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Enhancing embryogenesis efficiency in sweet pepper (*Capsicum annuum* var. grossum) via anther culture optimization

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ABSTRACT

Sweet pepper [*Capsicum annuum* var. grossum (L.)], valued for its nutritional benefits like high vitamin C and carotenoid levels, requires efficient breeding methods to meet demand. Double haploid (DH) technology provides a rapid alternative to traditional methods by generating homozygous lines in a single generation. The present study was carried out during 2021 and 2022 at ACSEN Agriscience Pvt. Ltd. Kullu, Himachal Pradesh to determine the ideal conditions for inducing embryo formation from anthers in 4 sweet pepper hybrids, viz. Asha, Sympathy, Nemalite, and Indra. The study investigated the impact of various factors on embryo development in sweet pepper anther cultures, including cold pre-treatment of buds, heat shock, different concentrations of sucrose and activated charcoal, and the application of plant hormones 2,4-D, and kinetin. Results indicated that a 24 h cold pre-treatment at 4°C accompanied by a 10-days heat shock at 35°C significantly enhanced embryogenic responses, particularly in genotype Asha, which showed a peak response of 39.3 embryos. The optimal sucrose (4%) and activated charcoal (0.50 gm/litre) concentrations further improved embryogenesis, with Asha achieving up to 48.7 embryos. The balance of 2, 4-D (0.50 mg/litre) and kinetin (4.0 mg/litre) was also crucial. Genotype Asha showed the highest response of 43.0 embryos under these specific conditions. Genotype-specific responses highlighted the importance of genetic variability in androgenesis studies, with Asha giving the best result, achieving the highest average of 34.7 embryos with a 23.1% embryogenic response in the combined experiment using the most effective treatment combinations. This study highlights how optimized anther culture enhances embryo formation, aiding the development of new sweet pepper cultivars and improving breeding efficiency with DH technology.

Keywords: Androgenesis, Anther culture, Doubled haploid, Embryogenesis, Genotype

Sweet pepper [*Capsicum annuum* var. *grossum* (L.)], also known as bell pepper, is a globally significant vegetable recognized for its nutritional value. It is particularly abundant in vitamin C and various carotenoids, contributing to its widespread use in culinary and medicinal practices (Irikova *et al.* 2011). To meet the growing need for sweet peppers, it is crucial to develop new and improved cultivars that can boost productivity. Conventionally, the creation of true breeding sweet pepper cultivars has relied on a labour-intensive and time-consuming process involving backcross breeding followed by multiple generations of self-pollination. The application of DH technology significantly streamlines the breeding process by allowing for the rapid generation of homozygous lines in just one generation. This bypasses the need for numerous selfing generations, significantly lowering production costs and proving invaluable for hybrid production (Segui-Simarro 2010). The DH technique significantly expedites the breeding process, enabling the rapid development of inbred lines with desirable traits, which can then be readily utilized in subsequent breeding programmes and new sweet pepper varieties (Kim *et al.* 2004, Koleva-Gudeva *et al.* 2007). Wang *et al.* (1973) conducted pioneering work on the successful development of haploid embryos from *Capsicum annuum* anthers in China, while George and Narayanaswamy (1973) did so in India. In Turkey, Abak (1983) initiated the initial studies on *in vitro* androgenesis in domestic pepper genotypes. Building upon these initial discoveries, numerous researchers have further investigated various aspects of androgenesis, encompassing techniques like anther culture, culture of microspores shed from anthers, and culture of individually isolated microspores (Sibi *et al.* 1979, Dumas de Vaulx *et al.* 1981, Kristiansen and Andersen 1993, Supena *et al.* 2006, Kim *et al.* 2008). Anther culture is the most commonly employed method for double haploid production in pepper among the three techniques. Previous studies have demonstrated that the frequency of androgenesis is influenced by multiple factors. In androgenesis, genotype consistently emerges as the foremost limiting factor, unaffected by growth

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conditions of donor and other cultural variables (Kristiansen and Andersen 1993, Wang and Zhang 2001, Rodeva *et al.* 2004, Buyukalaca *et al.* 2004, Nowaczyk and Kisiala 2006, Koleva-Gudeva *et al.* 2007). Furthermore, factors such as the microspore developmental stage, vitality of microspore, pre-treatment of anthers, composition of the medium, culture conditions, sources of carbon, and the presence of activated charcoal have been observed to affect androgenesis (Ciner and Tipirdamaz 2002, Buyukalaca *et al.* 2004, Nowaczyk and Kisiala 2006). The main objective of this study was to examine the influence of temperature treatments, nutrient levels (sucrose and activated charcoal), and plant hormones (2,4-D and kinetin) on embryo development in four sweet pepper hybrids.

MATERIALS AND METHODS

The present study was carried out during 2021 and 2022 at ACSEN Agriscience Pvt. Ltd., Kullu, Himachal Pradesh. During the study, anther culture experiments were conducted using four hybrids of sweet peppers, viz. Asha, Sympathy, Nemalite, and Indra. In February 2021, the hybrids were initially sown in plug trays containing a growth medium composed of cocopeat, vermiculite, and perlite in a 10:1:1 ratio. After 45 days, the seedlings were transplanted into a controlled greenhouse at the ACSEN Agriscience Private Limited, Kullu, Himachal Pradesh, in April 2021. The greenhouse was kept at ideal conditions, with a 16 h photoperiod and temperatures ranging from 25–30°C during the day and 15–20°C at night. Buds for experimentation were harvested during the initial flowering phase in May 2021. Throughout their growth, the plants were carefully cultivated to ensure they remained free from biotic and abiotic stresses such as pests, weeds, diseases, and nutrient deficiencies.

