

Nanobiochar and Nano-encapsulated ascorbic acid Synergistically Enhance Haploid and Doubled Haploid Induction and Production in Bell Pepper (*Capsicum annuum* L.)

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Abstract

Rapid generation of haploid and doubled haploid (DH) lines in *Capsicum annuum* is essential for accelerating breeding and genetic studies. This study introduces a novel approach that combines carbon-based adsorbents and nano-antioxidants to enhance in vitro androgenesis. Three commercial F₁ hybrids (Nirvin, LIAD, and Dicaprio) were cultured on solid anther induction medium. In the first phase, Activated charcoal (AC), Biochar (BC), and Nanobiochar (NBC) (0–2%) were evaluated as carbon-based adsorbents. Activated charcoal and Nanobiochar exhibited superior performance in embryo induction and plantlet regeneration and were therefore selected for the second phase. In this phase, conventional Ascorbic acid (AA) (20, 50, and 100 mg L⁻¹) and Nano-encapsulated ascorbic acid (NEAA) (3.33, 16.66, and 33.33 mg L⁻¹) were combined with 1% Activated charcoal or Nanobiochar. Higher concentrations of conventional Ascorbic acid inhibited embryogenesis, whereas moderate levels—particularly 16.66 mg L⁻¹ of Nano-encapsulated ascorbic acid significantly enhanced embryo number, embryogenesis frequency, and plantlet regeneration. In the LIAD genotype, Nanobiochar combined with 16.66 mg L⁻¹ Nano-encapsulated ascorbic acid produced the highest embryo count (5.6), embryogenesis rate (56%), and regenerated plantlets (4.5). Flow cytometry, cytogenetic examination, and simple sequence repeat (SSR) markers confirmed the successful recovery of true haploid and DH plants. This study provides the first experimental evidence that integrating Nanobiochar with Nano-encapsulated ascorbic acid synergistically improves haploid induction and regeneration efficiency in bell pepper, establishing a reproducible and efficient protocol for DH line development.

Key Message

Integration of Nanobiochar and nano-encapsulated antioxidants significantly improves androgenesis efficiency in *Capsicum annuum*, offering a reliable pathway for homozygous DH line production.

Introduction

Bell pepper (*C. annuum* L.) is a globally significant horticultural crop of the Solanaceae family, valued for its fleshy, flavorful fruits and rich nutritional composition, including provitamin A, vitamin C, and essential minerals (Chassy et al 2006; Agarwal et al 2007). Although predominantly self-pollinating, occasional cross-pollination events occur, rendering F₁ hybrids commercially important. The development of these hybrids requires genetically pure (homozygous) parental lines, which conventionally demand at least six to seven generations of time-intensive and laborious process of selfing (Seguí-Simarro 2010). In vitro haploid and doubled haploid (DH) production via androgenesis provides a rapid alternative for generating fully homozygous lines in a single generation, significantly reducing breeding cycles and associated costs (Ferrie et al 1994; Gómez-Campo 1999). Despite its potential, *C. annuum* remains recalcitrant to androgenesis, with high rates of abnormal embryo formation, poor shoot development, and strong genotype dependency limiting regeneration efficiency (Sibi et al 1979; Dolcet-Sanjuan et al 1997; Supena and Custers 2011). The success of microspore embryogenesis depends on multiple factors, including donor plant genotype, microspore developmental stage, pretreatments, culture medium composition,

and in vitro environmental conditions (Irikova et al 2011; Comlekcioglu and Ellialtioglu 2018). The application of bioactive compounds has been demonstrated to enhance embryogenic responses. Polyamines, such as putrescine, spermidine, and spermine, modulate gene expression under stress and promote somatic and androgenic embryogenesis across various plant species (Alcázar et al 2010; Kelley et al 2002). Ascorbic acid (AA), a potent antioxidant, regulates cell cycle progression, supports cell elongation, and facilitates embryonic development (Potters et al 2004; Hoseini et al 2014). However, its instability under in vitro culture conditions, including rapid degradation at low pH and elevated temperatures, limits its efficacy (Comunian et al 2022). Nanoencapsulation of Ascorbic acid has emerged as an effective strategy to enhance its stability and sustain its biological activity during microspore culture. Moreover, carbon-based additives and novel culture supports, such as Activated carbon (AC), Nanobiochar (NBC), and Biochar (BC), have been shown to improve nutrient availability, mitigate oxidative stress, and promote embryogenesis efficiency (Ahmadi et al 2014; Heidari-Zefreh et al 2019). These materials modulate the physicochemical properties of the culture medium, thereby facilitating the induction of the sporophytic developmental pathway in microspores and enhancing the production of haploid and DH plants. Considering the critical role of homozygous lines in pepper breeding and the persistent challenges of low embryo quality, this study aims to investigate the effects of AC, BC, NBC, and Nano-encapsulated ascorbic acid (NEAA) on haploid line production in bell pepper using the shed-microspore culture method. The findings are expected to provide optimized strategies for improving androgenesis efficiency and accelerating the generation of doubled haploid lines in pepper.

