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Development of a Unique Protocol for The Production of Doubled Haploids in Hot Pepper

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Abstract

Hot pepper ranks seventh among the most produced vegetables in the world. This crop is famous for its color and pungency. Hot pepper contains many important phytonutrients that enable it to fight against cancer. Conventional breeding in Hot Pepper is labor-consuming and a long-term process. This can be overcome by using novel approaches, such as, in vitro doubled haploid plant production. This method not only accelerate breeding programs but also facilitates recovery of recessive mutations. There are many articles published on doubled haploid development in Capsicum annuum; it reports both anther culture and shed-microspore culture methods, but both these methods require either subculture of anthers or the addition of new media, which makes both protocols laborious. The present study aims to develop a single-step direct embryogenesis protocol to produce doubled haploids in Capsicum annuum. We analyzed effectiveness of microspore embryogenesis on ten Hot pepper genotypes with six media combinations including shed-microspore culture protocol (Supena et.al 2006) and two-step anther culture protocol (Dumas de vaulx et.al 1981 and Para-Vega et.al 2016). Among six, one media combination responded very well to single-step direct embryogenesis compared to other protocols with more than 20 % plant regeneration frequency. We observed an average embryogenesis of 7.53% and plant regeneration frequency of 3.81%. We achieved a survival index of 97.61% after acclimatization of plants in the polyhouse. The results of these experiments show that, one-step direct embryogenesis can be achieved with unique combination of plant growth regulators.

Keywords: *Capsicum annuum, Doubled haploids, Ploidy Analysis, Androgenesis, Anther Culture.*

1. Introduction

Hot pepper ranks seventh among the most produced vegetables in the world. This crop is well-known for color and pungency. Hot pepper contains many important phytonutrients that enable it to fight against cancer. Due to these properties, hot pepper has ever-increasing demand in the world. Hot pepper is vulnerable to many diseases, for example, bacterial wilt, anthracnose, and leaf curl disease, and all these diseases, combinedly or solely, hinder crop yield worldwide. There needs to be more than the current pace of resistant hybrid development to combat diseases necessary to fulfill the demand. An extended period required for line development through the conventional method, usually seven to eight generations, can be reduced to one step by the intervention of DH technology.

Biotechnological techniques are economical to increase the efficacy of breeding projects compared to time required to develop a new variety conventionally (Bermejo et al. 2016; Niazian and Shariatpanahi, 2020). Microspore embryogenesis is one of the most efficient and widely used methods to introduce genetic variability (Asif 2013; Dunwell 2010; Popova et al. 2016). Several factors play important role for successful microspore embryogenesis and subsequent plant regeneration; however, genotype, stages

of microspore and culture conditions have the major impact (Irikova et al.2011a; Segui-Simmaro et al. 2011; Taskin et al. 2011). Compare to other *Solanaceae* species, Hot Pepper (*Capsicum annuum* L.) is highly heterozygous and shows higher rate of spontaneous genome duplication among regenerated plants.

George and Narayanaswami (1973) and Wang et al. (1973) reported the first successful haploid development in *Capsicum annuum* through another culture. However, researchers reported frequency of plant regeneration from cultured anthers was very low. To increase the efficiency of these protocols, Sibi et al. (1979) introduced pre-treatment of higher (42℃) and lower (4℃) to buds along with optimization microspore stage (uninucleate to late uninucleate), while Dumas de Vaulx et al. (1981) tried post-treatment to anther at 35℃. Both modifications worked well and resulted in increased plant regeneration efficiency. In 1997, Dolcet-Sanjuan et al. reported a double-layer method for producing DHs in Hot Pepper, currently well-known as shed-microspore culture.

There are many articles published on DH development in *Capsicum annuum*, reporting both anther culture (Para-Vega and Segui-simarro 2016, Grozeva et al. 2021), as well as shed-microspore culture methods (Supena EDJ 2021, Ari E 2021) but both these methods, require either subculture of anthers or addition of new media, which makes both protocols laborious. In the present study, we developed a unique single-step direct embryogenesis protocol to produce DHs in *Capsicum annuum* and compared it to shed-microspore culture protocol and two-step anther culture protocol.