Anther culture

Selection of flower bud size and developmental stage: Flower buds were selected based on their size, ranging from 4.75–5.50 mm in 0.25 mm increments, to capture diverse developmental stages and ensure optimal vitality. Each bud size was then stained with DAPI and FDA-PI to evaluate its developmental stage and microspore viability. Buds with microspores in the late uninucleate to early binucleate stage, exhibiting a 70:30 viability ratio under microscopic examination, were chosen for subsequent investigation (Kim *et al.* 2004, Rodeva *et al.* 2007).

Cold pre-treatment and surface sterilization: To prepare the flower buds for experimentation, the selected buds from each genotype were subjected to cold shock by storing them in a refrigerator at 4°C for periods of 0, 24, and 48 h. The buds underwent surface sterilization, starting with pre-sterilization in a 0.5% bavistin and tween 20 solution, followed by rinsing, then sterilization with 4% sodium hypochlorite, and finally, several rinses with autoclaved distilled water.

Anther culture experimentation: The anther culture experiments were divided into three sets, each with unique treatment combinations. Each treatment was repeated three times, utilizing 300 anthers per repetition. Throughout every experiment, the experimental conditions and growth media (CP) were maintained identical to those specified by Dumas de Vaulx *et al.* (1981), except for the factors under investigation. The culture medium underwent sterilization by autoclaving at 121°C under 15 psi pressure for 20 min. Before autoclaving, the *p*H of the medium was carefully adjusted to 5.8 using 1N HCl and NaOH solutions.

Relationship between cold pre-treatment and heat shock: To examine how the duration of cold pre-treatment and heat shock duration for inducing embryogenesis, anthers were extracted from flower buds that had been pre-treated at 4°C for varying durations: 0 hours (no pre-treatment), 24 h, and 48 h. These excised anthers were then cultured on plates (90 mm) having CP medium. The plates were incubated in darkness at 35°C for 8, 10, and 12 days of heat shock treatment. After each specific heat shock treatment, the plates containing the anther cultures were transferred to a controlled environment with a temperature of 25°C and a photoperiod consisting of 16 h of light and 8 h of darkness (Dumas de Vaulx *et al.* 1981).

Optimization of sucrose and activated charcoal concentrations: The influence of varying sucrose and activated charcoal levels on anther culture was investigated. The CP medium was enriched with three sucrose concentrations: 2, 3, and 4%, and four activated charcoal concentrations: 0.25, 0.50, 0.75, and 1.0 gm/litre. Anthers were cultured initially at 35°C in darkness for 8 days, then transferred to 25°C with a 16 h light and 8 h dark cycle (Dumas de Vaulx *et al.* 1981).

Optimization of 2,4-D and kinetin concentrations: To establish the ideal balance of 2,4-D and kinetin for anther culture, CP medium is supplemented with varying concentrations of these plant hormones. Three different concentrations of 2, 4-D (0.25, 0.50, and 0.75 mg/litre) and four concentrations of kinetin (1.0, 2.0, 3.0, and 4.0 mg/litre) were tested, resulting in a total of 12 unique media combinations. Anthers extracted from the selected flower buds were then cultured in petri-dishes containing these enriched media. The culture process involved two distinct phases. Initially, the anthers were incubated in darkness at 35°C for 8 days. Following this initial incubation, the cultures were transferred to a 25°C environment with a 16 h light and 8 h dark photoperiod (Dumas de Vaulx *et al.* 1981).

Integrated anther culture approach for enhanced embryo development: The most successful treatment combinations from three initial experiments was integrated into a single experiment with two replicates, each containing 150 anthers, for further analysis. Using the optimized CP induction medium, the combined treatment approach was then employed to evaluate the androgenic response of all four sweet pepper genotypes.

Statistical analysis: The collected data on embryo numbers and induction frequency underwent statistical analysis to assess the significance of the findings. The experiment was designed in a completely randomized design

(CRD) since it was conducted under laboratory conditions. Analysis of variance (ANOVA) was conducted using OPSTAT software to determine overall variation between groups. Subsequently, Duncan's multiple range tests were performed with SPSS-16 software to pinpoint specific significant differences between the various treatment groups.

RESULTS AND DISCUSSION

Flower bud selection: Bud size and microspore development varied among genotypes. Asha and Indra exhibited buds ranging from 4.75–5.25 mm, while Sympathy and Nemalite displayed slightly larger buds, ranging from 5.0–5.50 mm. In all genotypes, microspores were observed at the late uninucleate to early binucleate stages considered critical for androgenesis success (Fig. 1) (Parra-Vega *et al.* 2013). Sahana *et al.* (2024) identified buds measuring 5.39 mm in Orobelle and 4.8 mm in Bomby,two F1 hybrids of *Capsicum annuum* as optimal, containing uninucleate microspores. Similarly, Kim *et al.* (2004) emphasized the early binucleate stage as ideal for embryo induction, while Rodeva *et al.* (2007) confirmed that buds of 3.5–4.0 mm with microspores at these stages showed the highest androgenic response. Collectively, these studies highlight the critical importance of selecting buds at precise microspore developmental stages to maximize androgenesis success in sweet pepper.

