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Improvement of embryogenesis in cucumber (Cucumis Sativus L.) parthenogenesis by polyamine and cycocel treatments

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Abstract

This study evaluated the combined application of polyamines (putrescine, spermidine) and cycocel (CCC) to maternal plants in relation to the effect of pollen irradiation with gamma rays on seed formation, embryo formation and haploid production in three cucumber genotypes (Storm, Sahm, 547) using pollen irradiation at doses of 400 to 600 Gy. Maternal plants were treated with combination of putrescine (500 mg/L), spermidine (50 mg/L) and CCC (50 mg/L) from 12 days before to 12 days after pollination. Male flowers were irradiated with gamma rays (400, 500, 600 Gy) and pollination was performed. Embryos were rescued *in vitro* and regenerated plantlets were analyzed for ploidy level using flow cytometry and SSR marker to confirm haploid status. Haploid plants produced were treated with oryzalin for chromosome doubling. The combined treatment of polyamine and CCC significantly affected embryo induction and seed formation, with the Storm genotype producing the highest number of embryos and regenerated plants at a dose of 600 Gy. Embryogenesis and regeneration varied by genotype, with Storm and 547 showing the strongest responses; Sahm was the least responsive. Flow cytometry confirmed haploid/diploid status, and SSR validated homozygosity in doubled haploid (DH) lines. Higher gamma doses not only increased empty seeds but also embryo numbers, underscoring genotype-dependent radiation sensitivity. Controlled pollen irradiation combined with foliar polyamine/CCC pretreatment effectively induce haploid embryos and DH lines in cucumber, with strong genotype dependency and optimal effects at 600 Gy for certain genotypes. These findings support genotype-informed optimization of DH production and offer practical guidelines for accelerating cucumber breeding.

Key Message

The combined application of polyamines and “CCC“ on the maternal plant, plus gamma-irradiated pollen, yields genotype-dependent embryogenesis improvements and haploid production in cucumber, showing Storm and 547 as the responsive varieties.

Introduction

Cucurbitaceae is among the most widely cultivated and economically important vegetable families. *Cucumis sativus* L. (2n = 2x = 14) is a key cucurbitaceous crop valued commercially and widely used as a model in vegetable breeding and functional genomics (Dey et al. 2022). It is grown globally under diverse agroclimatic conditions, in both open-field and protected environments. The species originates from India, with a secondary center of diversity in China and the Near East. As an out breeding crop without inbreeding depression, cucumber provides an exemplary system for investigating a range of developmental and molecular pathways, aided by its relatively small genome (~ 372 Mbp) (Huang et al. 2009). The classification of Cucumis species into primary, secondary, and tertiary gene pools has been established through comprehensive assessments of cross-compatibility, genetic analyses, phylogenetic studies, and molecular evidence (Dey et al. 2022).

Application of doubled haploid (DH) technology offers a powerful route to accelerate conventional breeding by enabling the rapid production of completely homozygous lines, thus bypassing multiple generations of selfing (Germana 2006; Ahmadiet al., 2018). Traditional hybrid breeding relies on inbred parental lines, typically requiring 6–8 years of selfing in cucumber to achieve homozygosity. By contrast, DH induction via gynogenesis and androgenesis provides a single-step route to homozygous parental lines, with demonstrated applicability across several vegetable crops (Behera et al. 2022; Dey et al. 2022; Bhatia et al. 2018; Mineykina et al. 2021; Romanova et al. 2023; Mulyana et al. 2023; Zhang et al. 2023). Successfully produced in vitro DH plants, whether via androgenesis or gynogenesis, provide valuable resources for research and breeding, including the development of mapping among biotechnological strategies, androgenesis (*in vitro* anther/microspore culture), gynogenesis (*in vitro* ovule/ovary culture), and

parthenogenesis (egg cell induction by irradiated pollen followed by *in vitro* haploid embryo rescue) have proven more efficient and sustainable than conventional methods (Shariatpanahi and Ahmadi 2016). Notably, gynogenesis often yieldshaploids at higher frequencies than androgenesis in cucurbitaceous crops, as female haploid cells within the embryo sac/ovules tend to respond more readily than male haploid cells produced in the same flower. Structural differences haploid cells residing inside ovules and encased by nucellar and integument tissues versus male haploids confined to the anther wall contribute to the greater complexity and longer timelines of ovary/culture approaches, necessitating advanced technical expertise(populations, inbred lines, and expanded genetic diversity for breeding programs(Segui-Simarro 2021; Ferrie 2011).