Materials And Methods

Plant Material and Growth Conditions Seeds of three commercial F1 hybrids, Nirvin (Rijk Zwaan, The Netherlands), LIAD, and Dicaprio (Enza Zaden, The Netherlands), were sown in April 2024 at the greenhouse facilities of the Agricultural Biotechnology Research Institute of Iran (ABRII). The seeds were germinated in plastic pots filled with a growth medium composed of soil, peat, and perlite mixed in equal proportions (1:1:1, v/v/v). Plants were maintained under controlled greenhouse conditions with day/night temperatures of 25–30/18–22°C, respectively, and natural light regime. Irrigation and fertilization were carried out following standard horticultural practices recommended for pepper cultivation (Bosland et al 2012).

Experiment 1: Determination of the Optimal Microspore Developmental Stage for Androgenesis Induction in Bell Pepper

The identification of the appropriate microspore developmental stage is crucial for successful androgenesis in bell pepper. To establish the optimal stage and to develop a morphological marker for bud collection, floral buds ranging from 2 to 8 cm in length were harvested. The accumulation of anthocyanin pigments in the anthers was visually assessed as an additional indicator. Microspore developmental stages were analyzed using the fluorochrome-specific stain 4',6-diamidino-2-phenylindole

(DAPI), following the procedure described by Kusters (2003). Microspores were stained with DAPI, and their developmental stages were examined under an inverted fluorescence microscope (Nikon Eclipse TS100).

Experiment 2: Effect of Carbon-Based Adsorbents and Antioxidants on Androgenesis in Bell Pepper Anther Culture

Flower buds with uniform-sized sepals and petals, containing microspores at the mid- to late-uninucleate stages, were harvested early in the morning. The buds underwent a cold pretreatment at 4°C for 48 h to enhance androgenic response, followed by surface sterilization using 2.5% (v/v) sodium hypochlorite for 10 min and three rinses with chilled sterile distilled water. Anthers were aseptically excised from flower buds and placed in sterile disposable Petri dishes (300 × 80 × 80 mm), with six anthers from independent buds allocated per dish. Cultures were initiated on C basal medium (Dumas de Vaulx et al 1981), supplemented with 2 mg L⁻¹ 2,4-D, 2 mg L⁻¹ kinetin, 30 g L⁻¹ sucrose, and 0.7% plant agar, adjusted to pH 5.8.

Phase 1 – Carbon-Based Adsorbent Screening:

Three types of AC, BC and NBC were evaluated at 0 (control), 0.5, 1, and 2% (w/v) to determine their effects on androgenesis. These adsorbents were incorporated into the C basal medium prior to autoclaving to ensure uniform distribution throughout the medium. The most effective adsorbent was identified based on the androgenic response.

Phase 2 – Antioxidant Supplementation:

Following the selection of the optimal carbon-based adsorbent, antioxidant treatments were applied. AA was added at 20, 50, and 100 mg L⁻¹, and NEAA was applied at 3.33, 16.66, and 33.33 mg L⁻¹. Due to heat sensitivity, AA was sterilized via sterile filtration prior to incorporation, while NEAA was sterilized using UV irradiation. All antioxidant treatments were combined with the selected adsorbent and the same hormonal composition used in Phase 1, and added to the C medium. Anther cultures were incubated at 35°C in complete darkness for 8 days, followed by 4 days at 25°C under the same light conditions. Subsequently, anthers were transferred to R medium (Regeneration Medium) (Dumas de Vaulx et al 1981), containing 0.1 mg L⁻¹ kinetin, 30 g L⁻¹ sucrose, and 0.7% plant agar, adjusted to pH 5.8, and maintained at 25°C under a 16-h photoperiod (Heidari et al 2017). Plant regeneration after five weeks, the developed embryos were transferred to a hormone-free MS basal medium (Murashige and Skoog) supplemented with 30 g L⁻¹ sucrose and solidified with 0.7% plant agar. In this study, we assessed the influence of genotype together with two groups of treatments: (i) carbon-based adsorbents, including AC, BC, and NBC. Their effects on microspore embryogenesis efficiency during anther culture of bell pepper (*C. annuum* L.) were systematically evaluated.