Embryo induction response to cold pre-treatment and heat shock: For each genotype, the highest and lowest embryogenic responses, along with the mean values, resulting from varying durations of cold pre-treatment and subsequent heat shock treatments on CP medium (Table 1). The combination of a 24 h cold pre-treatment at 4°C and a 10-days heat shock at 35°C in darkness produced the highest average embryogenic response across all genotypes. Specifically, Asha exhibited the highest embryogenic response of 39.3 embryos, followed by Indra with 28.0 embryos, Nemalite with 20.3 embryos, and Sympathy with 4.7 embryos. This combination also resulted in the highest overall mean embryogenic response for Asha, 18.2 embryos, indicating the critical role of temperature treatments in enhancing embryogenesis. These findings align with earlier research highlighting the positive impact of temperature treatments on microspore development and embryogenesis (Sibi *et al.* 1979, Dumas de Vaulx *et al.* 1981). Cold

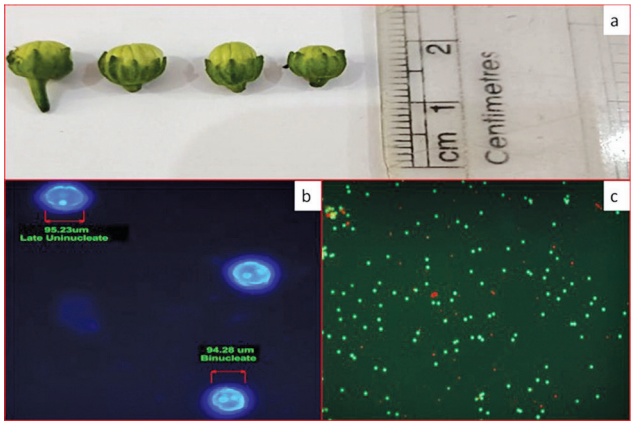

Fig. 1 Flower buds, developmental stages and viability of microspores; (a) various bud sizes at the microspore stage; (b) identification of late uninucleate and binucleate microspores with staining; and (c) evaluation of microspore viability using FDA/PI staining (green for viable cells and red for dead cells).

Table 1 Effect of cold pre-treatment on embryo induction with heat shock (average number of embryos per 300 anthers)

Genotype	Cold pre-treatment (Hours, at 4°C)	Heat shock				
		35°C (6 days dark) + 25°C continues	35°C (8 days dark)+ 25°C continues	35°C (10 days dark)+25°C continues	35°C (12 days dark) +25°C continues	Mean
Asha	0	4.7 ± 0.3^{lmn}	7.7 ± 0.9^{ij}	16.3 ± 0.3^{d}	1.0 ± 0.0^{rs}	7.4 ± 0.2^{d}
	24	10.3 ± 0.3^{fg}	16.0 ± 0.6^{d}	39.3 ± 0.3^{a}	7.0 ± 0.6^{jk}	18.2 ± 0.2^{a}
	48	0.0 ± 0.0^{s}	2.7 ± 0.7^{opq}	10.7 ± 0.3^{fg}	1.3 ± 07^{qrs}	3.7 ± 0.3^{g}
Sympathy	0	0.0 ± 0.0^{s}	$27.\pm0.3^{opq}$	4.7 ± 0.3^{lmn}	1.0 ± 0.0^{rs}	2.1 ± 0.2^{h}
	24	3.3 ± 0.7^{nop}	4.3 ± 0.3^{mno}	7.0 ± 0.6^{jk}	3.3 ± 0.7^{nop}	4.5 ± 0.1^{f}
	48	0.0 ± 0.0^{s}	3.3 ± 0.7^{nop}	4.0 ± 1.2^{mnop}	0.0 ± 0.0^{s}	1.8 ± 0.3^{h}
Nemalite	0	2.3 ± 0.3^{pqr}	7.3 ± 0.3^{ijk}	14.3 ± 0.7^{e}	0.0 ± 0.0^{s}	6.0 ± 0.0^{e}
	24	6.0 ± 0.6^{kl}	11.3 ± 0.3^{f}	20.3 ± 0.7^{c}	2.7 ± 0.3^{opq}	10.1 ± 0.3^{c}
	48	0.0 ± 0.0^{s}	$1.0.\pm0.6^{rs}$	3.0 ± 0.6^{nop}	0.0 ± 0.0^{s}	1.0 ± 0.3^{i}
Indra	0	4.0 ± 0.6^{mnop}	5.3 ± 0.7^{lm}	9.7 ± 0.7^{gh}	1.0 ± 0.6^{rs}	5.0 ± 0.0^{f}
	24	6.0 ± 0.6^{kl}	8.7 ± 0.7^{hi}	28.0 ± 0.6^{b}	5.3 ± 0.3^{lm}	12.0 ± 0.1^{b}
	48	0.0 ± 0.0^{s}	3.3 ± 0.7^{nop}	6.0 ± 0.6^{kl}	0.3 ± 0.3^{s}	2.4 ± 0.2^{h}
	Mean	3.1 ± 0.1^{c}	6.1 ± 0.1^{b}	13.6 ± 0.1^{a}	1.9 ± 0.0^{d}	

Mean values within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

pre-treatment is known to improve microspore embryogenesis by retaining pollen viability, delaying senescence, and preventing pollen abortion (Bajaj 1978). It stimulates sporophytic divisions and suppresses the gametophytic pathway through metabolic slowdown and starvation effects (Zheng 2003). Lazar *et al.* (1985) and Morrison *et al.* (1986) noted that cold pre-treatment from 24–100 h before culture enhanced callus yield and spontaneous chromosome doubling. Heat shock, vital for triggering embryogenesis, was most effective with an 8-day duration. It induces cytoskeletal changes, microtubule alterations, and heat shock protein synthesis, particularly HSP70, which inhibits apoptosis and supports embryogenesis (Hause *et al.* 1993, Jaattela *et al.* 1998, Segui-Simarro *et al.* 2003). However, excessive heat exposure (>12 days) suppressed embryogenesis by disrupting microspore pathways. These findings align with prior research emphasizing the importance of temperature stress for microspore development and embryogenesis in pepper (Shariatpanahi *et al.* 2006, Ozkum and Tipirdamaz 2011).