Polyamines are natural polycationic amines produced in living cells, including putrescine, spermidine, and spermine. Polyamines influence many biological processes, including cell division, differentiation, organogenesis, pollen formation, fertilization, gene expression, DNA and protein synthesis, apoptosis, and stress responses (reviewed by Tiburcio et al. 2014). There is compelling evidence for their crucial roles in somatic/gametic embryogenesis. Higher polyamine levels in unfertilized ovaries correlate with stronger gynogenesis (Wei et al. 2010; Ahmadi et al. 2014;), and adding polyamines to induction media markedly enhances somatic/gametic embryogenesis and subsequent embryo-to-plant conversion across multiple species (Kumar et al.2004;Ponce et al. 2006;Thiru-vengadam et al.2013; Ahmadi et al. 2014;Ebrahimzadeh et al. 2018). However, the underlying mechanism through which polyamines exert an effect is largely unknown. The plant growth regulator (CCC) is widely used to reduce stem elongation, promote flowering, and improve bud formation. CCC inhibits gibberellin production early in development (Zhang et al. 2009; Ebrahimzadehetet al. 2018). The regulatory role of CCC in *in vitro* somatic/gametic embryogenesis is less clear: CCC in induction media can retard embryo formation in some cases, while applying CCC to donor plants can markedly improve *in vitro* embryo formation, germination, and plant regeneration in certain species such as *Vitis vinifera* (Alifar et al. 2015; Ebrahimzadeh et al. 2018).

Application of irradiation to pollen as a means to induce haploidy remains a widely used and efficient approach in cucurbits, with ultraviolet (UV), gamma rays, and X-rays serving as recalcitrant agents to stimulate haploid formation. Among these, gamma irradiation is particularly favored in practical programs due to its simplicity, good penetrance, reproducibility, relatively high mutation frequency, and manageable abortion rates, making it the most commonly adopted method for haploid induction in many cucurbit breeding initiatives(Gałązka and Niemirowicz-Szczytt 2013; Chahal and Gosal 2002). Parthenogenesis has emerged as the most effective strategy to achieve complete homozygosity in *Cucumis sativus* L. in a single generation, although its routine deployment in breeding is limited by low haploid production rates(reviewed by Gałązka and Niemirowicz-Szczytt 2013; Dong et al. 2016). In irradiated pollen, observations include chromosome misbehavior and physio-biochemical alterations, with reports noting instances where a single male gamete is available for fertilization of either the egg cell or the fused polar nuclei; the former can lead to maternal homozygous diploids, while the latter may result in haploid parthenogenesis (pseudoembryony) triggered by irradiated pollen. Irradiated pollen can be still germinated on the stigma, extended through the style, and reached the embryo sac without fertilizing the egg cell or polar nuclei, allowing for parthenogenetic induction or gynogenic haploid production (Zhang 1991; De Witte 2000;Cuny 1993). Induction of haploid parthenogenetic embryos by *in situ* pollination with irradiated pollen has been reported in multiple Cucurbitaceae species, including *Cucumis melo*, *C. sativus*, *Citrullus lanatus*, Cucurbita pepo, *C. moschata*, and *C. maxima*, with varying efficiencies dependent on genotype, growth conditions, irradiation source and dose, and the developmental stage of embryos at excision (Sari et al. 1992; Taner et al.2000; Lotfi et al. 2003; Ebrahimzadeh et al. 2018; Ebrahimzadeh et al. 2013; Kurtar et al. 2009;Kurtar and Balkaya 2010). In this study, the concurrent effects of spraying maternal plants with putrescine, spermidine, and CCC from 12 days before to 12 days after pollination at 1-day intervals were examined, using three doses of gamma irradiation, on the frequency of haploid plant production.

Materials and Methods

Plant material, Pollen Irradiation and In Vivo Fertilization

This study was conducted in the greenhouse of the Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj. The"Sahm", "547" and "Storm" genotypes were used as maternal plants, with the "Beta Alpha" cultivar serving as the pollen donor. Plants were grown and maintained in a glasshouse under a 16-hour light/8-hour dark photoperiod, at a day temperature of 25 ± 2°C and a night temperature of 20 ± 2°C. To support plant growth, daily fertilization was performed with Hoagland's nutrient solution. Mother plants were sprayed with concentrations of putrescine, spermidine, and CCC each at 500, 50 and 50 mg/l, ranging from12 days before until 12 days after anthesis with 1-day intervals.

