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Production of Parental Lines using Temperature and Radiation Treatments in Greenhouse Cucumber (*Cucumis sativus* L.)

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ABSTRACT

Haploid and double-haploid technologies, facilitated by gametophytic embryogenesis and the modulation of gene expression linked to essential agricultural traits, provide an effective means of harnessing the genetic potential of various genotypes. These approaches offer a promising strategy for hybrid seed production. This study aimed to evaluate these technologies through a factorial experiment, implemented in a completely randomized design with three replications, conducted in research greenhouses of the Southern Agricultural and Natural Resources Research Center, Kerman province (2023). The experiments included five randomly selected cucumber genotypes as the first factor. The second factor comprised treatment groups at four levels, i.e., pollen irradiation (300 and 350 Gy) and temperature (35 °C for 6 and 12 h) to cause sterility in cucumber pollen. Results demonstrated that haploid seedlings could be successfully induced through parthenogenesis in these genotypes, with approximately one-third of the fruits pollinated with treated pollen producing at least one haploid parthenogenetic embryo. Optimal radiation dose was identified as a key factor in enhancing parthenogenetic embryo induction. The highest rates of haploid induction (10.67%) were observed in double-haploid seedlings exposed to 300 Gy of gamma radiation and subjected to a 6-h temperature treatment at 35 °C. Among the resulting plants, half exhibited a haploid chromosome number, while the remaining plants displayed either normal or mixoploid chromosome configurations. Chromosome doubling in haploid plants was achieved through colchicine treatment in the laboratory, using a concentration of 500 mg L^{-1} for 24 h. This method provided a reliable and efficient approach to generating homozygous lines, which are essential for advancing cucumber breeding programs.

Introduction

Cucumber (*Cucumis sativus L.*) belongs to the Cucurbitaceae family, which comprises 118 genera and 825 species. It is primarily cultivated in tropical and subtropical regions worldwide. Globally, Iran ranks third in greenhouse cucumber production after China, Turkey, and Russia. From 2002 to 2020, Iran's greenhouse cultivation area for vegetables and summer crops expanded from 3,380 to 13,000 hectares, of which 7,539 hectares were dedicated to cucumber cultivation, yielding 1.93 million tons annually (FAO, 2022). Promoting national priorities such as reducing dependence on imported agricultural inputs, enhancing technological self-sufficiency, and increasing competitiveness in strategic agricultural sectors is crucial for improving Iran's international standing and ensuring food security (Hooghvorst, 2020; Adkhamovich et al., 2019). The hybrid seed production industry is rapidly

growing, with significant commercial potential globally. Timely and reliable seed supply forms a critical pillar for sustaining agricultural productivity (Chaikam et al., 2019). However, 98% of the hybrid seeds required for Iran's greenhouse cucumber production are imported, making the country vulnerable to supply disruptions and external dependencies (Need Assessment of Iran's Agricultural Research, Education, and Promotion Organization, 1403). Addressing this reliance through domestic hybrid seed production could create jobs, prevent currency outflows, counteract sanctions, and produce climate-adapted seeds at lower costs, thus enhancing food security (Dadlani et al., 2023). Hybrid seed production technology exploits heterosis between genetically distinct parental lines to maximize hybrid vigor and is widely employed for crop improvement (Chaikam et al., 2019). Hybrid varieties offer significant agronomic advantages, including high yield and quality, resistance to biotic and abiotic stresses, adaptability, and marketability, making them integral to greenhouse production systems (Bi et al., 2024). The foundation of hybrid seed production lies in purebred parental lines with specific traits and high combining ability (Baktemar et al., 2024).

Double-haploid technology, a biotechnological breeding method, plays a critical role in producing homozygous parental lines. Double-haploid lines represent distinct genotypic and phenotypic classes with unique combinations of homozygous alleles. Methods for generating double-haploids include parthenogenesis (induced by irradiated pollen), gynogenesis (*in vitro* culture of ovules and ovaries), and androgenesis (Salehian et al., 2023). Advances in double-haploid technology have significantly enhanced breeding efficiency by reducing the time required to develop new cultivars while addressing economic and ecological goals (Verma and Kumar, 2020). In Cucurbitaceae, parthenogenesis induction using irradiated pollen is the most effective method for producing haploid plants. Irradiated pollen can germinate on the stigma, grow within the pistil, and reach the embryo sac but is unable to fertilize the oocyte or polar nuclei (Yali, 2022). Consequently, the resulting embryos lack endosperm, necessitating embryo rescue and in vitro culture to prevent early-stage embryo death (Wehner and Naegele, 2019). This technology facilitates the development of cultivars with improved yield, quality, disease resistance, and unique genetic and phenotypic traits (Tugce et al., 2017; Zou et al., 2018; Elena et al., 2020). The efficiency of double-haploid production depends on accurate ploidy level assessment, performed at least two to three times during the process. Common methods include direct chromosome counting in root apical meristems, flow cytometry, and morphological evaluation of chloroplast size and number in stomatal guard cells (Pradeep Kumara et al., 2023). The earliest reports of haploid production in Cucurbitaceae involved interspecific crosses with *Cucumis ficifolius*, where irradiated pollen successfully generated haploid embryos (Xu et al., 2022). Despite its potential, hybrid seed production in cucumbers faces numerous challenges that can impact efficiency at various stages of the process (Kurtar et al., 2020). Optimal pollination timing, typically during early to mid-flowering, is essential for achieving high success rates (Tian et al., 2023). Hybrid seed production technologies in Cucurbitaceae remain pivotal for addressing global agricultural demands and are widely utilized by leading seed production companies to develop superior cultivars.