Effects of sucrose and activated charcoal on embryogenic response: The influence of different concentrations of sucrose and activated charcoal on embryo formation in four sweet pepper genotypes was investigated (Table 2). Among the genotypes, Asha exhibited its highest embryogenic response, 48.7 embryos, with 4% sucrose and 0.50 g/ litre activated charcoal. Sympathy demonstrated a peak response of 10.7 embryos under the same conditions, while Nemalite and Indra showed their highest responses, 32.0 and 37.3 embryos, respectively. These results underscore the importance of optimizing nutrient levels for each genotype to maximize embryogenic response. Higher concentrations of sucrose (4%) and activated charcoal (0.50 g/litre) demonstrated the highest embryogenic responses, indicating that these components are crucial for supporting cellular metabolism and mitigating inhibitory substances, thereby enhancing microspore viability and embryogenesis (Johansson 1983, Ciner and Tipirdamaz 2002). The promotive effect of activated charcoal (AC) in anther culture has been attributed to its adsorption of inhibitory substances like phenolics and abscisic acid (Typyrdamaz and Ellialtyoulu 1998). Cheng *et al.* (2013) found that 0.05% AC significantly increased ELS yields in pepper genotypes. These findings align with Supena *et al.* (2006), who observed a significant improvement in *in vitro* androgenesis of pepper on a 1% AC medium, regardless of genotype responsiveness. AC's strong adsorptive properties are thought to remove growth inhibitors from the medium, but it may also absorb beneficial substances, potentially hindering embryogenesis (Weatherhead *et al.* 1979, Johansson *et al.* 1990). Supena *et al.* (2006) noted that increasing AC concentrations did not proportionally enhance embryo yield and that a 2% concentration impaired embryonic shoot development in pepper. Additionally, Dumas de Vaulx *et al.* (1981) reported that 3% w/v sucrose combined with 0.8% agar is an efficient carbon source in embryo induction media for pepper.

Effects of 2,4-D and kinetin on embryogenic response: The effects of varying combinations of 2,4-D (0.25, 0.50, and 0.75 mg/litre) and Kinetin (1.0, 2.0, 3.0, and 4.0 mg/litre) on embryo formation in four sweet pepper genotypes (Table 3). Among the varieties, Asha demonstrated the highest embryogenic response, reaching a peak of 43.0 embryos under the treatment of 2,4-D (0.50 mg/litre) and Kinetin (4.0 mg/litre). Similarly, Sympathy, Nemalite, and Indra exhibited their highest responses with the same concentration of 2,4-D and Kinetin, indicating the effectiveness of this hormone combination in promoting embryogenesis. These findings highlight the significance of the balance between

Table 2 Sucrose effects on embryo induction with activated charcoal (average number of embryos/300 anthers)

Genotype	Sucrose (%)	Activated charcoal (g/l)				
		(0.25)	(0.50)	(0.75)	(1.0)	Mean
Asha	2	0.0 ± 0.0^{u}	4.7 ± 0.3^{nop}	4.0 ± 0.6^{opq}	2.7 ± 0.3^{qrs}	2.8 ± 0.2^{h}
	3	7.7 ± 0.3^{kl}	22.0 ± 0.6^{d}	8.0 ± 0.6^{k}	6.0 ± 0.6^{lmn}	10.9 ± 0.2^{d}
	4	12.0 ± 0.6^{hi}	48.7 ± 0.7^{a}	23.3 ± 0.7^{d}	15.0 ± 1.0^{g}	24.8 ± 0.4^{a}
Sympathy	2	0.0 ± 0.0^{u}	2.0 ± 0.6^{rst}	0.3 ± 0.3^{tu}	0.0 ± 0.0^{u}	0.6 ± 0.1^{i}
	3	0.0 ± 0.0^{u}	4.3 ± 0.9^{nop}	4.0 ± 0.6^{opq}	2.0 ± 0.6^{rst}	2.6 ± 0.5^{h}
	4	10.0 ± 0.6^{j}	10.7 ± 0.9^{ij}	5.3 ± 0.3^{mno}	0.0 ± 0.0^{u}	6.5 ± 0.2^{f}
Namelite	2	0.7 ± 0.3^{tu}	5.3 ± 0.3^{mno}	3.0 ± 0.6^{pqr}	1.0 ± 0.6^{stu}	2.5 ± 0.3^{h}
	3	8.0 ± 0.6^{k}	19.0 ± 0.6^{e}	7.0 ± 1.0^{klm}	3.0 ± 0.6^{pqr}	9.3 ± 0.4^{e}
	4	12.3 ± 0.9^{hi}	32.0 ± 0.6^{c}	12.7 ± 0.7^{h}	9.7 ± 0.7^{j}	16.7 ± 0.5^{b}
Indra	2	0.0 ± 0.0^{u}	17.0 ± 0.6^{f}	2.0 ± 0.0^{rst}	0.0 ± 0.0^{u}	4.8 ± 0.1^{g}
	3	6.0 ± 0.6^{lmn}	22.0 ± 0.6^{d}	6.0 ± 0.6^{lmn}	5.0 ± 0.6^{no}	9.8 ± 0.1^{e}
	4	7.0 ± 0.6^{klm}	37.3 ± 0.3^{b}	12.3 ± 0.9^{hi}	3.0 ± 0.6^{pqr}	15.0 ± 0.4^{c}
	Mean	5.3 ± 0.21^{c}	18.8 ± 0.2^{a}	7.3 ± 0.0^{b}	3.9 ± 0.0^{d}	

Mean values within a column followed by the same lowercase letter are not significantly different at $P\leq0.05$ according to Duncan's multiple range test.