One day before the anthers opened and dehisced, male flowers (greenish-yellow) were collected and exposed to gamma radiation from a cobalt-60 source at a dose rate of 0.2 Gy/s with various gamma doses (400, 500 and 600 Gy). The female flowers of the F1 hybrids, serving as pollen recipients, were bagged to prevent contamination by unwanted pollen, up to dusk on the same day. After irradiation, the male flowers were kept at room temperature until pollination in the following day. Early the next morning, when the stigma was receptive to pollen, pollination was performed using pollen grains from the irradiated anthers. For each flower, 2–3 anthers were sufficient. The female flowers were re-bagged for 3–4 days with their petals closed to prevent cross-pollination.

Embryo Rescue

Growth sprouts were harvested after 35 days and surface-sterilized with 70% ethanol to reduce greenhouse contaminants. After washing, the fruits were opened and the seeds were extracted from running tap water and cleaned. Seed surface sterilization was performed under a laminar flow hood with 1% sodium hypochlorite for 10 minutes, followed by three washes with sterile distilled water for 5 minutes each.To facilitate identification and embryo extraction, the sterilized seeds were transferred to liquid medium E20 (Sauton and Dumas de Vaulx, 1987) supplemented with 3% sucrose and 0.01 mg/L indole-3-acetic acid (IAA) according to Lotfi et al. (2003). Approximately 25 seeds per dish containing 25 mL liquid E20 medium were used, ensuring seeds were submerged. Dishes containing immature embryos were sealed with Parafilm and placed in a growth chamber under standard conditions for tissue culture: a 16-hour photoperiod (light) and an 8-hour dark period, at a temperature of 22 ± 2°C. The dishes were gently agitated several times per week to maintain aeration. After 10–20 days, embryos were examined and isolated using a suitable light source. Isolated embryos were transferred to solid E20 medium (Fig. 1a,b).One to three weeks after culture, the developed plantlets (epigeal plantlets) were transferred to glass jars containing MS medium supplemented with BAP at 2.0 mg/L and NAA at 0.05 mg/L for further growth (Fig. 1c). After three weeks, for better growth of small plantlets, the culture was transferred to MS medium without supplements (Fig. 1d).

Ploidy Level Determination

Flow cytometry

Small section of young leaf tissue (0.5 cm) from diploid control plants (control) and plants with unknown ploidy, produced by induced apomixis, was separately excised with a fine scalpel in 500 µL of hypotonic propidium iodide (PI) lysis buffer, cooled on ice. The tissue was finely chopped.Subsequently, 2000 µL of nuclear staining solution and 6 µL of RNase were added, followed by 12 µL of propidium iodide (PI) to the crude mixture.To reduce the effects of cytosolic and phenolic compounds on PI fluorescence, 1% PVP-40 was also added to the buffer.The suspension was

filtered through a 50 µm filter to remove cell debris and isolate nuclei. Ploidy level of the samples was analyzed with a flow cytometer (Partec, Münster, Germany) using a 488 nm Argon laser to excite the fluorochrome PI and a FL-2 detector with a 585/42 nm emission filter. Samples were run at low pressure and a minimum of 10,000 nuclei per sample were analyzed. Subsequently, DNA fluorescence per nucleus was measured and results were displayed as histograms. Since the injected sample contained a mixture of haploid and diploid plant extracts, the ploidy level of the sample was determined based on the fixed peak positions for haploid and diploid references.

Induction of doubled haploids in regenerated haploid plants

Haploid plantlets regenerated were micro-propagated *in vitro* before chromosome doubling. Young shoots, about 1–3 cm in length and leafless, were excised and transferred to liquid medium E20 containing 0.2 mg/L BAP, 80 mg/L Fe-EDDHA, and 50 mg/L oryzalin. The antimutagenic agent (oryzalin) was dissolved in dimethyl sulfoxide (DMSO, 2% v/v) and then added to the culture medium. The samples were incubated for 18 hours in a incubator at a constant temperature of 27°C with a speed of 150 rpm. Subsequently, the samples were washed three times with sterile distilled water, and the microexplants were transferred to solid E20 medium containing 0.01 mg/L IAA, 0.2 mg/L BAP, and 80 mg/L Fe-EDDHA. All cultures were maintained in a growth chamber under standard light conditions (photoperiod:16 h light/8 h dark) and at a temperature of 22 ± 2°C. After 3 weeks, each regenerated plantlet was individually transferred to flasks containing MS medium.

