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Optimizing Androgenic Embryo Regeneration and Chromosome Doubling of Haploid Plants for Sweet Pepper (Capsicum Annuum Var. Grossum L.)

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Keywords

Capsicum annuum var. grossum, regeneration, FCM, genotype effect, Doubled haploid.

Abstract

The study evaluated the effect of various hormonal treatments on androgenic embryo regeneration and the efficacy of colchicine and trifluralin in for inducing chromosome doubling in four hybrid genotypes of sweet pepper: Asha (V1), Sympathy (V2), Namelite (V3), and Indra (V4). Results showed that the highest frequency of embryo regeneration was obtained with Metatopolin (0.5 mg/l) and IAA (0.1 mg/l) in genotype Indra (V4) (64.38), while the lowest response was observed using TDZ .5 mg/L in variety genotype Sympathy (V2) (6.67) (0.00%). FCM analysis revealed that 41.53% of the overall regenerated plants were doubled haploids, with the most responsive genotype, Sympathy (V2), having the most spontaneous duplication (57.78%). Furthermore, the study investigated the survival rate and chromosome doubling frequency in four different genotypes using colchicine and trifluralin. The results indicated that colchicine was more efficient in inducing diploidization compared to trifluralin. The combination of 0.50% colchicine for 10 days yielded the highest frequency of doubled haploids in all genotypes, with a maximum DH frequency of 61.80% in Indra (V4). It was found that prolonged treatment course and high colchicine concentration decreased survivability while simultaneously increasing the frequency of doubled haploids. Moreover, the combination of 0.0075% trifluralin for 10 days also resulted in good DH frequency, with a maximum rate of 34.80% in Asha (V1). Overall, this study provides important insights into the factors affecting androgenic embryo regeneration and chromosome doubling in capsicum, which could contribute to the development of improved breeding strategies in the future.

1. Introduction

The herbaceous flowering plant *Capsicum annuum* is a member of the *Solanaceae* family. Sweet pepper, scientifically known as Capsicum annuum L., holds great importance as a highly valuable vegetable worldwide, primarily due to its nutritional composition. The primary target of inducing androgenesis is to obtain homozygotic plants. Using traditional breeding methods, such genotypes can be obtained after 7-8 years of inbreeding. Anther culture has developed significantly over time and has been utilized with success in various capsicum genotypes [7, 15]. Capsicum is considered a recalcitrant crop in terms of its *in-vitro* regeneration potential, as reported in the literature. Several studies have indicated that while a considerable number of embryos derived from anthers can be generated, only a limited proportion of them are able to develop into healthy plants. [7]. Many researchers used hormone free MS medium for the regeneration of androgenic embryos but the regeneration frequency is low [6]. The main drawback of regeneration on hormone free MS medium is emergence of only single shoot. Multiple researchers found the positive results when MS Medium is supplemented with of BAP, IAA, TDZ, zeatin and GA3 at different concentration's and combinations on regeneration and multiple shoot induction [1,10,17] The application of a factor that induces chromosome duplication and restores fertility is necessary for plants that have not undergone spontaneous diploidization. Some researchers studied the *in-vitro* effect of colchicine and trifluralin treatment of haploid regenerants obtained by anther culture of *Capsicum annuum* L and other vegetable crops [3,4,5,9]. The efficiency of chromosome doubling highly depends on the nature of chemical, treatment time and explant type. Due to the controlled conditions, the *in-vitro* method is easier to use and more effective than the greenhouse method for inducing chromosome doubling of haploids [16]. The efficiency of

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chromosome doubling is still low due to which large population of DH is hard to obtain. The objective of this study is to adapt an effective protocol for androgenic plant regeneration using various PGR and chromosome doubling using colchicine and trifluralin.

2. Methodology

Plant material

From 2019-2022, the anther culture of sweet pepper (Capsicum annuum var. grossum L.) were undertaken. Genetic material for experimentation includes four varieties namely Asha (V1), Sympathy (V2), Namelite (V3), and Indra (V4). The donor plants were cultivated and grown in a controlled greenhouse environment at the Temperate Breeding and Research Station, which is managed by ACSEN HYVEG Pvt. Ltd. in Kullu, Himachal Pradesh, India. The greenhouse maintained a temperature of 25°C during the day and 20°C during the night, along with a 16-hour photoperiod.