Table 3 Auxin effects on embryo induction with kinetin (average number of embryos/300 anthers)

Genotype	2,4-Dichlorophenoxyacetic acid (2,4-D)	Kinetin (mg/litre)				
		1.0	2.0	3.0	4.0	Mean
Asha	0.25	2.3 ± 0.3^{stu}	5.0 ± 0.6^{mnop}	18.0 ± 0.6^{fg}	28.0 ± 0.6^{d}	13.3 ± 0.3^{c}
	0.50	5.7 ± 0.3^{mn}	10.3 ± 0.7^{jk}	24.0 ± 0.6^{e}	43.0 ± 0.6^{a}	20.8 ± 0.3^{a}
	0.75	0.0 ± 0.0^{v}	0.0 ± 0.0^{v}	2.0 ± 0.6^{tu}	6.0 ± 0.6^{m}	2.0 ± 0.1^{h}
Sympathy	0.25	0.0 ± 0.0^{v}	2.7 ± 0.3^{rst}	4.3 ± 0.3^{nopq}	6.0 ± 0.6^{m}	3.3 ± 0.21^{g}
	0.50	5.3 ± 0.3^{mno}	5.3 ± 0.3^{mno}	6.3 ± 0.3^{m}	11.7 ± 0.3^{ij}	7.2 ± 0.2^{d}
	0.75	0.0 ± 0.0^{v}	0.0 ± 0.0^{v}	2.0 ± 0.6^{tu}	3.0 ± 0.6^{qrst}	1.3 ± 0.3^{i}
Nemalite	0.25	2.0 ± 0.6^{tu}	4.0 ± 0.6^{opqr}	6.0 ± 0.6^{m}	13.0 ± 0.6^{i}	6.3 ± 0.3^{e}
	0.50	5.3 ± 0.3^{mno}	8.7 ± 0.3^{l}	19.3 ± 0.7^{f}	32.3 ± 0.3^{c}	16.4 ± 0.1^{b}
	0.75	0.0 ± 0.0^{v}	1.0 ± 0.6^{uv}	3.0 ± 0.6^{qrst}	5.7 ± 0.3^{mn}	2.4 ± 0.2^{h}
Indra	0.25	0.0 ± 0.0^{v}	1.0 ± 0.6^{uv}	3.3 ± 0.3^{qrst}	14.7 ± 0.9^{h}	4.8 ± 0.0^{f}
	0.50	4.0 ± 0.6^{opqr}	10.7 ± 0.9^{j}	16.7 ± 0.9^{g}	34.0 ± 0.6^{b}	16.3 ± 0.3^{b}
	0.75	0.3 ± 0.3^{v}	3.7 ± 0.3^{pqrs}	5.0 ± 0.6^{mnop}	9.0 ± 0.6^{kl}	4.5 ± 0.3^{f}
	Mean	2.1 ± 0.1^{d}	4.4 ± 0.0^{c}	9.2 ± 0.1^{b}	17.2 ± 0.2^{a}	

Mean values within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

auxins and cytokinins in influencing embryogenic potential and underscore the importance of genetic variability in androgenesis studies (Morrison *et al.* 1986, Buyukalaca *et al.* 2004). Kinetin and 2,4-D are commonly used PGRs in pepper embryogenesis. The findings align with studies by Dumas de Vaulx *et al.* (1981) and Koleva-Gudeva *et al.* 2007, which demonstrated the effectiveness of these hormones in promoting haploid embryo formation. Popova *et al.* (2016) also noted that low concentrations of 2,4-D (0.004–0.1 mg/litre) in combination with kinetin enhanced embryogenesis, which is in agreement with the results of this study where a concentration of 0.50 mg/litre 2, 4-D and 4.0 mg/litre kinetin proved most effective. In addition, Sibi *et al.* (1979) showed that the sequential use of 2,4-D followed by kinetin could improve embryoid development. For recalcitrant genotypes, Qin and Rotino (1993) highlighted the benefits of combining BA with 2, 4-D, while Ozkum and Tipirdamaz (2011) demonstrated that NAA with BA effectively induced microspore embryogenesis. The combination of IAA and zeatin (Supena *et al.* 2006, Supena and Custers 2011) and 2, 4-D with kinetin (Lantos *et al.* 2009) also yielded positive results, supporting the notion that a specific combination of hormones is crucial for optimal embryogenesis. Interestingly, Kim *et al.* (2008) found that androgenesis could be initiated even in hormone-free media, which highlights the genotype-specific nature of hormonal requirements. This variation in response across different genotypes is critical when optimizing PGR concentrations for each specific genotype, as demonstrated by the success in the study's selected genotypes.