Distinguishing doubled haploids from diploid plants

In this study, the SSR marker CMCCA145 was used to identify the embryo type (diploid or doubled haploid). More than 20 SSR markers taken from the cucumber genome (from the Soul genomic network) were screened and the CMCCA145 marker was selected due to its apparent polymorphism. DNA extraction from 200 mg of young leaf tissue was performed using CTAB reagent according to the protocol with minor modifications. The quality and quantity of DNA were assessed using gel electrophoresis and fluorometric assay. PCR amplification was performed in a 96-well Variti thermal cycler (Applied Biosystem, USA) using the following cycles: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. The amplified products were examined using a 8% acrylamide gel in TBE buffer stained with Gelred using vertical electrophoresis. They were then visualized under UV light (Gelduc., UK). The size of the amplified fragments was determined using a 50bp Gen Ruler DNA ladder (Thermo Scientific, USA).

Experimental design and data analysis method

The experiment was a factorial experiment in a completely randomized design (the first factor was genotype and the second factor was gamma ray dose) with three replications. Two fruits were examined from each replication. SPSS software was used for data normalization and SAS software version 9.4 was used for data analysis.

Results

Simultaneous spraying of putercine, spermidine and CCC on mother plants with concentrations of (500, 50 and 50 mg/L) respectively along with irradiation with different doses of gamma rays to sterilize pollen grains showed that the interaction effect of genotype with gamma ray dose had a significant effect at the 5% probability level on the number of seeds per fruit. The results showed that the highest number of seeds per fruit was in the Storm genotype with a gamma ray dose of 600 Gy (205 pcs.) and the lowest number of seeds per fruit was in the 547 genotype (66.88 pcs.) and the gamma ray dose treatment was 400 Gy.The results showed that with increasing dose from 500 to 600Gy, the

number of seeds per fruit increased in 3 tested cultivars (Table 1).Also, the genotype and gamma ray dose had a significant effect on the number and percentage of full and unfilled seeds in cucumber fruit at the 5% probability level. The results showed that in all 3 varieties, the control treatment had the highest number of seeds. The highest number of filled seeds (124.66) was related to the 547 genotype, and the lowest number was observed in the same genotype and gamma ray dose treatment at 600 Gy. Considering that the number and percentage of full seeds decreased with increasing gamma ray dose, the role of gamma ray, especially at higher doses, in the sterilization of pollen grains can be justified (Table 1). The response of each genotype under the same conditions was different, indicating that the effect of genotype is also effective in the success rate of parthenocarpy. Regarding the number and percentage of whole and empty seeds, the results showed that the interaction between genotype and gamma radiation dose had a significant effect at the 5% probability level. The Storm genotype pollinated with pollen irradiated with a dose of 600 Gy had the highest number of whole and empty seeds (153), and the lowest number (21.66) was observed in the Saham genotype and the control treatment. Our results showed that by increasing gamma radiation dose, the number of whole and empty seeds in the fruit increased, and also the response of different genotypes was different. Considering that the embryo is obtained from empty seeds, the increase in the number of empty seeds due to irradiation indicates the efficiency of gamma radiation in sterilizing anthers. Data on the number of embryogenesis and regenerated plants in cucumber fruit through pollen irradiation showed that the interaction effect of genotype with gamma radiation dose had a significant effect at the 5% probability level on the number of embryos and regenerated plants (Table 2), such that the Storm genotype with a radiation dose of 600 Gy showed the greatest effect on the number of embryos and regenerated plants (6 and 5, respectively), while in the control treatment no embryo formation was observed in any of the three cultivars. Examination of the main effect of gamma radiation dose showed that the dose of 600 Gy had the greatest effect on the number of embryos and regenerated plants in all three cultivars studied.

Table 1

Analysis of the comparison of means of the interaction effect of genotype and gamma radiation dose on the total number of seeds in the fruit, whole and full seeds, percentage of whole and full seeds, whole and empty seeds and percentage of whole and empty seeds in the three genotypes of cucumber (*Cucumis sativus* L.).

Genotype	Gamma irradiationdose (Gy)	Total seeds/fruit (TSF)	Filledseeds/fruit (FSF)	Filledseeds (%)	Emptyseeds/fruit (ESF)	Emptyseeds (%)
” sahm F1”	Control	138.33^{de}	88.33^{b}	63.85^{c}	21.66^{h}	15.68^{g}
	400	90.66^{g}	28.66^{cd}	31.64^{d}	43.33^{gh}	47.8^{f}
	500	126^{ef}	29.66^{cd}	23.53^{e}	69.33^{ef}	55.01^{e}
	600	133.33^{de}	20.66^{d-f}	15.5^{g}	83.66^{cd}	62.77^{d}
” stoorm F1”	Control	180.33^{bc}	117.66^{a}	65.13^{b}	31.33^{gh}	17.77^{g}
	400	156^{dc}	37.66^{c}	24.16^{e}	88^{c}	56.46^{e}
	500	189^{ab}	22.33^{de}	11.83^{h}	128.66^{b}	68.12^{c}
	600	205^{a}	16.66^{ef}	8.12^{j}	153^{a}	74.53^{a}
” 547 F1”	Control	187^{ab}	124.66^{a}	66.66^{a}	29.33^{h}	15.68^{g}
	400	95.33^{g}	19.66^{d-f}	20.62^{f}	60.33^{f}	63.21^{d}
	500	88.66^{g}	10.66^{f}	12.03^{h}	62^{ef}	70^{bc}
	600	103^{gf}	10^{f}	9.69^{i}	74.66^{de}	72.58^{ab}