The anther culture procedure was conducted following the protocol described by Dumas de Vaulx et al. in (1981) [2], with modifications provided by Jha et al. (2022) [8]. In summary, buds at an appropriate stage with maximum viability were selected and subjected to a cold pretreatment at 4°C for 24 hours. Following the cold pretreatment, anthers were excised and placed on a culture medium containing 4% sucrose, 0.50 g of activated charcoal, 0.50 mg/l of 2,4-D, and 4.0 mg/l of kinetin. The plated cultures were then incubated in the dark at 35°C for 10 days, after which the plates were transferred to a temperature of 25°C under a photoperiod of 16 hours of light and 8 hours of darkness.

Regeneration

Normal looking embryos start to emerge out from anthers after three to four weeks of start culture. These experiment. In the second experiment, trifluralin was added to MS medium at concentrations of 0.0060%, 0.0075%, and 0.0090% for three different durations (6, 8, and 10 days). To ensure uniformity in every combination designed sufficient numbers of haploid lines were propagated for all entries. Following the specific duration of treatment, the plants were subsequently transferred to a hormone-free MS medium. After the emergence of new leaves which may take 6 to 8 weeks after transfer to MS medium, were transferred to MS regeneration media. For testing maximum regeneration frequency MS medium was supplemented with 6 combinations of the hormones at different concentrations, poured in petri dishes and PTC boxes. The combinations used were, TDZ 0.5 mg/l (H2), BAP 5.0 mg/l + IAA 0.5 mg/l (H3), BAP 2.0 mg/l + GA3 1.0 mg/l (H4), Zeatin 0.2mg/l+ GA3 0.03mg/l (H5), Metatopolin 0.5 mg/l + IAA 0.1 mg/l (H6) followed by Control: No hormone addition (H1). Initially embryos were transferred in petri dishes and after attaining certain height were transferred to PTC boxes.

Ploidy Determination

Flow cytometry (FCM) analysis was used to determine the ploidy levels of the regenerated plants. For this analysis, small leaves measuring 2-3 mm in size were finely chopped and mixed with 400 µl of nuclei-extracting buffer (referred to as Solution A in the CyStain UV Precise P Kit from Sysmex, Japan). The mixture was then combined with 1,600 µl of DAPI staining buffer (Solution B of the Kit). To remove any solid particles, the resulting suspension was filtered through a 30 µm nylon mesh filter. The doubled haploid (DH) plants were subsequently transferred to the greenhouse for further acclimatization, while the haploid plants underwent an in-vitro treatment with colchicine and trifluralin to induce chromosome doubling.

Chromosome doubling

The haploid plants were cultured on MS medium (Murashige and Skoog, 1962), which was supplemented with 30 g/l of sucrose and solidified with 8 g/l of agar. The pH of the medium was adjusted to 5.8. Colchicine was added at concentrations of 0.3%, 0.4%, and 0.5% for three different durations (6, 8, and 10 days) in the first

the final cytometric analysis were made of survived plants. OPSTAT software was used to do an analysis of variance (ANOVA) on the experimental data. The SPSS-16 programme for Windows was also used to compare the data and find significant differences using Duncan's multiple range tests.

3. Results and discussion:

Regeneration

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In sweet pepper, androgenic embryo regeneration was significantly influenced by different genotypes and hormonal treatments. Among the varieties, genotype V4 had the highest regeneration of embryos (38.65%) while variety V2 had the least response (12.50%). In the case of hormones, the highest regeneration of embryos was recorded with H6 (Metatopolin: 0.5 mg/l + IAA: 0.1 mg/l) (48.05%). The interaction between genotypes and hormonal treatments (V × H) revealed that the highest regeneration of embryos (64.38%) was recorded in genotype V4 when supplemented with Metatopolin (0.5 mg/l) and IAA (0.1 mg/l) (H6). The minimum regeneration response (0.00%) was obtained with treatment V2 × H1 and found at par with V1 × H1 (2.50%). The embryo regeneration followed by multiple shoot induction is shown in Figure 1. The results of the study show that the successful regeneration of androgenic embryos requires a specific ratio of auxin and Cytokinins.

Table 1: The impact of various hormone treatments on embryo regeneration in sweet pepper.