2. Materials And Methods

Plant material:

Seeds of Hot Pepper to be used as donor plants, procured from the National Bureau of Plant Genetics and Resources (NBPGR), India. Ten genotypes were used as donor material; five were indigenous collections, and the remaining were exotic collections. Details of genotypes mentioned in Table – 1. Genotype numbers starting form IC are indigenous collection and genotypes numbers starting from EC are exotic collection.

Growing of donor plants:

Seeds of all ten genotypes sown in cocopeat in a tray and watered as needed. After one month, well, grown seedlings are potted in big pots containing cocopeat: soil (1:1)—all plants are grown under natural light conditions in a polyhouse with an average temperature of 23℃ (Figure 1). Flower buds were harvested 60 to 65 days after transplanting of plants.

Figure 1: Growing of Donor plants of Hot Pepper under protected polyhouse condition

Selection of bud and anther size:

Flower buds of different sizes (2.5mm, 3mm, 3.5mm & 4mm) were collected, and anthers were crushed and filtered through 45µm nylon mesh, this filtration process remove debris after this step, filtered microspore suspension stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Kim & Jang, 2000) and observed under fluorescent microscope. Anthers containing microspores with uninucleate to early binucleate stage selected for DH development (Figure-2).

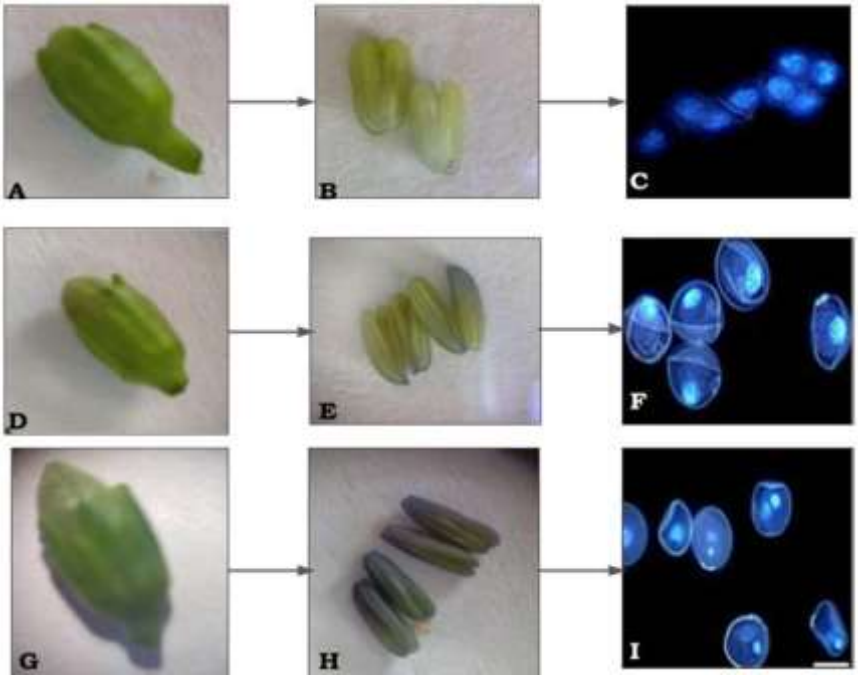

Figure 2: Flower buds of different sizes with different stages of microspores after DAPI staining: A, B & C – Flower bud with green anthers and early uninucleate stage of microspores. D, E & F- Flower bud with anther of purple tip and late uninucleate stage of microspores, G, H & I- Flower bud with purple anthers and binucleate stage of microspores