Shared superscrip tletters indicate that means do not differ significantly at the 5% probability level ($p \leq 0.05$).

Table 2
Analysis of the comparison of means of the interaction effect of genotype and gamma radiation dose on the number of embryos, percentage of embryogenesis, number of regenerated plants and resistance to regeneration in the three genotypes of cucumber (*Cucumis sativus* L.).

Genotype	Gamma irradiationdose (Gy)	Embryos (No.)	Embryogenesis (%)	Regeneratedplants (No.)	Regeneration (%)
” sahm F1”	Control	0^{g}	0^{d}	0^{f}	0^{d}
	400	3.66^{d-f}	2.75^{cb}	2.33^{de}	63.88^{bc}
	500	3.33^{ef}	3.57^{b}	3^{c-e}	93.88^{a}
	600	2.66^{f}	2.13^{c}	2^{e}	72.22^{bc}
” stoorm F1”	Control	0^{g}	0^{d}	0^{f}	0^{d}
	400	4^{c-e}	2.61^{cb}	3.33^{b-d}	83.33^{ab}
	500	4.66^{b-d}	2.46	3^{c-e}	65^{bc}
	600	6^{a}	2.92^{c}	5^{a}	83.8^{ab}
” 547 F1”	Control	0^{g}	0^{d}	0^{f}	0^{d}
	400	3.33^{ef}	3.45^{b}	2^{e}	58.33^{c}
	500	5^{a-c}	5.68^{a}	3.66^{bc}	73.88^{a-c}
	600	5.66^{ab}	5.52^{a}	4.33^{ab}	76.66^{a-c}
Shared superscrip tletters indicate that means do not differ significantly at the 5% probability level ($p \leq 0.05$).					

Ploidy analysis using a flow cytometry to determine the ploidy level of regenerated plants resulting from *in vitro* embryo rescue showed that all regenerated plantlets contained the gametic chromosome number (n = x = 7) of the parental plants. As shown in (Fig. 1), diploid and haploid samples showed a single, distinct peak, while in haploid plants, the number of peaks was approximately half that of diploid plants (Fig. 1j ,i). In contrast, a mixed sample containing diploid and haploid nuclei showed three distinct peaks. The first peak (which may be due to instrument noise), a second peak with a higher height indicating the G1 phase of the cell cycle, and a third peak indicating the G2/M phase, located approximately twice as far away as the previous peak (Fig. 1k).Oryzalin was applied to the nodal sections at a rate of 50 mg for 18 hours at a speed of 150 rpm and showed a favorable effect on the rate of chromosome doubling and the generation of doubled haploid plants(Fig. 1e).The SSR marker was also used to assess the ploidy status of plants for which chromosome doubling was performed. Banding profiles on 8% acrylamide gels showed that haploid samples produced a single band of low intensity and samples in which doubling was performed had a similar band but with stronger intensity, probably due to the homozygous nature of their genome, in contrast to diploid plants which had two distinct bands due to heterozygosity (Fig. 1i).

Discussion

We evaluated the immediate effectiveness of simultaneously applying polyamines and CCC. This approach resulted in high embryogenesis rates, though embryogenesis efficiency varied among genotypes. We found that embryo induction in *in vitro* cultures of the Storm, 547 and Sahm genotypes was significantly increased by the combined application of three compounds putrescine at a concentration of 500 mg/L, Spermidine and CCC (each 50 mg/L) on