In Iran, a lack of focused and well-structured breeding programs for cucumbers can be attributed to limited knowledge of the plant's germplasm. Ensuring an adequate and timely seed supply is critical for sustaining agricultural productivity. This study aimed to address these gaps by evaluating the commercial potential and parthenogenetic capacity of selected greenhouse cucumber genotypes. The findings can support the development of double-haploid lines, which are essential as parental lines for advanced breeding programs.

Material and methods

This study was carried out in a two-factor factorial experiment using a completely randomized design with three replications. The experiments were conducted in 2023 at the research greenhouses of the Southern Agricultural and Natural Resources Research Center in Kerman province. Five randomly selected cucumber genotypes were evaluated as the first factor. The second factor comprised treatment groups at four levels, i.e., pollen irradiation (300 and 350 Gy) and temperature (35 °C for 6 and 12 h) to cause sterility in cucumber pollen. These treatments were applied to assess and optimize the parthenogenetic potential of the selected genotypes (Wehner and Naegele, 2019).

The seedlings were grown in an isolated greenhouse with controlled conditions. The greenhouse covered an area of 200 m^2, maintained average temperatures of 25 °C during the day and 18 °C at night, and provided a light intensity of 350 µM m^{-2} s^{-1}. A relative humidity of 70% was maintained, and plants were irrigated using drip systems spaced 10 cm apart. Ventilation was achieved through side vents equipped with a 50% anti-insect net (Seguí-Simarro, 2021). Twenty plants of each genotype were cultivated as donor materials for the experiments.

Pollen sterility treatments were applied once the plants reached the flowering stage. Female flowers were isolated with paper envelopes 1 d prior to crossing to prevent unwanted pollination (Sari and Solmaz, 2021). Male flowers were harvested and

transported to the Nuclear Research Center for Agriculture, where they were irradiated using a gamma cell device at 300 and 350 Gy. For the temperature treatments, male flowers were placed in a laboratory oven at 35 °C for 6 and 12 h (Chaikam et al., 2019). The following morning, treated male flowers were used for pollinating the isolated female flowers, which were then re-covered with paper envelopes to maintain strict isolation. The envelopes were removed 3 d after pollination, and the female flowers were inspected for fruit formation or abscission. Additional analyses were conducted, including germination tests and assessments of pollen tube growth, to evaluate the effects of the treatments on pollen viability and function (Seguí-Simarro et al., 2021).

Parthenogenetic embryo rescue and plant renewal

The fruits obtained from the crosses were harvested 3–5 weeks after pollination and transferred to the laboratory for further analysis. Under sterile conditions in a laminar flow hood, the parthenocarpic fruits were thoroughly washed and disinfected with 70% alcohol. Individual seeds were then examined for the presence of haploid embryos.

To isolate the embryos, seeds containing parthenogenetic embryos were carefully opened using sterile forceps and scalpel blades on sterile filter paper. The extracted embryos were cultured on solid E20A medium and placed in a growth chamber maintained at 25 °C, with a light intensity of 3000 lux and a photoperiod of 16 h light and 8 h dark.

After 3–5 d, the resulting seedlings were transferred to MS culture medium supplemented with 0.101 mg L^{-1} of indole-3-acetic acid (IAA) to promote root induction and facilitate further growth (Ey, 2020).

Micropropagation

To obtain more plants *in vitro*, the rescued embryos were individually micropropagated at the 4-6 leaf stage to obtain the required number of clones from each plant for adaptation, ploidy level determination, and doubling (Bi et al., 2024).

Determining the genomic DNA content and the ploidy level of seedlings using flow cytometry

The ploidy level of the obtained seedlings was assessed both before and after chromosome doubling. Flow cytometry was utilized for this purpose, as it determines ploidy level and genomic DNA content by measuring the relative fluorescence intensity of nuclei. The genomic DNA content and ploidy level of an unknown sample can only be estimated accurately when compared to a standard plant with known ploidy and genomic DNA content (Sari, 2021; Nicoletti et al., 1991). In this study, a diploid tomato plant with a genomic DNA content of 2C DNA = 1.96 pg was used as the standard reference (Doležel et al., 2005; Loureiro et al., 2007). Leaf samples were collected from the seedlings obtained through embryo rescue, and their ploidy levels were analyzed using a flow cytometer (PAI, Partec GmbH, Germany). The device was equipped with an HBO lamp and an argon laser for precise measurements (Doležel and Bartoš, 2005).

Doubling and compatibility

Chromosome doubling was performed following Lim and Earl's protocol, which involved exposing the seedlings to 500 mg L^{-1} of colchicine under laboratory conditions for 24 h. Seedlings containing several lateral buds and stem tips were selected as plant material and placed in E20A liquid medium containing the mitotic inhibitor. After 24 h, the samples were thoroughly washed three times with sterile distilled water, and the surface moisture was removed using sterile filter paper. The treated seedlings were then transferred to MS solid culture medium for further development.

At the 4–6 leaf stage, the samples underwent an adaptation process (Hooghvorst, 2021). During this stage, the lids of the culture flasks were gradually opened, and the plants were carefully removed and placed into plastic cups filled with sterile peat soil. Thin plastic bags were used to cover the cups to maintain high relative humidity (approximately 85–95%). After acclimatization, which included gradual temperature adjustments in the afternoon, the plants were transferred to the greenhouse. They continued growing until flowering and were subsequently self-pollinated after inducing male flower production. Seeds were harvested approximately 5–6 weeks post-pollination (Badu, 2017). Key parameters were recorded 4 weeks after pollination. These included the growth ratio and fruit formation of female flowers pollinated with treated pollen, the number of seeds containing parthenogenetic embryos per fruit, the number of isolated and normal embryos, the number of rescued embryos, and the embryo regeneration rate.