Variety	Regeneration (%)				
	V1 (Asha)	V2 (Sympathy)	V3 (Namelite)	V4 (Indra)	Mean
H_1	2.50±0.50^{mn}	0.00±0.00^{n}	6.00±0.67^{lm}	10.00±1.25^{jl}	4.63±0.61^{e}
H_2	23.00±1.00^{g}	6.67±0.00l^{m}	13.34±1.34^{ij}	31.25±2.50^{ef}	18.56±0.54^{d}
H_3	30.50±1.50^{ef}	18.34±1.67^{h}	38.00±2.00^{d}	50.00±1.25^{bc}	34.21±0.98^{b}
H_4	29.50±0.50^{ef}	8.34±1.67^{l}	27.33±2.00^{fg}	29.38±1.88^{ef}	23.64±0.33^{c}
H_5	33.50±1.50^{e}	15.00±1.67^{hi}	33.34±1.34^{e}	46.88±1.88^{c}	32.18±0.00^{b}
H_6	52.50±0.50^{b}	26.67±3.34^{fg}	48.67±0.67^{bc}	64.38±0.63^{a}	48.05±0.95^{a}
Mean	28.59±0.09^{b}	12.50±0.83^{c}	27.78±1.11^{b}	38.65±0.32^{a}	

Mean values in each column (for each variety) and overall mean of each variety (in row) followed by the same lower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test

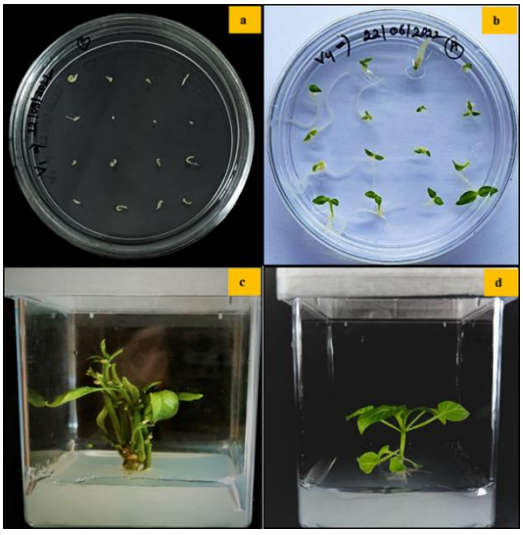

Figure 1: Embryo regeneration and multiple shoot induction in *capsicum annuum* L. a.) Embryo cultured on regeneration media, b.) Regeneration of embryos, c.) Multiple shoot induction, d.) Rooted plant on MS media.

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Combining these elements has a synergistic effect that increases the ability of androgenic embryos to regenerate and ensures their effective reestablishment. For example, Manoharan et al. (1998) [10] found that the inclusion of TDZ at a concentration of 0.5 mg/l in MS medium led to a high rate of shoot regeneration in capsicum. Similarly, Mathew (2002) [11] observed that the addition of BAP at 2.0 mg/l in MS medium supplemented with GA3 at 1.0 mg/l resulted in the maximum number of embryos in capsicum. Furthermore, Shivegowda et al. (2002) [17] successfully regenerated shoot-lets from cotyledonary explants of chilli using MS medium supplemented with Zeatin at concentrations ranging from 9-18 µM in combination with GA3 at 2.89 µM.

Ploidy Determination

Flow cytometry was used to examine the ploidy level of regenerated embryos and the results are shown in Table 2 and Figure 2. According to the FCM analysis, out of 1009 regenerated plants, 43.11% of the plants were haploids, 41.53% were doubled haploids, 5.45% were triploids, 4.76% were tetraploids, and 5.15% were mixoploids. Different genotypes had different proportions of plants with different ploidy levels. V2 (Sympathy), had maximum spontaneous duplication (57.78%) whereas, the least duplication rate was found in V1 (Asha) (34.11%)

Chromosome doubling

435 haploid plants obtained from anther culture of genotypes Asha (V1), Sympathy (V2), Namelite (V3), and Indra (V4) were used as materials. The genotype, concentration of diploidizing agent, and treatment duration were observed to have a significant impact on the survival rate and frequency of chromosome doubling in Capsicum. This can be seen in Tables 3 and 4, which present the results for colchicine and trifluralin treatments, respectively.

Table 2: Ploidy level of the four genotypes of capsicum plants generated from anther culture as determined by flow cytometry.