the mother plant from 12 days before to 12 days after flowering. Previous cucumber observations reported by Ebrahimizadeh et al. (2018) showed that separate applications of these substances at similar concentrations increased embryogenesis and plant regeneration in our assay. Furthermore, prior observations indicated that higher levels of these compounds were detrimental, with embryogenesis and regenerated plantlets from embryos grown at higher concentrations markedly reduced (Ebrahizadeh et al., 2018). Additionally, a high concentration of Putrescine at 5000 mg/L and CCC 500–5000 mg/L relative to fruit weight significantly reduced fruit set and seed formation (Ebrahimzade et al. 2018).Exogenous putrescine at low concentrations markedly enhances somatic embryogenesis across several species, including cotton (*Gossypium hirsutum* L.), spine gourd (*Momordica dioica* Roxb.), *Brassica napus* L., and wheat *(Triticum aestivum* L.). Polyamines are involved in a range of processes, including cell division, embryogenesis induction, flowering initiation, fruit development, and maturation. External application of polyamines has been reported to enhance fruit set and performance in several species, including *Cucumis sativus* L. (Ebrahimzade et al. 2018), *Pistachia vera* L. (Kamiab et al. 2015), *Phoenix dactylifera* L. (Abd El-Migeed et al. 2013), and *Gossypium hirsutum* L. (Bibi et al. 2012). Compared with polyamines, there is limited information on the role CCC in the induction of somatic/gametic embryogenesis. The enhancing effect of CCC and other inhibitors of gibberellin biosynthesis on the induction of somatic/gametic embryogenesis has been reported in species such as *Cucumis sativus* L. (Ebrahimzade et al. 2018), *Pinus taeda* L. (Pullman et al. 2005), A. *cepa* L. (Ponce et al. 2006)d *aestivum* L. (Miroshnichenko et al. 2009). Fruit weight and seed set were significantly improved by applying CCC low levels (50 mg L^{-1}) to mother plants in cucumber (Ebrahimzade et al. 2018). Similarly, foliar spraying (500 mg L^{-1} CCC) on autumn lettuce (*Lactuca sativa* L.) increased the number of inflorescence branches per plant and also seed yield (Pasam et al. 2008). In tomato (*Solanumlycopersicum*), the greatest fruit weight was obtained with 100–300 mg L^{-1} CCC (Altinas 2011).The results show that the simultaneous use of these mixtures varies in different cultivars. This phenomenon seems to be somewhat genotype-dependent, as the Sahm genotype showed lower embryogenesis compared to Storm and 547. Although spraying these mixtures was useful for inducing embryogenesis *in vitro*. Careful cultivation of Storm and 547 mother plants in greenhouse conditions followed by the use of 600 Gy gamma-irradiated pollen grains resulted in higher frequencies of embryogenesis. Gamma irradiation, which has been used in recent years, is one of the effective strategies for inducing embryogenesis through parthenogenesisin Cucurbitaceae. The results showed that the interaction effects of genotype and gamma radiation dose were significant on the total number of seeds, the number of whole and filled seeds, the number of whole and empty seeds in the fruit, and the number of embryos and regenerated plants. The highest number of seeds per fruit was obtained from the genotype "Storm" with an average of 205 at a dose of 600 Gy. These findings are consistent with previous studies showing that controlled pollen irradiation can enhance haploid induction and parthenocarpic embryogenesis (Pradeepkumara et al. 2023; Dey et al. 2022). These results highlight the importance of selecting the appropriate genotype and adjusting the irradiation dose for each genotype for seed production. Regarding the number of whole and filled seeds, the highest number was observed in the genotypes "547" and "Storm" with averaging 124.66 and 117.66 seeds respectively under the control treatment. In contrast, the genotype "547" exhibited the lowest number of whole and filled seeds, with an average of 10. The effect of radiation dose showed that the control treatment produced the highest number of whole and filled seeds, and increasing radiation doses led to a significant decrease in this trait. In contrast, the highest number of whole and empty seeds in all 3 cultivars was in the treatment with the highest radiation dose of 600 Gy. The results of this study show that radiation dose and genotype had significant effects on the percentage of full and empty seeds. Increasing the radiation dose to 600 Gy increased the number of whole and empty seeds in the Storm genotype, but lower doses reduced this trend, indicating a stimulating effect of higher doses and an effect on pollen and mother cells. Gamma radiation has a distinct negative effect on full seed production, and the response of genotypes to radiation dose is different. Lower doses of irradiation were able to maintain the number of full seeds, while higher doses caused a severe reduction in the number of seeds. The findings of this study are consistent with previous results in cucumber and other Cucurbitaceae, which showed that the use of controlled pollen irradiation can improve