Analysis of the survival and germination power of pollen

Based on the Lim and Earl protocol, germination and pollen tube growth measurements were recorded during 24 h in darkness at 25-26 °C. To evaluate the *in vitro* germination ability, 20 mm of liquid culture medium containing the elements in Table 1, was poured into Petri dishes, and pollen were dissipated in the medium, and the water lid was closed to prevent water loss. The germination ability was observed using a laboratory microscope at 100x magnification.

Table 1. Culture medium affecting the germination percentage of pollen grains treated for parthenocarpy induction in greenhouse cucumber.

Materials	Weight to volume (%)
KNO_3	0.01
$Ca(Na_3)_2$	0.1
$MG(SO_4)$	0.02
H_3BO_3	0.01
Sucrose	15
Agar	0.5
pH	6.5

Statistical analysis

The data was analyzed with IBM SPSS v.21 software, and mean values were compared using the LSD test.

Results

The top five genotypes were studied for parthenogenesis traits. The results of ANOVA indicated a significant difference for the measured factors at the 1% significant level. The ploidy level of seedlings obtained from embryo rescue in cucumber was determined using tomato leaf samples as a standard plant. The leaves of seedlings obtained from embryo rescue were used as an unknown sample, and the results were analyzed using flow cytometry equipment. Based on the results of flow cytometry analysis, the genomic DNA content was 0.95 picogram in the cucumber plant (Fig. 1 and Table 2). The DNA 2C-value of the cucumber seedlings obtained from embryo rescue was calculated by comparing the average peak value of the diploid plant and the standard plant.

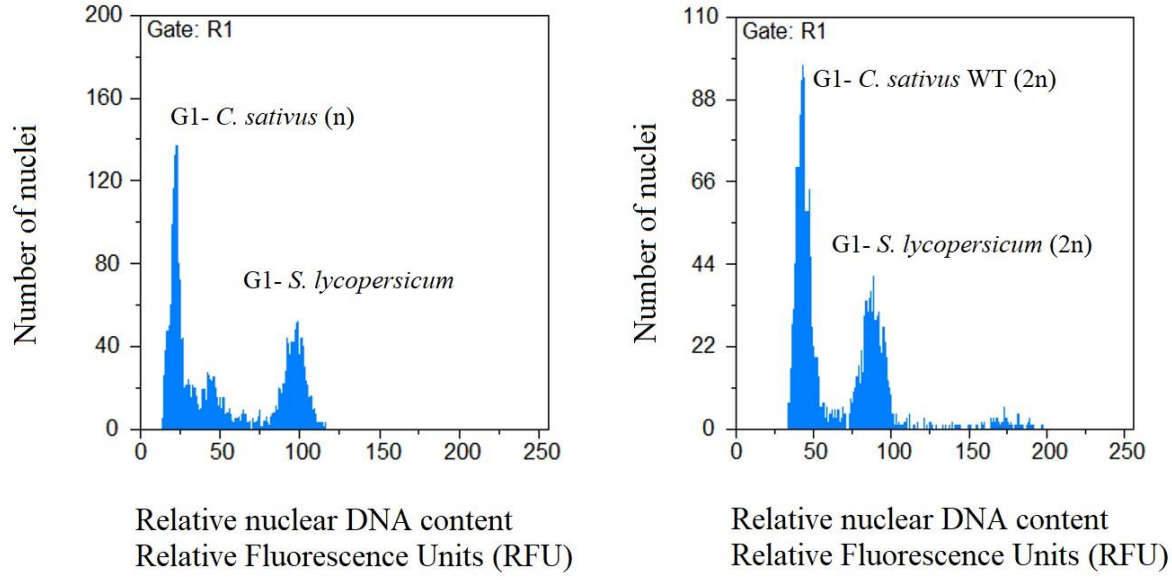

Fig. 1. Histogram of flow cytometric analysis of cucumber plant nuclei in the diploid and haploid state along with the standard tomato plant (*Solanum lycopersicum* 'Stupicke' 2C DNA = 1.96).

Table 2. Results of flow cytometry examination of seedlings obtained from parthenogenesis in greenhouse cucumber.

Ploidy level	Genomic DNA content (pg)
Haploid seedlings	0.47
Diploid mother plants	0.95

The data analysis revealed significant variation in the parthenogenetic responses of the genotypes across different treatments, which were influenced by growth conditions and seasonal changes. The number of clones produced per genotype, as well as the germination and growth conditions in both laboratory and greenhouse environments, were critical factors affecting the regeneration process. Optimal pollination time in the south of Kerman was determined to be November and December,

requiring precise control of greenhouse and laboratory conditions to maximize results. Approximately 80 flowers from each experimental plot were pollinated using temperature-treated and irradiated pollen, resulting in an average of 45 fruits per treatment. Analysis of the five genotypes showed a significant difference ($P < 0.01$), as outlined in Table 3. The five cucumber genotypes served as donors in this study and were evaluated for haploid induction. Their parthenogenetic potential was assessed through a series of procedures, including temperature and radiation treatments, parthenogenetic embryo rescue, seedling performance evaluation under laboratory conditions, and chromosome doubling.

Table 3. ANOVA results for genotypes and haploid levels on studied traits in the haploid induction experiment in the greenhouse cucumber.