Synthesis of Biochar, Nanobiochar, and Nano-encapsulated ascorbic acid (NEAA)

Commercial BC derived from pistachio hard shells was obtained from the local market and used as the carbon precursor for NBC synthesis. The purchased BC was thoroughly washed with deionized water to remove impurities, oven-dried at 80°C for overnight, and subsequently subjected to high-energy ball milling to achieve nano-dimensional particles (Hosseinpour Azad et al 2022; Pradhan et al 2025). Then, ascorbic acid-loaded mesoporous silica nanoparticles (NEAAs) were synthesized by a sol-gel process employing tetraethyl orthosilicate (TEOS) as the silica precursor. TEOS was hydrolyzed and condensed in an ethanol-water-ammonia medium (molar ratio 1:40:10:0.1) under vigorous stirring, while an aqueous ascorbic acid solution was progressively incorporated into the reacting sol, facilitating molecular entrapment within the evolving silica network. The colloidal product was purified by repeated centrifugation and washing with ethanol and deionized water, followed by drying to yield fine white powders. Morphological and structural analyses of nanomaterials (NBC and NEAA) were performed using SEM and Brunauer-Emmett-Teller (BET) surface area characterization through nitrogen adsorption-desorption at 77 K (Rashidi et al 2013; Bahrami et al 2015).

Ploidy Determination of In Vitro Regenerated Plants

Ploidy Determination

The ploidy level of regenerated plants were assessed using two complementary approaches: flow cytometry and chromosome counting.

For flow cytometric analysis, approximately 50 mg of young leaf tissue from each regenerated plant was co-chopped with an equal amount of diploid bell pepper leaf tissue (used as an internal reference) in 1 mL of Otto I buffer (0.1 M citric acid and 0.5% Tween-20) using a sharp razor blade. The resulting suspension was filtered through a 30-µm nylon mesh and incubated for 5 min at room temperature. Subsequently, 1 mL of Otto II buffer (0.4 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) supplemented with propidium iodide (50 µg/mL) and RNase A (50 µg/mL) was added. Samples were analyzed using a Partec CyFlow cytometer (Sysmex Partec, Germany), and histograms were generated using FlowMax software. Ploidy levels were determined by comparing the relative fluorescence peaks of regenerated plants with those of the diploid control. For chromosome counting, actively growing root tips (~1–2 cm) were excised from regenerated plants and pretreated with saturated α -bromonaphthalene for 4 h at 4°C to arrest cells at metaphase. Root tips were then fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid) for 24 h at 4°C and stored at –20°C until further analysis. For slide preparation, root tips were first hydrolyzed in 1 N HCL at 60°C for 10 min, followed by staining with 4% hematoxylin for 16 h. Subsequently, the root tips were incubated with 2% (w/v) cellulase at 37°C for 20 min to facilitate cell wall digestion. The softened root tips were gently squashed on clean glass slides and mounted in a drop of 45% acetic acid. Chromosome spreads were observed under a light microscope (Olympus BX51), and at least 10 well-spread metaphase plates

were examined per plant. The expected chromosome number ($2n = 2x = 24$) was used as a reference to confirm ploidy levels.

Identification of Doubled Haploids and Diploid Plants

To differentiate between doubled haploid and diploid plants, a Simple Sequence Repeat (SSR) marker assay was conducted using the locus Hpms1-117. Out of more than 30 SSR markers derived from the pepper genome (Sol Genomics Network), this marker was selected due to its high polymorphic potential.

Polymerase Chain Reaction (PCR) reactions were performed in a total volume of 15 μL , consisting of 4.8 μL nuclease-free water, 2 μL genomic DNA, 1.5 μL of 10 $\times$ PCR buffer, 0.2 μL of Taq DNA polymerase (5 U/ μL), 1 μL dNTP mix (2.5 mM), 1.5 μL MgCl_2 (25 mM), and 2 μL of each primer (forward and reverse, 10 μM). The amplification conditions included an initial denaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 55–57°C for 30 s, and extension at 72°C for 30 s, with a final elongation step at 72°C for 3 min and a hold at 4°C.