Enhanced embryo formation with optimized anther culture combinations: This experiment focused on enhancing embryo formation in sweet pepper genotypes by employing the most effective combinations identified from previous results. The optimized culture medium included sucrose (4%), activated charcoal (0.50 g/litre), 2, 4-D (0.50 mg/litre), and kinetin (4.0 mg/litre). Anthers were subjected to a 10-day heat shock at 35°C in darkness and then transferred to a culture room at 25°C under a 16:8 hour photoperiod. Among the sweet pepper genotypes studied, Asha achieved the highest average of 34.7 embryos, followed by Indra with 27.3 embryos, Nemalite with 22.3 embryos, and Sympathy with 7.3 embryos. These results demonstrated the effectiveness of the optimized culture conditions in enhancing embryo formation and highlight the potential for improving breeding methods in agriculture. Fig. 2 visually presents the anther inoculation process onto the CP medium, featuring a detailed view of embryos developing within the anthers and illustrating various stages of their growth. Fig. 3 provides a comparative analysis of embryo formation among different sweet pepper genotypes under the optimized culture conditions, further emphasizing the variations in embryogenic response.

Genotype effect: The genotype effect was evident in all treatments. Asha consistently showed higher embryogenic response rates compared to the other genotypes across varying concentrations of growth regulators, temperature treatments, and nutrient levels. For instance, in the embryo induction response to cold pre-treatment and heat shock, Asha had the highest embryogenic response of 39.3 embryos, indicating a strong genotype effect. In the sucrose and activated charcoal treatments, Asha again exhibited the highest embryogenic response of 24.8 embryos. Additionally, under the influence of 2, 4-D and Kinetin, Asha demonstrated the highest response of 43.0 embryos. These findings underscore the importance of genetic variability in androgenesis studies, highlighting that different genotypes respond uniquely to specific treatments, which is crucial

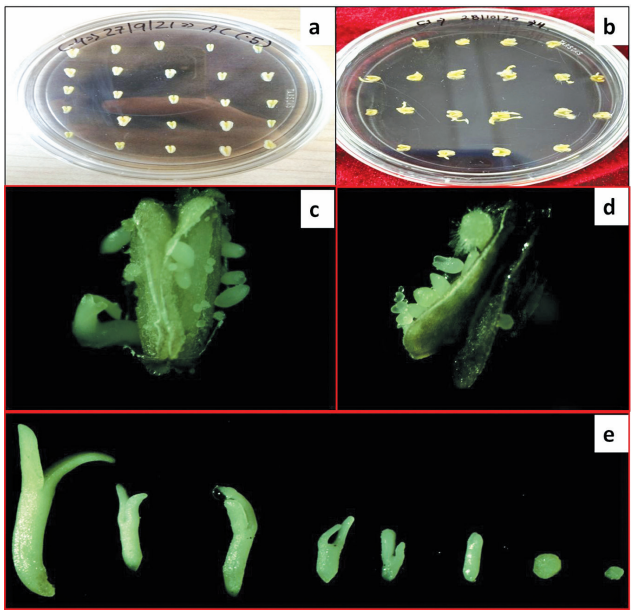

Fig. 2 The anther culture process comprised several crucial stages: (a) Anthers were placed on media for inoculation; (b) embryos emerged from these cultured anthers; (c) and (d) embryos were closely examined under magnification (1.5x) as they emerged from the anther, and (e) different developmental stages of embryos were observed, progressing from globular to cotyledonary forms.

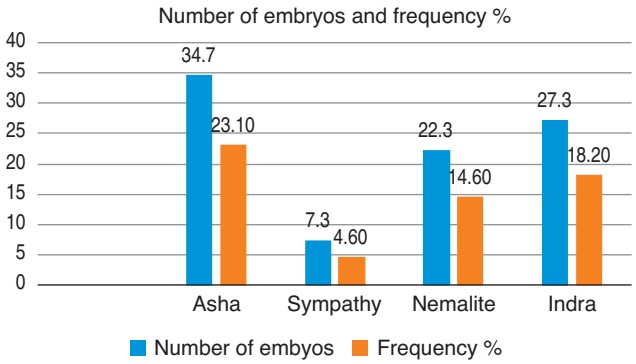

Fig. 3 The frequency of embryogenesis among different genotypes using the most effective treatment combinations.

for optimizing breeding strategies (Morrison *et al.* 1986, Buyukalaca *et al.* 2004).

Despite the differences in genotypes, a similar effect of factors was observed across all four hybrids, which can be attributed to shared physiological mechanisms driving androgenesis. Growth regulators like 2, 4-D and kinetin, along with stress treatments such as cold pre-treatment and heat shock, stimulate biochemical responses that promote androgenesis. Supena *et al.* (2006) found that, although there are genotype-specific responses, growth regulators consistently affected androgenic responses in pepper species. The uniformity in treatment conditions, such as temperature and nutrient media, ensures that observed differences in embryogenic response are primarily genotype-driven. Dumas de Vaulx *et al.* (1981) and Cheng *et al.* (2013) noted that successful androgenesis requires both genotype-specific protocols and consistent environmental conditions. These results reflect the intricate relationship between genotype and environment in androgenesis, as highlighted by Mityko *et al.* (1995) and Nowaczyk *et al.* (2009), suggesting that once certain thresholds are met, treatments may exert similar effects across different genotypes.