Sources of variance	Pollinated flowers	Developed flowers	Number of formed fruits	Number of parthenocarpic embryos	Number of cultured embryos	Losses (%)	Survival of embryos (%)
Genotype	125.900*	311.067**	4.067^{ns}	139.43**	54.233**	748.53^{ns}	14.233**
Haploid level	105.800^{ns}	164.356*	0.556^{ns}	69.68**	33.800**	54.08^{ns}	6.422*
Genotype × haploid level	36.078*	37.911*	1.333^{ns}	20.96*	6.552*	223.91^{ns}	1.589*
Error	60.900	36.867	3.467	9.633	2.700	656.193	1.567
(CV)	8.59	14.41	29.14	11.74	9.54	26.89	12.23

*, ** significant at $P < 0.05$, $P < 0.01$; ^{ns} non-significant.

About one-third of the harvested fruits from each treatment contained 1–5 embryos, while some fruits yielded empty seeds. The number of embryos per fruit varied depending on the treatment and genotype. The ANOVA results indicated significant differences in several traits, including survival percentage, embryo loss, the number of cultivated embryos, the number of fruits formed, and the number of pollinated and developed flowers across all genotypes. These findings underscore the complex interplay between genotype, treatment, and environmental conditions in determining parthenogenetic success.

The data in Table 4 reveals that the highest number of parthenocarpic embryos was induced by the temperature treatment of 35 °C for 6 h combined with a radiation dose of 300 Gy, while the lowest induction was observed with the 35 °C treatment for 12 h and a radiation dose of 350 Gy. This indicates that the combination of 300 Gy and 35 °C for 6 h was more effective than 350 Gy and 35 °C for 12 h in inducing parthenocarpic embryos. According to Table 5, the highest embryo survival percentage (5.66) was achieved with the 6-h 35 °C treatment. Interestingly, no significant difference in embryo survival was observed between the radiation doses of 300 Gy and 350 Gy, regardless of whether the embryos were obtained from temperature or radiation treatments. The number of haploid embryos varied among genotypes, with genotypes 1, 2, and 5 producing the highest numbers (5.66, 5.5, and 3.33, respectively). These findings emphasize the critical role of the donor plant genotype in determining the success of haploid induction.

Previous studies have demonstrated that ploidy levels can significantly influence morphological parameters, including leaf and flower morphology as well as fruit characteristics such as diameter and length. In this research, distinct differences in leaf and flower shape and size were observed between haploid and doubled haploid plants (Figs. 2 and 3). The variation in ploidy levels was associated with factors such as diploid anther wall tissue, spontaneous diploidy, and irregular meiosis. Some genotypes displayed a strong parthenocarpic response, while others showed a weaker response, highlighting the influence of genetic variation on this parameter (Table 6).

Table 4. Number of parthenogenetic embryos of cucumber genotypes during temperature treatments and gamma radiation doses.

Treatment	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5
6h/35 °C	**8.67^{ab}**	**10.67^{a}**	5.00^{cd}	4.00^{cde}	**4.67^{cd}**
12h/35 °C	**6.33^{bc}**	**4.00^{cde}**	5.33^{cd}	1.335^{e}	**3.00^{de}**
300 gry	**8.67^{ab}**	**10.67^{a}**	5.00^{cd}	4.00^{cde}	**4.67^{cd}**
350 gry	**6.33^{bc}**	**4.00^{cde}**	5.33^{cd}	1.33^{e}	**3.00^{de}**

Genotypes with similar letters had no significant difference.

Table 5. Embryo survival percentage of temperature and exposure treatments in greenhouse cucumber cultivars.

	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5
35 °C/6h	5.66^{a}	5^{ab}	3^{bcd}	2.66^{bcd}	3.33^{a-d}
35 °C/12h	3.66^{abc}	3^{bcd}	4^{abc}	1^{d}	2.33.0^{cd}
300 gry	5.66^{a}	5^{ab}	3^{bcd}	2.66^{bcd}	3.33^{a-d}
350 gry	3.66^{abc}	3^{bcd}	3.13^{bc}	1^{d}	2.33^{cd}

Genotypes with similar letters had no significant difference.