Variety	No of tested plants	Haploids (%)	Doubled haploids (%)	Triploids (%)	Tetraploids (%)	Mixoploids (%)
V_1	343	46.06	34.11	6.12	7.58	6.12
V_2	45	22.22	57.78	2.22	11.11	6.67
V_3	250	59.20	39.20	0.00	0.80	0.80
V_4	371	32.08	47.98	8.89	4.04	7.01
Total	1009	43.11	41.53	5.45	4.76	5.15

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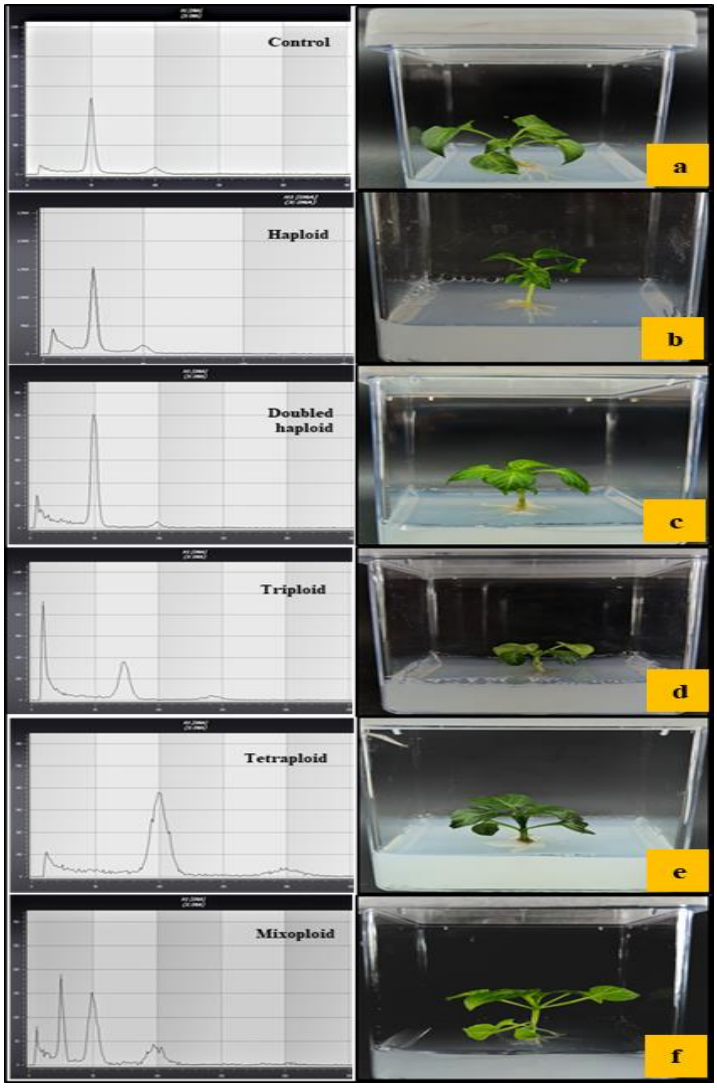

Figure 2: Ploidy estimation of Regenerated plantlets: a.) Diploid Control, b.) Haploid, c.) Doubled haploid, d.) Triploid, e.) Tetraploid, f.) Mixoploid.

Table 3: The efficacy of colchicine in inducing diploidization in different haploids.

Variety	Conc. of Colchicine (%)	Treatment Duration (Days)	Treated haploid plants	No. of plants survived	DH frequency (%)	Survival (%)
Asha (V_1)	0.3	6	30	25.5	0.0 ± 0.0^{m}	85.0 ± 1.7^{c}
		8	30	21.0	4.8 ± 0.0^{ki}	70.0 ± 0.0^{ef}
		10	30	19.0	10.5 ± 0.0^{h}	63.3 ± 0.0^{gh}
	0.4	6	30	24.5	4.1 ± 0.1^{l}	81.7 ± 1.7^{d}
		8	30	22.0	9.1 ± 0.0^{hij}	73.3 ± 0.0^{e}
		10	30	20.5	14.7 ± 0.4^{g}	68.4 ± 1.7^{f}
	0.5	6	30	19.0	15.8 ± 0.0^{g}	63.2 ± 0.1^{gh}
		8	30	18.0	30.6 ± 2.8^{c}	60.0 ± 0.0^{hij}
		10	30	16.5	48.6 ± 1.5^{b}	55.0 ± 1.7^{k}

