtered through 30µm nylon mesh and the samples were analysed and results documented.

D. Colchicine Treatment

The plants which were identified to be haploid were taken and treated with (0.5%) Colchicine for 8 hours and were grown in a growth chamber for a month. Then the ploidy levels were rechecked by all the three methods, i.e. Chromosome count, chloroplast count and Flow cytometry.

III. RESULTS and DISCUSSION

A. Chromosome Counting in Root Tips

The basic chromosome number of the genus *Capsicum* is x=12, all the species are diploid, most are 2n=2x=24, including the cultivated ones. Out of 100 androgenic plantlets that were raised from anther culture technique 51 plantlets were found to be haploid with 2n=12 chromosomes, 45 plantlets were double haploids with 2n=24, indicating spontaneous chromosome doubling (Table I). Somatic or gamatophytic origin of anther culture derived plants was determined by SSR markers. The haploid and double haploid ratio of 1:1 in anther culture regenerated plants was reported by [6] and [7] found 47.3% of spontaneous diploidization of haploids. The plants which were confirmed as haploids were treated with 0.5% Colchicine. 2 plantlets with 2n=36 chromosomes and 2 plantlets with 2n=15 and 2n=16 (Table II) were also found among androgenic plantlets, indicating a very low level of trihaploid & Aneuploid plant generation. This type of analyses is time consuming and was studied by [8], [9] and [10].

B. Chloroplast Count

The number of chloroplasts in a stomatal guard cell varied from 8 to10 (Fig:A) in 51 plantlets (Table. I) that were haploid (2n=12) as determined by chromosome count 16 to 20 chloroplasts (Fig:B) in a stomatal guard cell were observed in 45 plantlets determined to be double haploids (2n=24) by chromosome count. This indicates that ploidy level is highly correlated with number of chloroplast count. [11], [12] and [13] have also reported that studies in melon, potato and alfalfa showed that numbers of chloroplasts in the tetraploid variety was nearly double that of the diploid variety.

Each chloroplast possesses an independent and circular DNA. This organelle has special genes for the purpose of division, growth and biochemical action and are directly related to expressed genes of the nucleus [14] and hence polyploidy increases the number of such gene transcripts that express to form as many chloroplasts. Hence this study confirms the correlation between number of chloroplasts in stomata guard cells and ploidy levels. Chromosome counting method is time consuming and time specific, but chloroplast count of stomatal guard cells a fast and economical means for ploidy determination. Chloroplast count cannot precisely determine the chromosome number; it can only give a confirmatory determination of ploidy level.

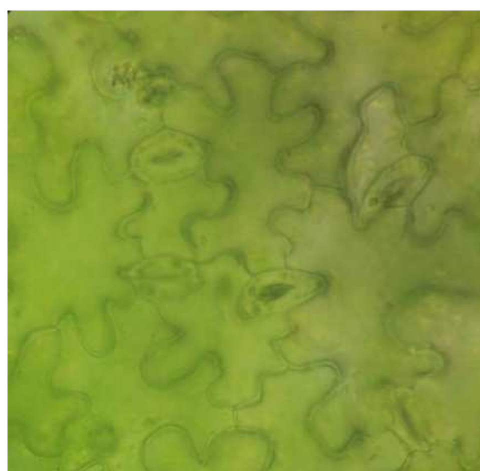

Fig (A). Haploid Chloroplast

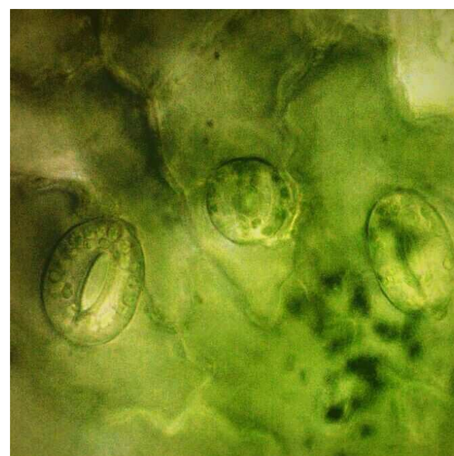

Fig (B). Double haploid Chloroplasts

C. Flowcytometry

In flow cytometry, DNA analysis is based on fluorescence parameter whose number will depend on the number of optical detectors with which the flow cytometer is equipped. If a one-parameter histogram shows how many cells contain DNA or number of antibody molecules bound to DNA. All cells having almost equal quantities of DNA form a peak. Histogram with one peak represents the