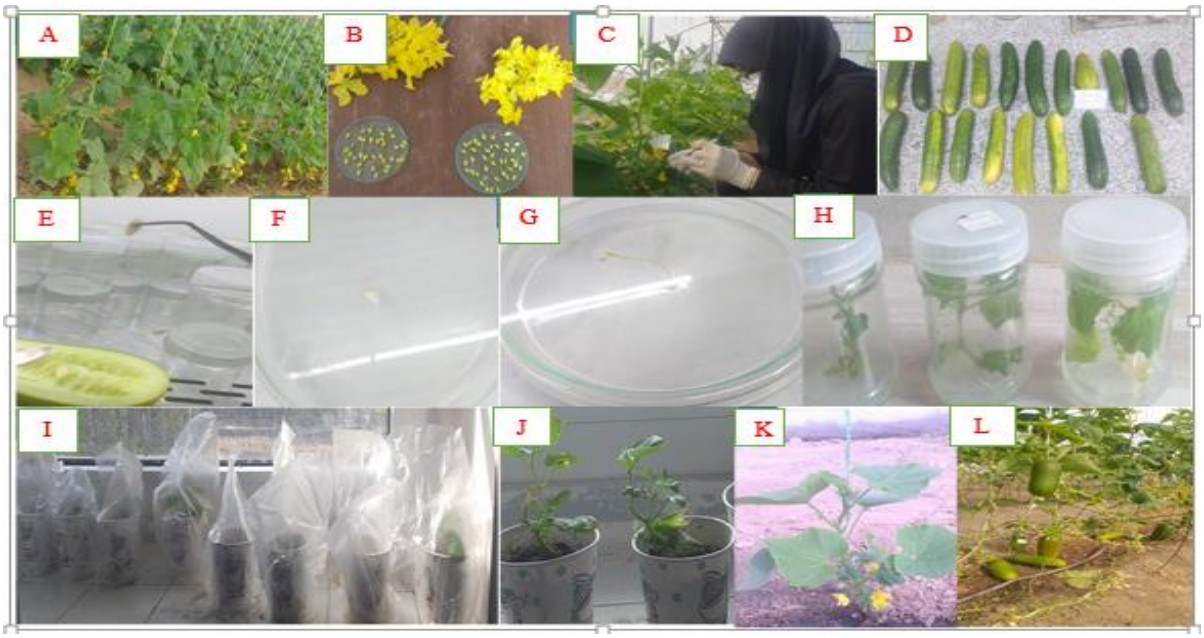

Fig. 2. (A) Stages of induction of parthenogenesis in selected greenhouse cucumber genotypes for the production of parental lines: growth of cucumber donor genotypes in greenhouse; (B) preparation of male flowers for radiation treatment; (C) crossing of isolated flowers with treated pollen; (D) parthenocarpic fruits produced; (F and G) parthenogenesis embryo diagnosis (E), separation and cultivation of parthenocarp embryos ; (H) growth of parthenocarp embryos in the culture medium; (I) adaptation of double haploid seedlings ; (J) growth of double haploid seedlings in laboratory conditions; (K) cultivation of double haploid plants in the greenhouse; (L) preparation of seeds from productive plants.

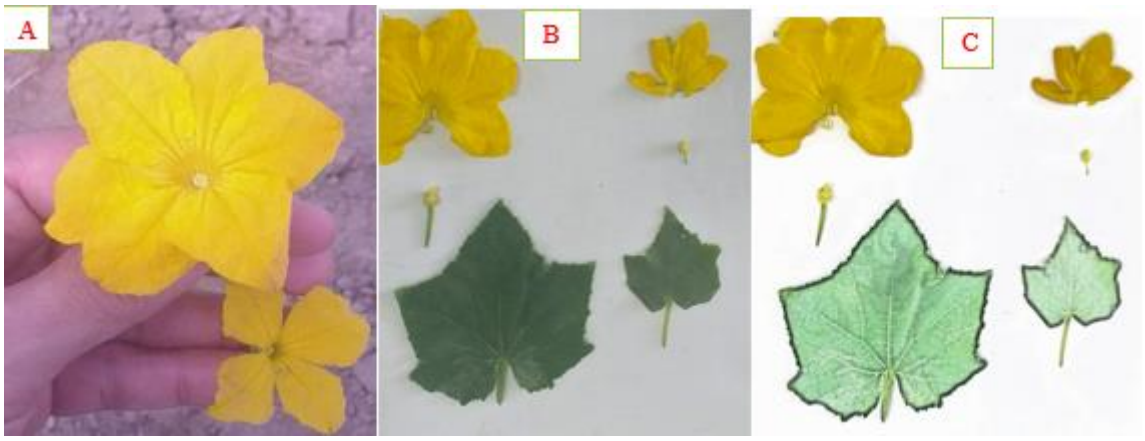

Fig. 3. (**A**) The difference between the flowers of haploid and double haploid plants (A). (**B** and **C**) The difference in the shape of the petals and leaves of haploid and double haploid plants of greenhouse cucumbers obtained from parthenogenesis technology.

Correlation analysis revealed that the number of cultured embryos was positively and significantly correlated with the survival percentage of embryos, indicating that a greater number of cultured embryos resulted in higher survival rates (Table 7). Conversely, a negative and significant relationship was observed between the number of cultured embryos and the percentage of embryo losses,

suggesting that higher numbers of cultured embryos were associated with a proportionally higher loss rate. A similar negative and significant relationship was found between the survival percentage of embryos and the percentage of losses, reinforcing this trend. Additionally, the number of parthenocarpic embryos showed a positive and significant relationship with the survival percentage of embryos, with haploid embryos exhibiting a higher survival percentage compared to other ploidy levels. The number of parthenocarpic embryos also positively correlated with the number of cultured embryos, indicating that a higher count of identified and cultured parthenocarpic embryos increased the likelihood of obtaining more haploid seedlings.

Table 6. Fruits produced from double haploid lines in selected genotypes of greenhouse cucumber.

Double-haploid lines	Number of clones	Number of crosses	Phenotypic ploidy	Double-haploid flower phenotype	Fruit from self-pollination
G-DH C1	3	11	Diploid	Normal	7
G- DHC2	2	9	Diploid	Normal	5
G- DHC4	4	5	Haploid	Normal	3
G-DHC5	2	10	Diploid	Normal	5
G-DHC3	2	7	Haploid	Small with limited pollen	2

Table 7. Correlation coefficients of the studied traits obtained from temperature and irradiance treatments in selected greenhouse cucumber cultivars.