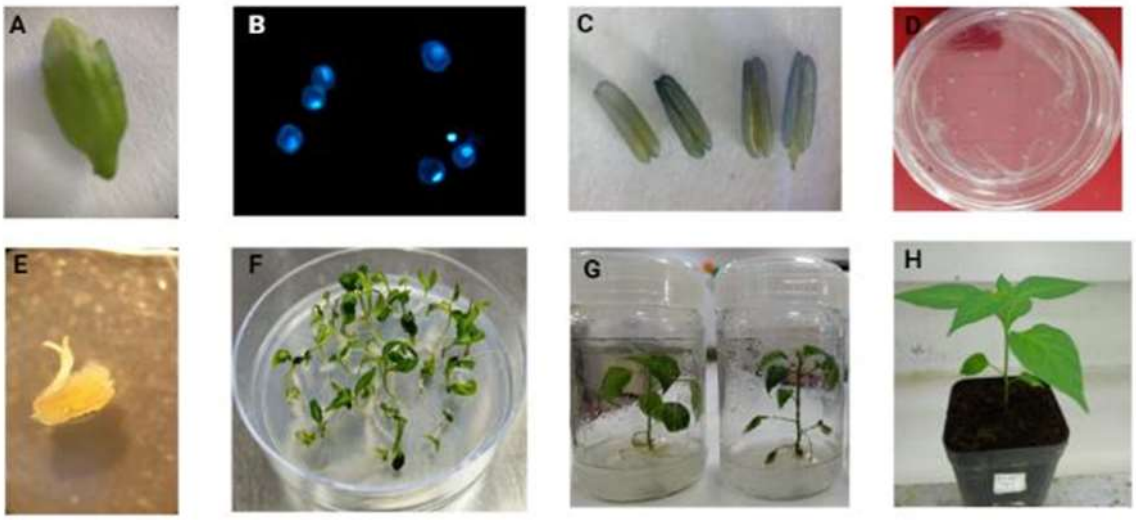

Figure 3: Stages of androgenesis in Capsicum annuum. A) Selected bud for DH development B) DAPI stained microspores of uninucleate stage C) Selected anthers with slight purple color/ purple tip D) Cultured anthers on embryo germination media E) Germinated embryo from anther F) Plantlet development from embryos G) Plant regeneration H) Plant hardening in a small cup.

Optimization of media for direct embryogenesis:

Anthers were inoculated on six different media combinations/ protocols (Table 1) in 90X20mm Petri plates (Make-Tarson) and incubated in complete dark condition at 25°C for embryo formation and as per protocol. The first observation of cultured anthers on different media combinations was taken 50 to 60 days after anther inoculation, petri plates which contains embryo are sorted for embryo harvesting. All remaining petri plates which did not respond for embryo formation, again kept for incubation at 25°C in dark condition. We repeated this process each 15 days interval up to 120 days after anther inoculation. All embryos were transferred in 90X20mm Petri plates (Make-Tarson) containing plant conversion media (MS medium with 3% sucrose and 8gm/lit agar) and incubated at 25°C in light. Normal shoots with cotyledons were transferred glass bottles containing plant regeneration media (MS medium with 2% sucrose and 8gm/lit agar) for plantlet development media (Figure-1 D, E, F & G).

Ploidy analysis of regenerants:

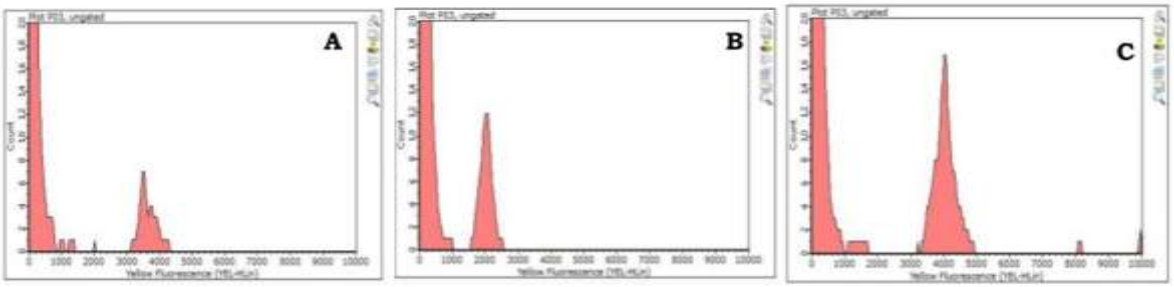

Figure 4: Ploidy analysis of regenerated plants A) Histogram of standard diploid (2n) B) Histogram of haploid (n) sample C) Histogram of DH (2n) sample

The ploidy level of all well-developed plants was determined through flow cytometric ploidy analysis. Leaf samples were chopped in Galbraith buffer (Galbraith *et al.*, 1983) to isolate the nuclei. These crude nuclei suspensions were filtered through 45µm nylon mesh. Filtered nuclei solution was stained using propidium iodide staining buffer (1X PBS buffer, 100 ug/ml RNase, 50 ug/ml Propidium Iodide) and incubated at room temperature for 15 to 20 minutes. These stained nuclei solutions are read on the Merck-Guava® flow cytometer using Guava® 2.7 software. Plants showing histogram resembles standard (2n) considered as DHs, and samples showed histogram on the half scale of the standard considered as haploids (Figure-4). Only DH plants are carried forward for further process.