This study underscores the importance of optimizing temperature treatments, nutrient levels, and hormone combinations to enhance embryo formation in sweet pepper. The genotype effect plays a significant role, with Asha consistently showing superior responses across different treatments. The findings provide valuable insights for improving breeding methods, contributing to the development of a more efficient and effective anther culture protocol. This study is highly relevant to advancing the field of capsicum breeding by addressing the challenges of doubled haploid (DH) production. DH technology is a powerful tool in plant breeding, enabling the development of genetically uniform lines, which are critical for creating high-yielding, stress-resistant, and disease-tolerant varieties. By exploring genotype-specific responses and optimizing protocols for androgenesis, this research contributes to a deeper understanding of the factors influencing successful DH production. It highlights the importance of tailoring pre-treatments, growth regulator combinations, and cultural conditions to specific genotypes, thereby improving the efficiency and reliability of DH technology in pepper breeding. Furthermore, these findings have practical implications for breeding programmes by reducing the time and cost associated with developing pure lines, thereby accelerating the production of superior hybrids. The study supports sustainable agricultural practices by promoting the development of robust, high-quality cultivars capable of thriving under diverse environmental conditions. Ultimately, this research bridges critical knowledge gaps and lays the groundwork for innovations in crop improvement and food security.

REFERENCES

Abak K. 1983. Study on the anther culture *in vitro* of pepper (*Capsicum annuum*). *Capsicum Newsletter* **2**: 72–73.

Bajaj Y P S. 1978. Regeneration of haploid tobacco plants from isolated pollen grown in drop culture. *Indian Journal of Experimental Biology* **16**: 407–09.

Buyukalaca S, Comlekcioglu N, Abak K, Ekbic E and Kilic N. 2004. Effect of silver nitrate and donor plant growing conditions on production of pepper (*Capsicum annuum* L.) haploid embryos via anther culture. *European Journal of Horticultural Science* **69**(5): 206–09.

Cheng Y, Ma R L, Jiao Y S, Qiao N and Li T T. 2013. Impact of genotype, plant growth regulators and activated charcoal on embryogenesis induction in microspore culture of pepper (*Capsicum annuum* L.). *South African Journal of Botany* **88**: 306–09. https://doi.org/10.1016/j.sajb.2013.08.012

Ciner D O and Tipirdamaz R. 2002. The effects of cold treatment and charcoal on the *in vitro* androgenesis of pepper (*Capsicum annuum* L.). *Turkish Journal of Botany* **26**: 131–39.

Dumas de Vaulx R, Chambonnet D and Pochard E. 1981. *In vitro* anther culture in red pepper (*Capsicum annuum* L.):

Improvement of the rate of plant production in different genotypes by treatments at 35°C. *Agronomie* **1**: 859–64.

George L and Narayanaswamy S. 1973. Haploid capsicum through experimental androgenesis. *Protoplasma* **78**: 467–70.

Hause B, Hause G, Pechan P and Van Lammeren A A M. 1993. Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* (L.). *Cell Biology International* **17**(2): 153–68.

Irikova T, Grozeva S and Rodeva V. 2011. Anther culture in pepper (*Capsicum annuum* L.) *in vitro*. *Acta Physiologiae Plantarum* **33**(5): 1559–70. https://doi.org/10.1007/s11738-011-0736-6

Jaattela M, Wissing D, Kokholm K, Kallunki T and Egeblad M. 1998. Hsp70 exerts its anti-apoptotic function downstream of caspase 3-like proteases. *The EMBO Journal* **17**(21): 6124–34. https://doi.org/10.1093/emboj/17.21.6124

Johansson L. 1983. Influence of silver nitrate on androgenesis in *Capsicum annuum* L. *Physiologia Plantarum* **59**(3): 397–403. https://doi.org/10.1111/j.1399-3054.1983.tb04221.x

Johansson L B, Calleberg E and Gedin A. 1990. Correlations between activated charcoal, Fe-EDTA and other organic media ingredients in cultured anthers of *Anemone canadensis*. *Physiologia Plantarum* **80**: 243–49.

Kim M, Kim J and Yoon M. 2004. Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). *Plant Cell, Tissue and Organ Culture* **77**(1): 63–72. https://doi.org/10.1023/B:TICU.0000016506.02796.6a

Kim M, Jang I C, Kim J A, Park E J, Yoon M and Lee Y. 2008. Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. *Plant Cell Reports* **27**(3): 425–34. https://doi.org/10.1007/s00299-007-0442-4

Koleva-Gudeva L R, Spasenoski M and Trajkova F. 2007. Somatic embryogenesis in pepper anther culture: the effect of incubation treatments and different media. *Scientia Horticulturae* **111**(2): 114–19. https://doi.org/10.1016/j.scienta.2006.10.013

Kristiansen K and Andersen S B. 1993. Effects of donor plant temperature, photoperiod, and age on anther culture response of *Capsicum annuum* (L.). *Euphytica* **67**: 105–09. https://doi.org/10.1007/BF00022732

Lantos C, Juhasz A G, Somogyi G, Otvos K, Vagi P, Mihaly R, Kristof Z, Somogyi N and Pauk J. 2009. Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. *Plant Cell, Tissue and Organ Culture* **97**: 285–93. https://doi.org/10.1007/s11240-009-9527-9

Lazar M D, Schaeffer G W and Baenziger P S. 1985. The physical environment in relation to high-frequency callus and plantlet development in anther cultures of wheat (*Triticum aestivum* L.) cv. *Chris*. *Journal of Plant Physiology* **121**(2): 103–09. https://doi.org/10.1016/S0176-1617(85)80034-1

Mityko J, Andrasfalvy A, Csillery G and Faril M. 1995. Anther-culture response in different genotypes and F1 hybrids of pepper (*Capsicum annuum* L.). *Plant Breeding* **114**: 78–80.