haploid induction and parthenocarpic embryo formation in cucumber, while simultaneously increasing the quality and number of seeds under the optimal dose. However, high doses reduce the quality and number of full seeds (Pradeepkumara et al. 2023; Dey et al. 2022).The findings related to embryogenesis showed that the genotypes "Storm" and 547 produced the highest number of embryos (6 and 66.5), respectively, while the control treatments did not produce any embryos. Also, the radiation dose of 600 Gy had the greatest effect on the number of embryos and regenerated plants. These results indicate that gamma radiation has an effective role in embryo induction from empty seeds and the selection of the optimal dose can facilitate the production of haploid lines. This finding is consistent with the results reported in other species such as cucumber and cucurbits, which have shown that genotypic sensitivity and radiation dose play an important role in the success of parthenocarpic embryogenesis (Khoshkam et al. 2024). These results are in contrast to the findings of Ebrahimzadeh et al. (2018) and Gonzalo et al. (2011), who reported that the percentage of embryo induction decreases with increasing radiation dose. The possible reason for this discrepancy could be the dose range used and the difference in genotypic sensitivity to radiation, which could be an important factor in this difference in results. Flow cytometry analysis confirmed the accuracy of the regeneration data. DNA content index values in haploid samples were approximately half those of diploid samples. Despite the utility of flow cytometry in estimating DNA content, this method is inherently unable to distinguish between homozygous diploids and heterozygous diploids, especially when both show the the same ploidy level. To overcome this limitation, co-dominant SSR markers were included as a complementary tool to confirm genetic uniformity. In particular, the 3B27marker consistently showed single-band patterns in gel electrophoresis assays and provided molecular confirmation of homozygosity in selected DH lines. Therefore, the integration of nuclear DNA quantification and molecular marker-based genotyping is a comprehensive and reliable strategy for DH identification, providing critical support for accelerating breeding programs and ensuring genetic stability in restored populations (Keleş et al. 2015; Pareeth 2015).

Among antimitotic agents, oryzaline has shown better efficacy in producing doubled haploid plants, which highlights that choosing the right agent and carefully adjusting the dose and contact time are essential for successful chromosome doubling (Ebrahimzadeh et al., 2018). Furthermore, animal cells are immune to this agent and it has less negative effect on humans.

Conclusion

The present study aimed to produce pure lines of cucumber (*Cucumis sativus* L.) through pollen irradiation. Results showed that increasing the gamma radiation dose, particularly at level 600 Gy, had a significant inhibitory effect on the formation of whole and fill seeds and was associated with an increase in the number of whole and empty seeds as well as embryos, identifying 600 Gy as the optimal dose for this study. To mitigate the adverse effects of radiation and improve reproductive conditions, foliar spraying of polyamines including putrescine (500 mg/L), spermidine (50 mg/L)and CCC (50 mg/L) was applied 12 days before and 12 days after pollination on an alternate-day basis. Foliar application of polyamines and CCC increased the proportion of fruits formed and preserved viable embryos. Overall, combined treatment with a high radiation dose (600 Gy) and polyamines plus CCC showed the greatest efficiency in inducing haploid embryos. Thus, these results indicate that controlled levels of gamma radiation can be an effective tool for pollen-induced haploidy in cucumber, though dose and genotype play decisive roles in the success of the process. Additionally, polyamines and CCC can serve as an ancillary strategy to reduce radiation-induced oxidative stress and to improve the growth and viability of gametic cells.

Statements & Declarations

Abbreviations

BAP: 6-Benzylaminopurine

CCC: Cycocel

CTAB: CetylTerimetil Ammonium Bromide

DH: Doubled haploid

DNA: Deoxyribonucleic acid

EDTA: Ethylene diamine tetra acetic acid

Gy Gamma irradiation

IAA: Indole-3-acetic acid

MS: Murashige and Skoog medium

NAA: 1-Naphthaleneacetic acid

PI: Propidium iodide

PCR: Polymerase chain reaction

Rpm: Revolutions per minute

SSR: Simple sequence repeat

SAS: Statistical Analysis System

SPSS: Statistical Package for the Social Sciences

TBE : Tris- borate -EDTA buffer

Declarations

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Competing Interests

The authors declare that they have no conflict of interest.

Authors' contributions:

Authors' contributions: MES conceived of the presented idea and planned the experiments. SFT developed the theory conducted the research experiments and wrote the manuscript with the support of all co-authors. MHS analysis of related data preparation. MT assisted in project implementation. Zahra Hosseini provided laboratory materials. All authors discussed the results and contributed to the final manuscript

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Data availability:

The data that support the findings of this study is available

Ethices decleration: not applicable

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Figures

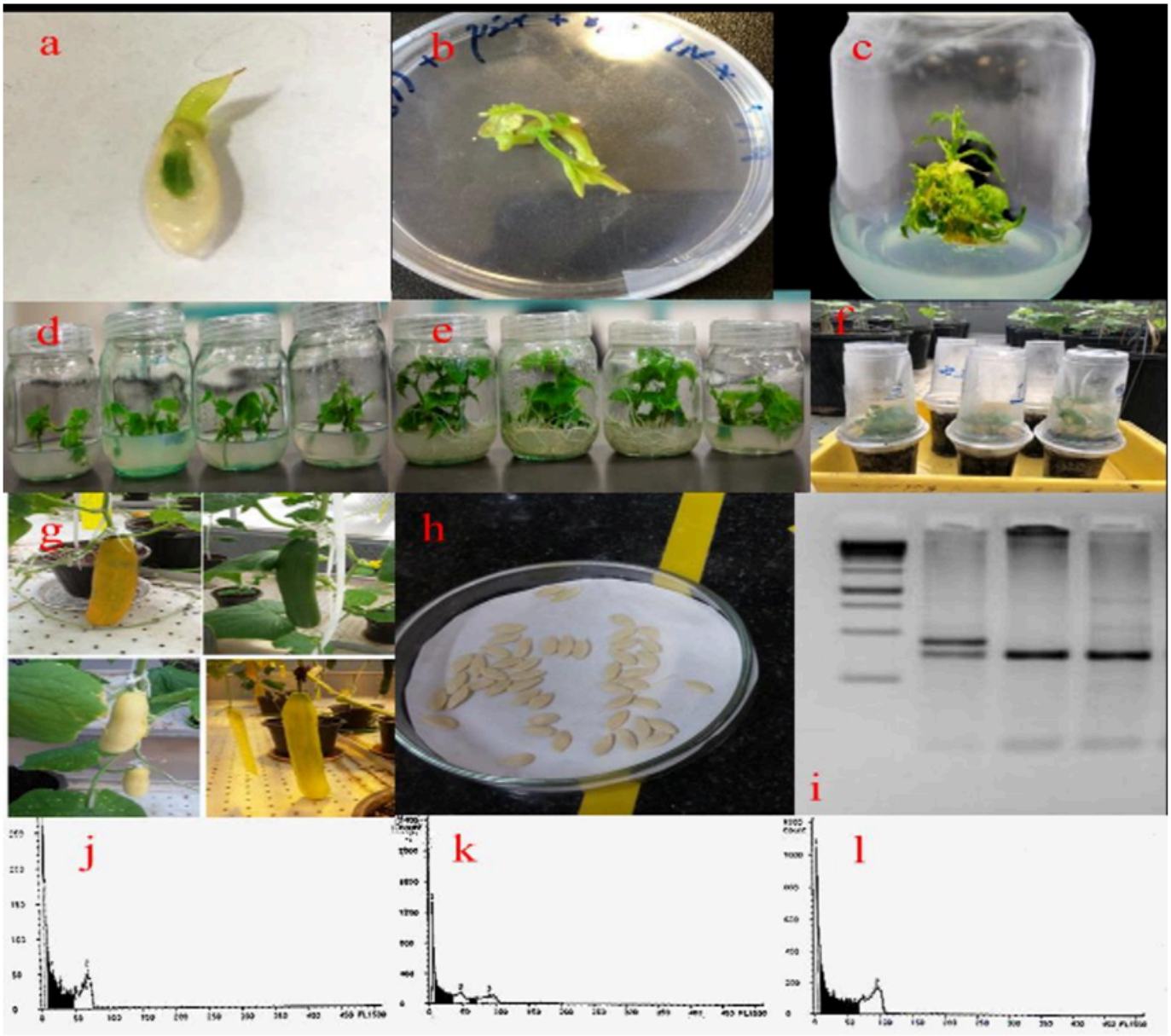


Figure 1

Selected steps in Parthenogenesis procedurein cucumber (*Cucumis sativus* L.); a) embryo rescue; b) embryo growth and its transformation into a seedling, c) transfer of the seedling into a glass for further growth, d) micropropagation of haploid plants, e) regeneration of plants after doubling treatment, e) acclimatization; f-g)seeding from the fruit;i)SSR marker profiles on a 8% acrylamide gel used for distinguishing haploid, spontaneously doubled haploid, and diploid,Lane M: molecular size marker (DNA ladder,Lane 1: diploid,Lane 2: haploid;Lane 3: spontaneously doubled haploid, Flowcytometry histogram of a haploid sample, showing a single peak representing C DNA content;j)Flow cytometry analysis of ploidy levels in regenerated plants:k) Flowcytometry histogram of a mixed sample containing both diploid and haploid nuclei, with the third peak corresponding to diploid, the second to haploid, and the first to

background noise; l) Flowcytometric histogram of a diploid sample, showing a single peak representing 2C DNA content