Acclimatization of DH plants:

All DH plants are potted in a small cup containing sterile cocopeat. For acclimatizing to the outer environment, plants were covered with transparent polybags to create humidity and a safer climate as it was derived through the plant tissue culture process. Covered polybags are removed after eight to ten days of potting. Approximately 30 to 35 days after potting, plants got acclimatized to the outer environment (Figure-1 H); these well-acclimatized plants were transplanted in big pots containing soil and cocopeat (1:1) for fruit setting and seed collection.

3. Results and Discussion

Another Culture:

A total of 13538 anthers forms ten genotypes cultured on six different media combinations/protocols. Approximately 200 to 250 anthers were cultured per genotype- per media combination/protocol (Table 1). Out of six, only four media combinations responded for one step of direct embryogenesis including shed-microspore clture protocol and two-step anther culture protocol. CAEI-1 media showed the highest embryo formation frequency (40.79%) as well as the highest plant regeneration frequency (20.62%), while CAEI-6 media showed the lowest embryo formation (0.81%) and plant regeneration frequency (0.41%) (Figure-3). Media codes CAEI-2 & CAEI-3 did not respond to one-step direct embryogenesis. However, shed-microspore culture protocol (media code -CAEI-5) also responded for microspore embryogenesis with 2.63% embryo formation frequency and plant regeneration frequency of 1.4%. The two-step anther culture protocol responded with 1.39% embryo formation frequency and

plant regeneration frequency of 0.65%. Compared to these shed-microspore and two-step anther culture protocol, our media combination resulted with very high frequency of embryogenesis as well as plant regeneration frequency.

Embryo harvesting and Plant development:

We were very keen at the time of embryo harvesting, and confirmed the androgenic origin of all harvested embryos; there is no involvement of somatic cells in embryo development. When embryo was observed under microscope at 4X magnification at that time the anther wall was fully opened and a microspore developed in to an embryo and few microspores besides the developed embryo also present in that anther and those all are in process to develop as an embryo. This microscopic observation confirms that embryos were developed from microspores and not from somatic cells. We gently detached embryo from anther and inoculated on plant conversion media. Detaching embryo from anther needs very skillful hand, because, if the embryo got broken during harvesting process, then chances of broken embryo to convert in to normal plant are very less. More care was taken while harvesting embryos from another culture protocols, whereas in case of shed-microspore culture protocol microspores bursted in liquid layer and converted in to embryos. We have harvested a total of 1020 embryos derived from all genotypes and developed on four different media combinations/protocols (CAEI-1, CAEI-4, CAEI-5, CAEI-6). Out of 1020 only 516 embryos developed in to normal plants with an average frequency of 50.85%, remaining embryos were abnormal, hence discarded.

Table 1: Frequencies obtained during media optimization for one-step direct embryogenesis in *Capsicum annuum*

	Genotype Numbers															
Media Codes	IC-1120	IC-1121	IC-1122	IC-1123	IC-1124	EC-2212	EC-2213	EC-2214	EC-2215	EC-2216	No of anthers cultured	No of Embryos germinated	No of plants developed	No of DH plants	Embryo germination frequency	Plant regeneration frequency	Anther to DH frequency
CAEI-1	2110	2112	2220	2225	2255	2552	2332	2110	2005	2400	2231	910	460	186	40.79 %	20.62 %	8.34 %
CAEI-2	2200	2332	2110	2110	2665	2550	2005	2220	2400	2110	2262	0	0	0	0.00	0.00	0.00
CAEI-3	2442	2440	2110	2115	2440	2110	2110	2445	2115	2114	2241	0	0	0	0.00	0.00	0.00
CAEI-4	2552	2445	2225	2112	2445	2332	2225	2220	2110	2400	2306	32	15	6	1.39 %	0.65 %	0.26 %
CAEI-5	2005	2665	2005	2552	2555	2330	2225	2110	2000	2332	2279	60	32	13	2.63 %	1.4%	0.57 %
CA	2112	2005	2665	2442	2220	2220	2110	2225	2005	2115	2219	18	9	5	0.81 %	0.41 %	0.23 %

EI-6																
Total	1349	1399	1335	1356	1450	1394	1307	1330	1275	1351	13538	1020	516	210	7.53 %	3.81 %	1.55 %