PCR products were resolved on a 3.5% MetaPhor agarose gel prepared in 0.5 $\times$ TAE buffer. For gel preparation, 3.5 g MetaPhor agarose was dissolved in 100 mL 0.5 $\times$ TAE buffer by heating, cooled to $\sim 60^\circ\text{C}$, and supplemented with GelRed (Biotium) or ethidium bromide for DNA staining. The molten gel was cast in a tray with combs and allowed to solidify for 30–45 min at room temperature.

Electrophoresis was performed at 80–100 V for 2–2.5 h to achieve clear separation of fragments. DNA bands were visualized under a UV transilluminator, and fragment sizes were estimated using a 50 bp DNA ladder.

Statistical Analysis

Data were analyzed using factorial experiments arranged in a completely randomized design (CRD). In the first phase, the effects of genotype and different concentrations of carbon-based adsorbents on androgenesis were evaluated. In the second phase, the combined effects of genotype, adsorbent type, and concentrations of AA and nano-encapsulated AA were assessed. Each treatment was replicated five times, with each Petri dish considered as an independent experimental unit. Data normality was checked using SPSS®, and transformations were applied as needed. Analysis of variance (ANOVA) was conducted using SAS® version 9.4 (SAS Institute Inc., Cary, NC), and mean comparisons were performed using the Least Significant Difference (LSD) test at $P \leq 0.05$.

Results

DAPI staining confirmed the reliability of morphological markers for bud selection.

Flower buds with sepals approximately equal to or slightly shorter than the petals (Fig. 1a) had anthers 5–6 mm long (Fig. 1b) and exhibited faint purple pigmentation at the apical region, particularly on the

distal side facing the petals (Fig. 1c). At this stage, vacuolated microspores (VMs) transitioned to young binucleate pollen (YBP), with nuclei gradually migrating from the central region toward the cell periphery (Fig. 1d).

Effect of carbon-based materials and concentrations on embryo induction and plantlet regeneration in bell pepper

Carbon-based materials and their concentrations significantly affected embryo induction and plantlet regeneration (Table 1). In all genotypes, the number of embryos, embryo induction percentage, and the number of embryos converted to plantlets increased with AC and BC. In NBC, these traits peaked at 1% concentration and then declined. The highest values for embryo number (2), embryogenesis percentage (20%), and regenerated plantlets (1.66) were observed in 1% NBC, whereas the lowest values were recorded in the control. The highest plantlet regeneration percentage was recorded in 1% AC (96.66%). No significant three-way interaction among genotype, carbon source, and concentration was detected, indicating consistent effects across genotypes.

Table 1
Effects of carbon-based materials and their concentrations on embryo induction and plantlet regeneration in bell pepper.

Carbon Source	Concentration (%)	Embryos per Petri dish (mean)	Embryo induction (%) (mean)	Embryos converted to plantlets (mean)	Plantlet regeneration (%) (mean)
AC	Control	0 ^f	0 ^f	0 ^f	0 ^e
	0.5%	0.6 ^{de}	6 ^{de}	0.46 ^{de}	46.66 ^{cd}
	1%	1.4 ^b	14 ^b	1.33 ^a	96.66 ^a
	2%	1.8 ^{ab}	18 ^{ab}	1.33 ^a	72.22 ^{ab}
BC	Control	0 ^f	0 ^f	0 ^f	0 ^e
	0.5%	0.4 ^e	4 ^e	0.26 ^{ef}	26.66 ^d
	1%	0.8 ^{cd}	8 ^{cd}	0.66 ^{cd}	66.66 ^{bc}
	2%	0.93 ^c	9.33 ^c	0.8 ^{bc}	73.3 ^{ab}
NBC	Control	0 ^f	0 ^f	0 ^f	0 ^e
	0.5%	1.46 ^{ab}	14.66 ^{ab}	1.13 ^{ab}	76.66 ^{ab}
	1%	2 ^a	20 ^a	1.46 ^a	75.55 ^{ab}
	2%	1.66 ^{ab}	16.66 ^{ab}	1.33 ^a	73.33 ^{ab}
different letters indicate significant differences among treatments according to LSD ($p \leq 0.05$).					

Synthesis and Characterization of Nanobiochar and Ascorbic Acid Nanocapsules

SEM analysis of the synthesized Nanobiochar revealed irregular, angular nanoparticles with heterogeneous morphology, primarily within the 80–300 nm range, and occasional aggregates up to 500 nm (Fig. 2). BET analysis confirmed a high specific surface area of 362 m² g⁻¹, a total pore volume of 0.28 cm³ g⁻¹, and an average pore diameter of 2.2 nm. Additionally, SEM observations showed that the NEAAs possessed predominantly spherical to slightly elongated shapes with a relatively uniform morphology, and particle sizes mainly distributed between 90 and 160 nm (Fig. 2). BET surface analysis indicated that NEAAs exhibited a specific surface area of 449.6 m² g⁻¹, a total pore volume of 0.39 cm³ g⁻¹, and an average pore diameter of 2.66 nm.