	Survival of embryos (%)	Victims (%)	Number of cultured embryos	Number of parthenocarpic embryos	Number of fruits formed	Developed Parthenocarpic fruit	Pollinated flowers
Survival of embryos (%)	1						
Victims (%)	-0.124	1					
Number of cultured embryos	0.869**	-0.046	1				
Number of parthenocarp embryos	0.812**	-0.016	0.952**	1			
Number of formed fruits	0.028	0.147	0.093	0.213	1		
Developed Parthenocarpic fruit	0.059ns	-0.232	0.102	0.142	0.057	1	
Pollinated flowers	0.199	-0.297*	0.129	0.231	0.292*	0.790**	1

*, ** significant at $P < 0.05$, $P < 0.01$; ns non-significant.

A positive and significant relationship was identified between the number of pollinated flowers and the percentage of embryo losses. A greater number of pollinated flowers corresponded to the development of more parthenocarpic fruits, which aligns with the findings of numerous studies referenced in this research. In this study, parthenogenetic efficiency was assessed by the number of embryos per fruit for each genotype. Various methods for diagnosing parthenogenetic embryos are available; however, one-by-one seed evaluation was employed in this research as it caused less damage to embryos compared to liquid medium culture.

The efficiency of this study was assessed by the number of parthenogenetic embryos produced per fruit for each genotype. For embryo diagnosis, a one-by-one seed evaluation method was utilized to minimize damage to the embryos, as liquid medium culture was found to reduce the success of the process, as outlined in a previous study. The use of liquid medium culture for embryo detection and rescue was found to reduce the success of the process. Despite thorough initial disinfection of the seeds, bacterial and fungal contamination presented significant challenges to the successful rescue of parthenogenetic embryos. Seedlings derived from the doubled haploid processes exhibited varying

ploidy levels, including haploid, diploid, and mixoploid. Germination and growth under controlled laboratory conditions were identified as critical steps to increase the number of clones within each genotype and improve the efficiency of the regeneration process. The observed inhibition of germination and seedling growth may be attributed to the combination of deleterious genes in homozygosity, which regulate vegetative growth and lead to the survival failure of embryos carrying deleterious recessive alleles in homozygous form.

The study successfully produced 22 fruits from 11 independent parthenogenetic doubled haploid plants. These plants represent a valuable resource for use as parental lines in combinability and compatibility tests aimed at hybrid seed production. This highlights the potential of doubled-haploid technology in advancing cucumber breeding programs by providing genetically stable and efficient parental lines.

Significant variation was observed in the measured parameters among the five evaluated genotypes. The highest number of parthenogenic embryos was obtained under the temperature treatment of 35 °C for 6 h combined with 300 Gy radiation, while the lowest number was recorded for the temperature treatment of 35 °C for 12 h. These results indicate that the 6-h treatment at 35 °C is more effective than the 12-h treatment. Among the genotypes, genotype 5 produced the highest number of fruits from doubled-haploid lines, with seven fruits, whereas genotype 3 had the lowest yield with only two fruits. Phenotypic analysis of male flowers revealed a small size and limited pollen production in self-pollinated plants of genotype 2.

The plant growing season, age, physiological stage, and greenhouse conditions, including light, temperature, humidity, and the presence of pests or diseases, were crucial factors influencing the success rate of the parthenogenesis process. Notably, irradiated male flowers stored in plastic envelopes in the dark for 1–2 d retained their viability, while longer storage periods resulted in reduced pollen survival and failure to form cucumber fruits. The efficiency of parthenogenetic embryo production was assessed by the number of embryos generated per treatment. However, the detection and isolation of parthenogenetic embryos proved to be a labor-intensive and time-consuming process. The success of this process is heavily skill-dependent. In line with previous research, this study demonstrated the effectiveness of colchicine, an anti-synthesis agent of microtubules, at a concentration of 500 mg L^{-1} for 24 h in inducing diploidy *in vitro*. This optimized protocol offers a promising approach for accelerating breeding programs and developing new cultivars with desirable traits, particularly in Iran, where vegetable seed production remains reliant on imports.

Discussion

The findings of this study provide critical insights into the complex interplay of genotypic, environmental, and procedural factors influencing haploid induction and embryo rescue in cucumber. Significant variation among the five genotypes highlights the role of genetic diversity in parthenogenetic responses, consistent with studies on haploid induction efficiency in various crops (Hooghvorst et al., 2020; Salim et al., 2023). This variation underscores the importance of genotype-specific protocols in optimizing haploid production.

The optimization of temperature and radiation treatments emerged as a decisive factor in achieving high efficiency. The 35 °C temperature treatment for 6 h combined with a 300 Gy radiation dose was the most effective, inducing the highest number of parthenocarpic embryos. This finding corroborates the work of Salim et al. (2023) and reinforces the role of precise environmental control in enhancing haploid induction efficiency. Similarly, Liu et al. (2021) highlighted the significance of balancing physiological stress from such treatments with their stimulatory effects on embryogenesis.

The results of this study underscore the pivotal role of temperature and radiation treatments in parthenogenetic haploid induction. Among the tested conditions, the combination of a 35 °C temperature treatment for 6 h with a radiation dose of 300 Gy emerged as the most effective, yielding the highest number of parthenocarpic embryos. This combination demonstrated a delicate balance between inducing the stress necessary for parthenogenesis and avoiding cellular damage that could hinder embryo formation. Temperature treatments, particularly short-duration exposure to elevated temperatures (e.g., 35 °C for 6 h), appear to play a significant role in priming the physiological pathways required for parthenogenetic development. Elevated temperatures can influence cytoplasmic streaming, hormonal activity, and membrane stability, all of which are critical for embryo induction. The effectiveness of the 6-h treatment aligns with findings by Salim et al. (2023), who noted that brief, controlled heat stress enhances the parthenogenetic response by stimulating cellular and hormonal mechanisms. Prolonged exposure to high temperatures (e.g., 35 °C for 12 h), on the other hand, was less effective in this study, likely due to heat-induced damage to cellular structures, enzyme denaturation, or oxidative stress that impairs embryogenesis. Radiation treatments, such as the application of 300 Gy gamma radiation, contribute to parthenogenetic induction by inducing double-strand breaks in DNA, which can activate repair mechanisms leading to parthenogenetic development.