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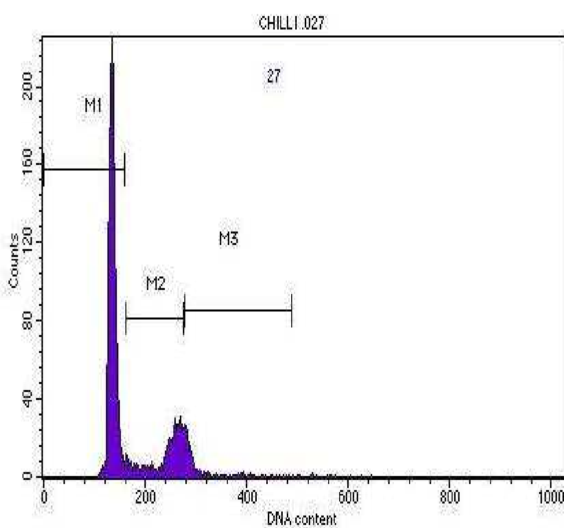
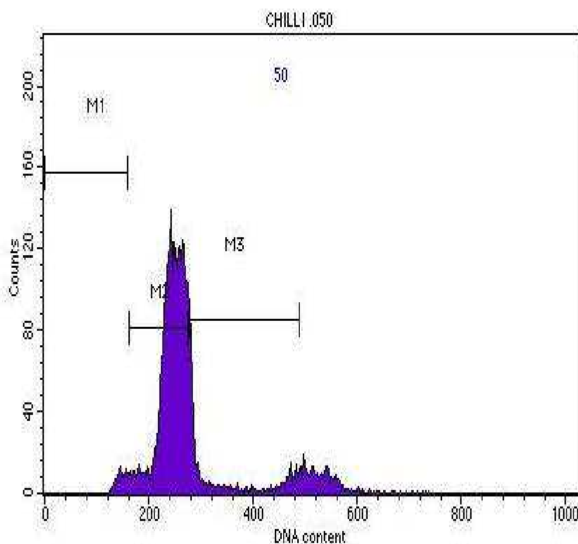
G1Phase and G2/M phase of cell cycle, when twice the channel value is present. In a cell population the distribution of nuclear DNA content is assessed by comparing the number of cells in different peaks (Fig:C&D). In pepper regenerants ploidy analysis is done by measuring the quantity of DNA in nuclei of cells from young leaves with flow cytometric analysis [15], [16] The correlation between haploid to doubled haploid regenerants depends on genotype was studied by [17] The spontaneous diploidization of haploid varies from 1:1 to 1:2 in large fruit pepper and from 3:1 to 2:1 in pepper used for spices.

In our study we found 45% Spontaneous double haploids, 51% Haploids, 2% Trihaploid and 2% Aneuploid Plants (Table.I& II).



Fig(C). Flow cytometric analysis of Haploid plant



Fig(D) Flow cytometric analysis of Double Haploid plant

D. Colchicine Reaction

Few plants undergo spontaneous chromosome doubling, but the frequency is very low but for few of them chromosome doubling may be induced by treating haploid plants with an antimitotic agent such as Colchicine which inhibits spindle formation and segregation of sister chromatids during mitosis. This is done after ploidy analysis before hardening stage. [18] Colchicine is highly toxic and results in stunting, delay in flowering, mutagenesis, ploidy chimeras and poor seed set. Standardization of Colchicine treatment for doubling the chromosome number in Diploid and haploid plants in vivo has been initiated and checked for doubling.

Treatment of Colchicine has given rise to diploid plants. As we see from fig..E we can observe that the haploid plants are usually dwarf and having small leaves and sterile flowers (fig .F), where as diploid plants are tall having fertile flowers (fig G), and broader leaves. These were the morphological differences seen in the plant.

After the treatment with colchicine 23 haploid plants were found to be diploid (2n=2x=24) proving the plants to be induced double haploids. The remaining plants had a varied number and thus we could segregate 5 chimeras and 1 Anuploid from them, the remaining 10 plants could not survive because of the stress induced by colchicine treatment (Table.III). [19] found in 24 androgenic regenerants 20 haploids, 2 diploids and 2 triploids plantlets. High frequency of haploid- diploid or diploid chimera's regenerated plants was reported by [20].