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Sympathy (V_2)	0.3	6	10	9.0	0.0±0.0^{m}	90.0±0.0^{a}
		8	10	8.0	0.0±0.0^{m}	80.0±0.0^{d}
		10	10	6.0	0.0±0.0^{m}	60.0±0.0^{hij}
	0.4	6	10	8.0	6.3±0.0^{jkl}	80.0±0.0^{d}
		8	10	7.0	14.3±0.0^{g}	70.0±0.0^{ef}
		10	10	6.0	16.7±0.0^{fg}	60.0±0.0^{hij}
	0.5	6	10	6.0	16.7±0.0^{fg}	60.0±0.0^{hij}
		8	10	5.0	20.0±0.0^{e}	50.0±0.0^{i}
		10	10	4.0	25.0±0.0^{d}	40.0±0.0^{m}
Namelite (V_3)	0.3	6	30	26.5	0.0±0.0^{m}	88.4±1.7^{ab}
		8	30	21.0	0.0±0.0^{m}	70.0±0.0^{ef}
		10	30	18.5	5.5±0.2^{kl}	61.7±1.7^{ghi}
	0.4	6	30	24.5	0.0±0.0^{m}	81.7±1.7^{d}
		8	30	21.5	7.0±2.5^{ijkl}	71.7±1.7^{ef}
		10	30	19.5	10.3±0.3^{hi}	65.0±1.7^{g}
	0.5	6	30	18.5	5.5±0.2^{kl}	61.7±1.7^{ghi}
		8	30	17.0	14.7±2.9^{g}	56.7±0.0^{jk}
		10	30	15.5	19.4±0.6^{ef}	51.9±1.9^{i}
Indra (V_4)	0.3	6	30	26.0	5.8±2.0^{kl}	86.7±0.0^{bc}
		8	30	21.0	9.5±0.0^{hij}	70.0±0.0^{ef}
		10	30	18.5	10.8±0.3^{h}	62.2±1.2^{gh}
	0.4	6	30	24.5	8.2±0.2^{hijk}	81.7±1.7^{d}
		8	30	21.0	9.5±0.0^{hij}	70.0±0.0^{ef}
		10	30	20.5	14.7±0.4^{g}	68.9±1.2^{f}
	0.5	6	30	19.0	21.1±0.0^{e}	63.3±0.0^{gh}
		8	30	17.5	45.8±1.4^{b}	58.4±1.7^{ij}
		10	30	16.5	61.8±3.0^{a}	56.7±0.0^{jk}

Mean values in each column followed by the same lower case letters were not significantly different at P≤0.05 according to Duncan's multiple range test

Table 4: The efficacy of Trifluralin in inducing diploidization in different haploids

Variety	Conc. of Colchicine (%)	Treatment Duration (Days)	Treated haploid plants	No. of plants survived	DH frequency (%)	Survival (%)
Asha (V_1)	0.3	6	30	25.5	0.0±0.0^{m}	85.0±1.7^{c}
		8	30	21.0	4.8±0.0^{ki}	70.0±0.0^{ef}
		10	30	19.0	10.5±0.0^{h}	63.3±0.0^{gh}
	0.4	6	30	24.5	4.1±0.1^{l}	81.7±1.7^{d}
		8	30	22.0	9.1±0.0^{hij}	73.3±0.0^{e}
		10	30	20.5	14.7±0.4^{g}	68.4±1.7^{f}
	0.5	6	30	19.0	15.8±0.0^{g}	63.2±0.1^{gh}
		8	30	18.0	30.6±2.8^{c}	60.0±0.0^{hij}
		10	30	16.5	48.6±1.5^{b}	55.0±1.7^{k}