The radiation dose must be carefully calibrated to provide sufficient stress to trigger these mechanisms without causing excessive damage that would compromise embryo viability. This study's findings that a 300 Gy dose was more effective than 350 Gy highlight the importance of identifying an optimal threshold for radiation exposure. Higher doses, such as 350 Gy, may lead to excessive damage, resulting in lower embryo induction rates. Liu et al. (2021) reported similar observations, emphasizing that moderate doses of radiation can stimulate embryogenesis through controlled induction of cellular stress and activation of repair pathways. The interplay between temperature and radiation is particularly noteworthy. When combined, these treatments likely exert a synergistic effect, with heat stress modulating the cellular environment and radiation inducing genetic changes that favor parthenogenesis. This synergy was evident in the significant improvement in embryo production under combined treatments compared to either stress applied alone. These findings support earlier work by Zhou et al. (2022), who demonstrated that the interaction of physical and genetic stressors can enhance developmental pathways involved in haploid embryo formation. Additionally, the genotype-specific responses to these treatments further highlight the complex dynamics involved. While genotypes 1, 2, and 5 showed strong responses under the 35 °C and 300 Gy treatment, other genotypes were less responsive, suggesting that genetic predispositions play a critical role in determining sensitivity to these treatments. Exploring the molecular basis of these genotype-specific responses could reveal key regulatory genes and pathways that influence parthenogenetic success.This study also noted the importance of precise control over treatment parameters. For instance, the duration and intensity of temperature treatments, as well as the exact radiation dose, require careful calibration. The findings suggest that small deviations in these parameters can significantly impact outcomes, reinforcing the need for standardization and optimization in experimental protocols.

Environmental conditions, including light, temperature, and humidity, as well as the physiological stage of the plants, were critical determinants of success. The identification of optimal pollination times in the southern Kerman region aligns with earlier findings that underscore the influence of climatic and growth conditions on haploid induction (Hooghvorst et al., 2020). Furthermore, the viability of irradiated male flowers stored under controlled conditions, as observed in this study, parallels the results of contemporary research, which demonstrates the importance of storage conditions for maintaining pollen efficacy (Singh et al., 2021). Correlations between cultured embryo numbers, survival rates, and embryo losses offer practical insights into the challenges of embryo rescue. A positive correlation between parthenocarpic embryo numbers and survival rates emphasizes the need for robust culture protocols to maximize embryo viability. This aligns with findings by Seguí-Simarro (2021), who discussed the critical role of culture media and environmental controls in embryo survival. However, the negative correlation between cultured embryo numbers and loss rates highlights the complexity of managing high embryo counts, which may lead to resource competition or contamination risks (Zhou et al., 2022). Distinct morphological differences between haploid and doubled haploid plants, observed in this study, validate previous research on the impact of ploidy levels on plant morphology and reproduction (Dadlani, 2023). The findings further reveal that chromosome doubling through colchicine treatment is effective, providing a reliable tool for advancing breeding programs. This aligns with the work of Chaikam (2021), who demonstrated the potential of colchicine in stabilizing genotypes for hybrid seed production.

Challenges such as bacterial and fungal contamination during embryo rescue remain significant. Despite stringent aseptic protocols, these challenges highlight the need for continued refinement of disinfection methods, as noted by recent studies focusing on contamination management in tissue culture (Wang et al., 2021). Furthermore, the inhibition of seedling growth due to deleterious recessive alleles in homozygous form warrants genetic interventions to mitigate such limitations, echoing concerns raised by Zhang et al. (2022). The successful production of 22 fruits from 11 independent parthenogenetic doubled haploid plants represents a significant milestone, providing genetically stable parental lines for hybrid seed production. This outcome is particularly relevant for regions like Iran, where local vegetable seed production depends heavily on imports. The integration of efficient parthenogenesis protocols with advanced breeding techniques could address such dependency, as suggested by Liu et al. (2021) and Salim et al. (2023).

The observations regarding ploidy variation and its effects on genetic stability provide a comprehensive understanding of the challenges and opportunities in haploid and doubled haploid production. Morphological distinctions between haploid and doubled-haploid plants, such as differences in leaf size, flower shape, and fruit characteristics, underscore the profound influence of ploidy levels on phenotypic expression. These findings align with studies like those of Dadlani (2023), which emphasize that variations in ploidy can significantly impact plant morphology, vigor, and reproductive potential. Ploidy variation in this study was

influenced by factors such as irregular meiosis, diploid anther wall tissue, and spontaneous chromosome doubling. Haploid plants displayed reduced vigor and smaller vegetative and floral organs compared to their double-haploid counterparts. This is consistent with prior findings that haploids, with a single set of chromosomes, lack the genetic redundancy present in diploids, making them more susceptible to deleterious recessive alleles. Such alleles can manifest as reduced growth rates, lower germination percentages, and limited reproductive success, as reported by Zhang et al. (2022).