Media combinations / protocols-

1. CAEI-1 – MS medium with vitamins + 0.1mg/lit TDZ + 0.5mg/lit IAA + 30gm/lit Sucrose + 3gm/lit Gelrite®, pH-5.8
2. CAEI-2 – MS medium with vitamins + 0.5mg/lit BAP + 1mg/lit NAA + 100gm/lit Sucrose + 3gm/lit Gelrite®, pH-5.8
3. CAEI-3 – MS medium with vitamins + 1mg/lit 2,4-D + 0.1mg/lit NAA + 100gm/lit Sucrose + 3gm/lit Gelrite®, pH-5.8
4. CAEI-4 – Induction medium – C medium supplemented with 0.01 mg/lit kinetin + 0.01 mg/lit 2,4, D +30gm/lit Sucrose + 8gm/lit Agar, pH-5.9 Regeneration medium – R medium supplemented with 0.1 mg/lit kinetin 30gm/lit Sucrose + 8gm/lit Agar, pH-5.9 (Dumas de Vaulx et.al 1981) and (Para-Vega et.al 2016)
5. CAEI-5 – Upper layer liquid medium (Nitsch medium with vitamins + 2.5 muM Zeatin + 5 muM IAA + 20gm/lit Maltose) Under layer solid medium (Nitsch medium with vitamins +20gm/lit Maltose + 10gm/lit activated charcoal+ 8gm/lit Micro Agar®, pH-5.8 (Supena et.al.2006)
6. CAEI-6 – MS medium with vitamins + 1mg/lit BAP + 1mg/lit Kinetin + 0.5mg/lit IAA + 30gm/lit Sucrose + 8gm/lit Agar, pH-5.8

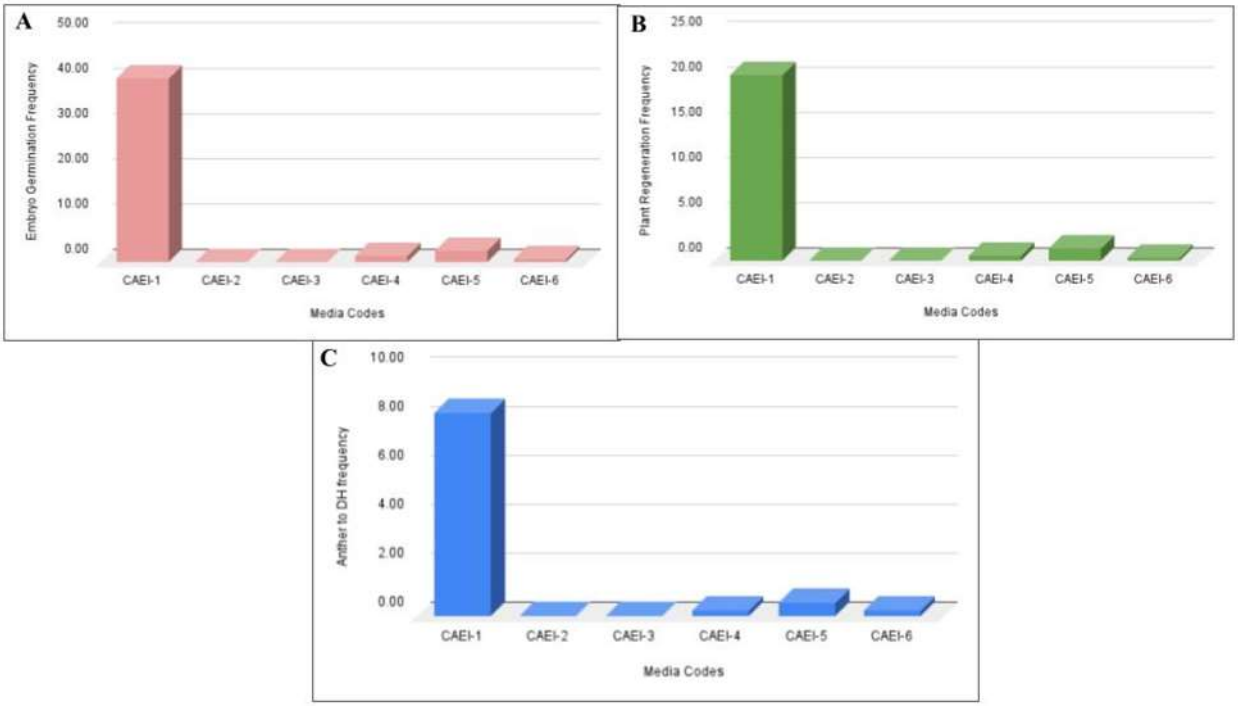

Figure 5: Graphical representation of media optimization for direct embryogenesis A) Embryo germination frequency B) Plant regeneration frequency C) Anther to DH frequency.