Fig(E). Haploid and Double haploid plants

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Table I. Details of ploidy levels in different androgenic plants

Genotype	Number of chloroplasts	Chromosome Numbers	Genotype	Number of chloroplasts	Chromosome numbers
PH 8-2	16	24	PH 8-3		12
PH 8-6	16	24	PH 8-4	8	12
PH 8-8	16	24	PH 8-5	8	12
PH 11-1	16	24	PH 8-7	8	12
PH 11-6	16	24	PH 8-9	8	12
PH 11-7	17	24	PH 8-10	8	12
PH 11-8	16	24	PH 11-2	8	12
PH 11-9	16	24	PH 11-3	9	12
PH 11-10	16	24	PH 11-4	10	12
PH 17-5	16	24	PH 17-2	8	12
PH 17-6	16	24	PH 17-3	10	12
PH 17-7	16	24	PH 17-4	8	12
PH 17-10	17	24	PH 17-8	8	12
PH 43-4	16	24	PH 17-9	8	12
PH 43-6	16	24	PH 43-1	9	12
PH 43-7	17	24	PH 43-2	10	12
PH 43-9	16	24	PH 43-3	8	12
PH 50-1	18	24	PH 43-5	8	12
PH 50-2	16	24	PH 43-8	10	12
PH 50-6	16	24	PH 43-10	8	12
PH 50-7	16	24	PH 50-3	8	12
PH 51-2	16	24	PH 50-4	8	12
PH 51-4	16	24	PH 50-5	8	12
PH 51-6	16	24	PH 50-8	9	12
PH 51-8	18	24	PH 50-9	8	12
PH 51-10	16	24	PH 50-10	8	12
PH 53-3	16	24	PH 51-1	10	12
PH 53-5	16	24	PH 51-3	9	12
PH 53-8	16	24	PH 51-5	8	12
PH 53-9	16	24	PH 51-7	9	12
PH 53-10	17	24	PH 51-9	9	12
PH57-2	16	24	PH 53-1	8	12
PH 57-4	18	24	PH 53-2	9	12
PH 57-6	16	24	PH 53-4	10	12
PH 58-1	16	24	PH 53-6	8	12
PH 58-3	18	24	PH 53-7	9	12
PH 58-6	17	24	PH 57-1	8	12
PH 58-8	16	24	PH 57-5	8	12
PH 58-10	17	24	PH 57-7	9	12
PH 59-2	16	24	PH 57-8	8	12
PH 59-4	17	24	PH 57-9	8	12
PH 59-7	16	24	PH 57-10	8	12
PH 59-8	16	24	PH 58-2	10	12
PH 59-9	16	24	PH 58-4	8	12
PH 59-10	16	24	PH 58-5	9	12
			PH 58-7	9	12
			PH 58-9	8	12
			PH 59-1	8	12
			PH 59-3	9	12
			PH 59-5	8	12
			PH 59-6	9	12

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Table II. Details of Triploid and Aneuploid plants

Triploids			Aneuploids		
Genotype	**Numbers of chloroplasts**	**Chromosome numbers**	**Genotype**	**Numbers of chloroplasts**	**Chromosome numbers**
PH 11-5	24	36	PH 8-1	12	15
PH 57-3	25	36	PH 17-1	12	16

Table III. Details of ploidy levels after 0.50% Colchicine treatment

Genotype	Number of Chloroplast	Chromosome numbers	Flow cytometry results
PH 8-3, PH 8-5,PH 8-7,PH 8-10,PH 11-2, PH 11-3, PH 17-2, PH 17-3, PH 43-2, PH 43-3, PH 43-5, PH 43-8,PH 50-4, PH 50-5,PH 50-10, PH 51-1,PH 51-9, PH 53-1,PH 53-6, PH 53-7 and PH 58-4	16 to 20	24	Double haploids
PH 11-4,PH 17-9,PH 43-1,PH 51-5,PH51-7,PH 57-9, PH 57-10,PH 58-5,PH 58-7,PH 58-9,PH 59-1, PH 59-10	8 to 10	12	Haploids
PH8-9,PH 17-4,PH 43-10,PH 50-8, PH 50-9,PH 53-2, PH 53-4 PH 57-1,PH 58-2, PH 59-3, PH			
PH 8-4,PH 17-8, PH 51-3, PH 57-8, PH 59-5	8 to 20	12,24	Haploid, Double haploid
PH 50-3	12	15	Aneuploid

Fig (F).Haploid flower-Sterile

Fig(G) Double haploid flower-Fertile

IV. Conclusion

From our study we have found that all the three different approaches to determine the ploidy level in regenerant plants have been useful. The morphological features were also found to vary in haploid and diploid plants. Chromosome counting, Chloroplast count and flow cytometry, all these methods have been reliable as all of them have found to give precise results.some of the advantages and disadvantages are; Chromosome counting in root tips squash method should be done in samples collected at specific time, but is found to be most acurate. Chloroplast count method is very simple, fast and economical for ploidy level analysis. Flow cytometry method is expensive and labour intesive but accurate. Colchicine tretment has been proved to cause chromosome doubling when used in 0,5%concentration, but care should be taken about the toxicity and stress to the plant.

V. Acknowledgement

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