Morrison R A, Kning R E and Evans D A. 1986. Anther culture of an interspecific hybrid of Capsicum. *Journal of Plant Physiology* **126**(1): 1–9. https://doi.org/10.1016/S0176-1617(86)80210-3

Nowaczyk P and Kisiała A. 2006. Effect of selected factors on the effectiveness of *Capsicum annuum* L. anther culture. *Journal of Applied Genetics* **47**: 113–17. https://doi.org/10.1007/BF03194609

Nowaczyk P, Olszewska D and Kisiała A. 2009. Individual reaction of Capsicum F2 hybrid genotypes in anther cultures. *Euphytica* **168**: 225–33. https://doi.org/10.1007/s10681-009-9909-4

Ozkum D and Tipirdamaz R. 2011. Effects of L-proline and cold treatment on pepper (*Capsicum annuum* L.) anther culture. *Survival and Sustainability, Environmental Earth Sciences*, pp. 137–43. Springer, Berlin. https://doi.org/10.1007/978-3-540-95991-5_14

Parra-Vega V, Gonzalez-Garcia B and Segui-Simarro J. 2013. Morphological markers to correlate bud and anther development with microsporogenesis and microgametogenesis in pepper (*Capsicum annuum* L.). *Acta Physiologiae Plantarum* **35**(2): 627. https://doi.org/10.1007/s11738-012-1085-6

Popova T, Grozeva S, Todorova V, Stankova G, Anachkov N and Rodeva V. 2016. Effects of low temperature, genotype and culture media on *in vitro* androgenic response of pepper (*Capsicum annuum* L.). *Acta Physiologiae Plantarum* **38**: 273. https://doi.org/10.1007/s11738-016-2294-4

Qin X and Rotino G L. 1993. Anther culture of several sweet and hot pepper genotypes. *Capsicum and Eggplant Newsletter* **12**: 59–62. https://doi.org/10.17660/ActaHortic.1995.402.51

Rodeva V, Irikova T and Todorova V. 2004. Anther culture of pepper (*Capsicum annuum* L.): Comparative study on effect of the genotype. *Biotechnology and Biotechnological Equipment* **18**(1): 34–38. https://doi.org/10.1080/13102818.2004.10817117

Rodeva V, Koleva-Gudeva L, Grozeva S and Trajkova F. 2007. Obtaining haploids in anther culture of pepper *Capsicum annuum* (L). and their inclusion in the breeding process. *Journal of Agricultural Plant Science* **7**(1): 7–18.

Sahana K P, Srivastava A, Khar A, Jain N, Jain P K, Bharti H, Harun M and Mangal M. 2024. Anther-derived microspore embryogenesis in pepper hybrids Orobelle and Bomby. *Botanical Studies* **65**(1): 1. https://doi.org/10.1186/s40529-023-00408-6

Segui-Simarro J M, Testillano P S and Risueno M C. 2003. Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. *Journal of Structural Biology* **142**(3): 379–91. https://doi.org/10.1016/S1047-8477(03)00067-4

Segui-Simarro J M. 2010. Androgenesis revisited. *Botanical Review* **76**: 377–404. https://doi.org/10.1007/s12229-010-9056-6

Shariatpanahi M E, Bal U, Heberle-Bors E and Touraev A. 2006. Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. *Physiologia Plantarum* **127**: 519–34. https://doi.org/10.1111/j.1399-3054.2006.00675.x

Sibi M, Dumas de Vaulx R and Chambonnet D. 1979. Obtention de plantes haploïdes par androgenese *in vitro* chez le piment (*Capsicum annuum* L.). *Annales de lAmelioration des Plantes* **29**: 583–606.

Supena E, Suharsono S, Jacobsen E and Custers J. 2006. Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). *Plant Cell Reports* **25**: 1–10. https://doi.org/10.1007/s00299-005-0028-y

Supena E and Custers J. 2011. Refinement of shed-microspore culture protocol to increase normal embryo production in hot pepper (*Capsicum annuum* L.). *Scientia Horticulturae* **130**: 769–74. https://doi.org/10.1016/j.scienta.2011.08.037

Typyrdamaz R and Ellialtyoulu P. 1998. The effects of cold treatments and activated charcoal on ABA contents of anthers and *in vitro* androgenesis in eggplant (*Solanum melongena* L.). Tsekos I and Moustakas M (Eds.). *(In) Proceedings of the Progress in Botanical Research, 1st Balkan Botanical Congress*. Kluwer Academic Publishers, Netherlands. https://

doi.org/10.1007/978-94-011-5274-7_139

Wang L H and Zhang B X. 2001. Advancement in the anther culture of *Capsicum annuum* (L.) *China Vegetables* **3**: 52–53.

Wang Y Y, Sun C S, Wang C C and Chien N F. 1973. The induction of the pollen plantlets of triticale and *Capsicum annuum* from anther culture. *Scientia Sinica* **16**(1): 147–51.

Weatherhead M A, Burdon J and Henshaw G G. 1979. Effects of activated charcoal as an additive in plant tissue culture media: Part 2. *Zeitschrift für Pflanzenphysiologie* **94**: 399–405. https://doi.org/10.1016/S0044-328X(79)80223-8

Zheng M Y. 2003. Microspore culture in wheat (*Triticum aestivum*)-Doubled haploid production via induced embryogenesis. *Plant Cell, Tissue and Organ Culture* **73**(3): 213–30. https://doi.org/10.1023/A:1023076213639