Table 2: Genotype wise plant regeneration frequency obtained on CAEI-1 media combination

Genotype Numbers	No. of Anthers Cultured	No. of Plants Regenerated	Plant Regeneration Frequency
IC-1120	210	80	38.10%
IC-1121	212	28	13.21%
IC-1122	220	35	15.91%
IC-1123	225	45	20%
IC-1124	225	42	18.67%
EC-2212	252	18	7.14%
EC-2213	232	60	25.86%
EC-2214	210	45	21.43
EC-2215	205	44	21.46
EC-2216	240	63	26.25
Total	2231	460	20.62

Genotypic Variation in Androgenesis:

We also studied genotype-wise frequencies and found that genotype number IC-1120 showed the highest plant regeneration frequency (6.41%), and genotype number EC-2214 showed the lowest plant regeneration frequency (2.41%). When we observed data of anthers cultured on the highest responding media, i.e., CAEI-1 (Table-2), the highest plant regeneration frequency is observed in genotype number IC-1120 (38.1%), which is relatively higher compared to reported frequencies. The lowest plant regeneration frequency is observed in genotype number EC-2212 (7.14%), which is also suitable for commercial DH production. This data reveals that there is variation in plant regeneration frequencies of different genotypes; this is predominantly regulated by the expression of transcriptional factors, namely BABY BOOM (Boutilier et al., 2002), LEAFY COTYLEDON1 (LEC1; Lotan et al., 1998), LEC2 (Stone et al., 2001), and FUSCA3 (To et al., 2006), present in the genome of plants.

Table 3: Genotype wise plant regeneration frequency obtained on CAEI-6 media combination

Genotype Numbers	No. of Anthers Cultured	No. of Plants Regenerated	Plant Regeneration Frequency
IC-1120	212	4	1.9%
IC-1121	205	0	0
IC-1122	265	0	0
IC-1123	242	1	0.4%
IC-1124	220	0	0
EC-2212	220	0	0
EC-2213	210	2	1%
EC-2214	225	0	0
EC-2215	205	1	0.5%
EC-2216	215	1	0.5%
Total	2219	9	0.4%

On the other hand, when we observed the data of the lowest responding media, i.e., CAEI-6 (Table-3), and found that the highest plant regeneration frequency is observed in genotype number IC-1120 (1.9%). The lowest plant regeneration frequency is observed in genotype number IC-1123 (0.4%), and five genotypes (IC-1121, IC-1122, IC-1124, EC-2212, and EC-2214) did not respond for

embryogenesis on this media combination. These results suggest that media code CAEI-6 is low-responding and highly genotype-dependent.

Ploidy Analysis:

We have analyzed the ploidy level of 516 regenerated plants and found that histograms of 210 plant samples resemble histograms of standard diploid samples and are considered DHs. As all plants were regenerated from microspores, we observed spontaneous chromosome doubling in 40.70 % of the total analyzed plants (Figure-6 & Table-4). Spontaneous chromosome doubling frequencies among all ten genotypes ranges between 23.26% to 53.42%, highest rate of spontaneous chromosome doubling observed in genotype number-EC 2213 and genotype number-IC 1124 showed lowest rate of spontaneous chromosome doubling. Samples from 300 plants showed histograms on the half scale of standard diploid considered as haploid as it contains only half nucleic acid content (one set of chromosomes) compared to standard diploid. Six plants considered as mixoploids as histograms of those samples showed two peaks, one resembles to standard scale as well as on half scale of standard diploid sample. As per analysis of histogram of mixoploid samples, we concluded that it contains diploid/doubled haploid as well as haploid cells. Besides artificial chromosome doubling using anti-mitotic agents, the spontaneous doubling of chromosomes in plant cells takes place due to three mechanisms- nuclear fusion, Endoreduplication, and Endomitosis (Segui-Simarro & Nuez, 2008). Out of these three mechanisms of spontaneous chromosome doubling, 90% times endoreduplication will takes place. We have not done any artificial chromosome doubling using any anti-mitotic agent such as colchicine, oryzaline, triflurine etc.