Interestingly, mixoploidy, where plants exhibit cells with varying ploidy levels, was also observed in some seedlings. This phenomenon is often attributed to incomplete chromosome doubling during colchicine treatment or spontaneous mitotic errors. Mixoploids pose challenges for breeding programs as they can lead to phenotypic instability and unpredictable reproductive behaviors. Effective strategies to address mixoploidy include optimizing colchicine concentrations, extending treatment durations, or employing alternative doubling agents, as suggested by Chaikam (2021). Chromosome doubling through colchicine treatment proved to be a reliable method for stabilizing haploid lines, with a concentration of 500 mg L^{-1} for 24 h yielding consistent results. Doubled haploids exhibit significant advantages over haploids, including enhanced vigor, larger organ size, and improved fertility. The genetic stability of doubled-haploid plants is attributed to their homozygous diploid nature, which eliminates the genetic variability seen in heterozygous populations and provides a uniform genetic base for breeding programs (Dadlani, 2023). However, the process of chromosome doubling is not without its challenges. The study highlighted that some double-haploid plants exhibited deleterious traits due to homozygosity for recessive alleles that are normally masked in heterozygous states. This phenomenon, known as inbreeding depression, limits the viability and growth potential of some double-haploid lines. Strategies to overcome these limitations include identifying and selectively breeding out deleterious alleles or employing genome editing techniques such as CRISPR-Cas to modify or eliminate harmful genes (Zhang et al., 2022). The genetic stability of double-haploid lines makes them invaluable as parental lines in hybrid seed production. The uniformity and homozygosity of double-haploids allow for predictable inheritance patterns, ensuring consistent performance in subsequent hybrid generations. The 22 fruits produced from 11 parthenogenetic double-haploid plants in this study represent a significant milestone for cucumber breeding programs, as they provide a reliable genetic base for developing high-yielding, stress-tolerant, and disease-resistant cultivars. This aligns with the findings of Liu et al. (2021), who emphasized the utility of double-haploid technology in accelerating crop improvement.

The use of double-haploid plants also minimizes the dependency on traditional inbreeding cycles to achieve homozygosity, significantly shortening the breeding timeline. This is particularly beneficial in regions like Iran, where vegetable seed production relies heavily on imports. By establishing local double-haploid-based breeding programs, countries can enhance their self-sufficiency and reduce reliance on imported germplasm. The study revealed critical correlations between the number of cultured embryos, survival rates, and embryo losses. A positive correlation between the number of cultured embryos and survival percentage suggests that increased embryo availability enhances the likelihood of successful rescue. However, the negative correlation between cultured embryo numbers and embryo losses underscores the importance of managing resource allocation and contamination risks during culture. These findings align with Zhou et al. (2022), who highlighted the challenges of balancing embryo density with resource optimization in tissue culture systems.

The methodology employed, including one-by-one seed evaluation, was pivotal in minimizing damage to embryos compared to traditional liquid medium culture, as previously noted by Chaikam (2021). However, the study also reported challenges with bacterial and fungal contamination, which affected embryo viability despite thorough disinfection. These challenges emphasize the need for improved aseptic techniques and antifungal treatments in embryo rescue protocols, as supported by Wang et al. (2021). This study demonstrated the potential of combining optimized environmental conditions, genotype-specific treatments, and robust procedural interventions to enhance haploid and doubled haploid production. The findings not only advance the understanding of parthenogenetic processes but also provide practical tools for improving cucumber breeding programs, with significant implications for sustainable agricultural development globally.

Conclusions

Approximately one-third of the fruits pollinated with treated pollen contained at least one parthenogenesis-derived embryo. Among the treatments, 300 Gy of gamma radiation (10.67%) and the temperature treatment of 35 °C for 6 h (8.68%) demonstrated the highest efficacy in haploid induction. The chromosome number in haploid plants was successfully doubled using colchicine at a concentration of 500 mg L^{-1} for 24 h under laboratory conditions. This process resulted in the production of 29 fruits from 17 independent parthenogenetic double haploid plants, which hold

potential as parental lines for compatibility and combinability tests in hybrid seed production. Given that haploid plant production and chromosome multiplication are the fastest methods for obtaining cucumber parental lines, the application of haploid and double haploid induction technology represents an effective strategy for breeding programs. This approach not only saves time but also facilitates the production of pure parental lines, which are essential for large-scale hybrid seed production. The method offers significant advantages for hybrid seed producers, particularly in semi-industrialized countries like Iran, which still relies on imports for greenhouse vegetable seed production. To enhance the efficiency of double haploid production, it is crucial to optimize environmental growth conditions, determine the best pollination timing, properly apply hormonal treatments, and achieve a high level of proficiency in embryo rescue and chromosome doubling techniques. Mastery of these factors can significantly improve the overall success and productivity of double haploid-based breeding programs.

Further research is needed to optimize chromosome doubling protocols, particularly for minimizing mixoploidy and enhancing the efficiency of double-haploid production. Exploring alternative antimitotic agents or developing non-chemical methods for chromosome doubling, such as temperature shock or endopolyploidy induction, could provide safer and more reliable alternatives to colchicine. Additionally, integrating high-throughput phenotyping and genotyping technologies can aid in the rapid identification and elimination of lines carrying deleterious alleles, ensuring the production of robust double-haploid plants. Molecular studies focusing on the regulatory mechanisms governing chromosome doubling, ploidy stability, and their effects on gene expression could also provide valuable insights. For instance, transcriptomic and epigenomic analyses of haploid and double-haploid plants could reveal how chromosomal and epigenetic changes influence phenotypic traits and stress responses.

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Data Availability Statement
The data related to the results of this study are available upon request from the corresponding author.

Conflict of Interest
The authors indicate no conflict of interest in this work.

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