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	0.3	6	10	9.0	0.0±0.0^{m}	90.0±0.0^{a}
		8	10	8.0	0.0±0.0^{m}	80.0±0.0^{d}
		10	10	6.0	0.0±0.0^{m}	60.0±0.0^{hij}
	0.4	6	10	8.0	6.3±0.0^{jkl}	80.0±0.0^{d}
Sympathy		8	10	7.0	14.3±0.0^{g}	70.0±0.0^{ef}
(V_2)		10	10	6.0	16.7±0.0^{fg}	60.0±0.0^{hij}
	0.5	6	10	6.0	16.7±0.0^{fg}	60.0±0.0^{hij}
		8	10	5.0	20.0±0.0^{e}	50.0±0.0^{i}
		10	10	4.0	25.0±0.0^{d}	40.0±0.0^{m}
	0.3	6	30	26.5	0.0±0.0^{m}	88.4±1.7^{ab}
		8	30	21.0	0.0±0.0^{m}	70.0±0.0^{ef}
		10	30	18.5	5.5±0.2^{kl}	61.7±1.7^{ghi}
	0.4	6	30	24.5	0.0±0.0^{m}	81.7±1.7^{d}
Namelite		8	30	21.5	7.0±2.5^{ijkl}	71.7±1.7^{ef}
(V_3)		10	30	19.5	10.3±0.3^{hi}	65.0±1.7^{g}
	0.5	6	30	18.5	5.5±0.2^{kl}	61.7±1.7^{ghi}
		8	30	17.0	14.7±2.9^{g}	56.7±0.0^{jk}
		10	30	15.5	19.4±0.6^{ef}	51.9±1.9^{i}
	0.3	6	30	26.0	5.8±2.0^{kl}	86.7±0.0^{bc}
		8	30	21.0	9.5±0.0^{hij}	70.0±0.0^{ef}
		10	30	18.5	10.8±0.3^{h}	62.2±1.2^{gh}
	0.4	6	30	24.5	8.2±0.2^{hijk}	81.7±1.7^{d}
Indra		8	30	21.0	9.5±0.0^{hij}	70.0±0.0^{ef}
(V_4)		10	30	20.5	14.7±0.4^{g}	68.9±1.2^{f}
	0.5	6	30	19.0	21.1±0.0^{e}	63.3±0.0^{gh}
		8	30	17.5	45.8±1.4^{b}	58.4±1.7^{ij}
		10	30	16.5	61.8±3.0^{a}	56.7±0.0^{jk}

Mean values in each column followed by the same lower case letters were not significantly different at P≤0.05 according to Duncan's multiple range test.

In accordance with the information presented in Table 3, the combination of a prolonged treatment course and a high colchicine concentration decreases survivability while simultaneously increasing the frequency of DH. In all genotypes, the combination of 0.5 percent colchicine for 10 days yielded the highest DH frequency, with a maximum of 61.8 % in V4, while the least DH frequency (0.0 %) was found in V2 using 0.3 percent colchicine in all treatment durations taken, which was comparable to seven other combinations. The combination of 0.3 percent colchicine for six days yielded the highest survivability percentage in V2 (90.0%), which was found at par (88.4%) in V3 using same concentration and the lowest was found in V2 (40.0%) using 0.5% colchicine for 10 days. These results are much higher than the results obtained by other researchers. Olszewska et al. (2015) [14] reported his findings for effective rate of diploidization in capsicum and recommended the use of 400 mg dm^{-3}[3] colchicine for 6 days in in-vitro conditions by obtaining 17-27% of DH frequency in four genotypes. Our experiment showed that upon increasing the treatment durations can increases the effective diploidization of haploids. [5,12].

According to the data in Table 4, the genotypes with the highest DH frequency were treated with 0.0075% trifluralin for 10 days, with a maximum DH rate of 34.80% in V1. On the other hand, the genotype with

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the lowest DH frequency was V1 (0.00%) when treated with 0.0060% trifluralin for 6 days and found to be on par with 8 other combinations. The combination of 0.0075% trifluralin for eight days yielded the highest survivability percentage (100%) in (V_2), while the lowest was recorded in (V_2) (50.00%). According to Wang et al (2021) [18] the application of trifluralin on growth points of pepper haploid's seedling (*in-vivo*) resulted in 100 % survivability but chromosome doubling frequency is 0.00%

4. Summary and Conclusion

This experiment revealed that androgenic embryo regeneration in sweet pepper is significantly influenced by genotype and hormonal treatments. The regeneration of embryos was found best using H6 hormonal treatment in all genotypes. The genotype found most responsive towards regeneration was V4 (Indra) with regeneration rate 38.65. The FCM analysis revealed that different genotypes had different proportions of plants with different ploidy levels; V2 genotype had maximum spontaneous duplication. The genotype, concentration of the diploidizing agent, and treatment duration were all found to have a significant impact on the survival rate and frequency of chromosome doubling in Capsicum, according to the study. The combination of 0.50% colchicine for 10 days yielded the highest DH frequency in all genotypes, with a maximum of 61.8% in V4 (Indra), while the combination of 0.0075% trifluralin for 10 days yielded the highest DH rate of 34.80% in V1 (Asha) genotype. These findings have practical implications for crop improvement programs as they provide insights into the optimal conditions for inducing androgenic embryo regeneration and chromosome doubling in Capsicum. Overall, the study highlights the importance of genotype selection and hormonal treatments in the efficient regeneration of plants and the production of doubled haploids in sweet pepper (Capsicum annuum var. grossum L.).

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