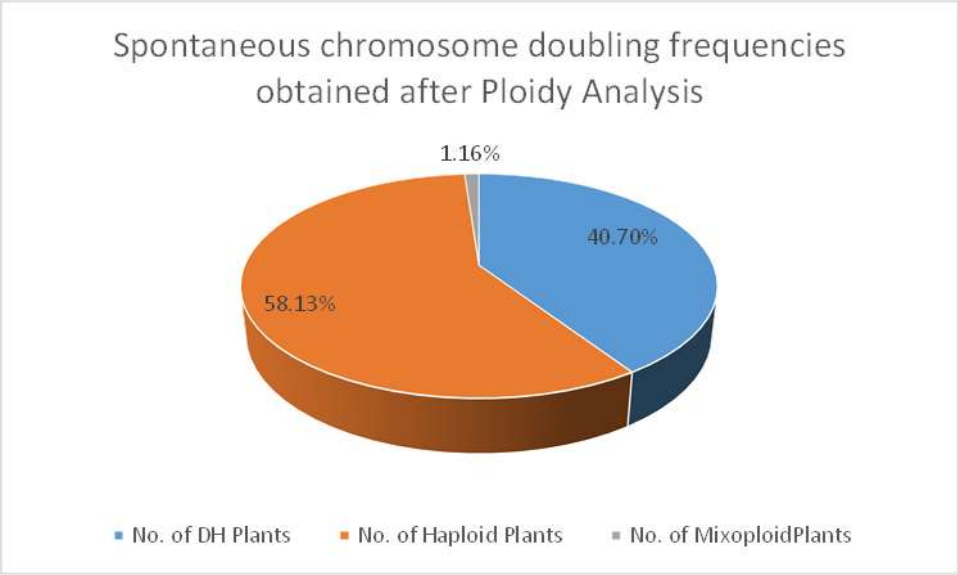

Figure 6: Spontaneous chromosome doubling frequencies obtained after flow cytometric ploidy analysis of regenerated plants

Table 4: Data of ploidy analysis with Genotype wise spontaneous chromosome doubling frequency

Genotype Numbers	No. of Plants Analyzed	No. of DH Plants	No. of Haploid Plants	No. of Mixoploid Plants	Spontaneous chromosome doubling Frequency
IC-1120	89	38	50	1	42.70
IC-1121	31	13	17	1	41.94
IC-1122	39	15	24	0	38.46
IC-1123	47	15	31	1	31.91
IC-1124	43	10	33	0	23.26
EC-2212	19	5	14	0	26.32
EC-2213	73	39	32	2	53.42
EC-2214	56	20	36	0	35.71

EC-2215	48	23	25	0	47.92
EC-2216	71	32	38	1	45.07
Total	516	210	300	6	40.70

Plant acclimatization:

We planted all 210 confirmed DH plants in sterile cocopeat and 205 plants were survived after acclimatization process with survival index of 97.61%. During planting process, only well rooted and well grown plants were selected. All process of plant acclimatization was strictly followed to achieve maximum survival rate.

4. Conclusion

In the present study, we studied response of six media combinations with the same basal media and several plant growth hormones for one step of direct embryogenesis from anthers of *Capsicum annuum*. We observed that plant growth hormones play a very important role in androgenesis. Media combination containing 2,4-D showed more callusing than embryo formation; on the other hand, media combination containing a high concentration of sucrose results in the blackening of anthers. Media code CAEI-1, which contains a lower concentration of TDZ, showed the highest embryo formation, plant regeneration, and anther to DH frequency among all six media combinations and compared to shed-microspore culture and two-step anther culture protocols. More importantly, the same media combination responded to direct embryogenesis in all genotypes, which makes it quite genotype-independent.

We have developed a unique one-step anther culture protocol for direct embryogenesis, which can be utilized for commercial DH production in *Capsicum annuum*. This protocol will support plant breeders to accelerate breeding programs by producing 100% homozygous lines in one step.

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Conflict of Interest:

On the behalf of all authors, corresponding author declares that they have no known competing financial interests or personal relationships, that could have appeared to influence the work reported in this paper.

Author Contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sachin Misal and Arunava Das. The first draft of the manuscript was written by Sachin Misal and Arunava Das commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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