Effects of AA and NEAA concentrations on embryo induction and plantlet regeneration in bell pepper with 1% AC and NBC

In this experiment, 1% AC and NBC were selected based on their superior performance in the previous experiment. The three-way interaction among the studied factors was not statistically significant. However, the type of carbon adsorbent as well as the concentrations of AA and NEAA significantly affected the number of embryos, embryogenesis percentage, the number of embryos converted into plantlets, and the percentage of regenerated plantlets. Increasing the concentration of AA reduced these traits, whereas NEAA showed an increasing trend at lower concentrations followed by a decline at higher levels. The highest mean values for the number of embryos (5), embryogenesis percentage (50%), and the number of regenerated plantlets (4.2) were obtained in the treatment of NBC combined with NEAA at 16.66 mg L⁻¹. Furthermore, the highest regeneration percentage of plantlets was observed when NBC was combined with NEAA at 33.33 mg L⁻¹ (Table 2).

Table 2
Effects of AA and NEAA on embryo induction and plantlet regeneration in bell pepper.

Carbon Source	Treatment (compound and concentration (mgL ⁻¹) (mean)	Embryos per Petri dish (mean)	Embryo induction (%) (mean)	Embryos converted to plantlets (mean)	Plantlet regeneration (%) (mean)
AC	0.5% AA	3.26 ^{cd}	32.66 ^{cd}	2.66 ^{cd}	83.77 ^{abc}
	1% AA	2.66 ^e	22.66 ^e	1.66 ^e	76.11 ^{bc}
	2% AA	0.53 ^g	5.33 ^g	0.53 ^f	53.33 ^d
	3.33% NEAA	3.33 ^c	33.33 ^c	2.66 ^{cd}	82 ^{bc}
	16.66% NEAA	4.2 ^b	42 ^b	3.6 ^b	87.09 ^{abc}
	33.33% NEAA	2.73 ^{de}	27.33 ^{de}	2.4 ^d	89.44 ^{abc}
NBC	0.5% AA	3.66 ^{bc}	36.66 ^{bc}	3 ^c	83 ^{abc}
	1% AA	2.2 ^{ef}	22 ^{ef}	1.6 ^e	74.44 ^c
	2% AA	0.4 ^g	4 ^g	0.13 ^f	13.33 ^e
	3.33% NEAA	3.2 ^{cd}	32 ^e	2.86 ^{cd}	91.77 ^{ab}
	16.66% NEAA	5 ^a	50 ^a	4.2 ^a	86.34 ^{abc}
	33.33% NEAA	1.66 ^f	16.66 ^f	1.66 ^e	100 ^a
different letters indicate significant differences among treatments according to LSD (p ≤ 0.05).					

As illustrated in Fig. 3, anthers obtained from the selected treatments were transferred from culture medium C, supplemented with NBC and NEAA, to medium R (Fig. 3a). The arrows in the Petri dish indicate the formation of embryos, while the magnified panels beside the dish show microscopic views of the developing embryos. Figure 2b demonstrates the germination of the formed embryos, and Fig. 2c shows the regenerated plantlets derived from these embryos. These observations visually confirm the quantitative results reported in Table 2. Embryo induction and plantlet regeneration under different AA treatments.

Effect of genotype and AA / NEAA concentrations on embryo induction and plantlet regeneration in bell pepper

The effects of genotype and different concentrations of AA and NEAA were significant on the number of embryos, embryogenesis percentage, and the number of regenerated plantlets, whereas these factors did not significantly affect the percentage of plantlet regeneration. In all three genotypes, increasing the concentration of AA reduced the measured traits, while NEAA initially increased these traits at lower concentrations, followed by a decline at 33.33 mg L⁻¹. The highest values for embryo number (5.6), embryogenesis percentage (56%), and the number of regenerated plantlets (4.5) were observed in the LIAD genotype treated with NBC combined with NEAA at 16.66 mg L⁻¹ (Table 3).

Table 3

Effects of genotype and different concentrations of AA and NEAA on embryo induction and plantlet regeneration in bell pepper with 1% AC and NBC.

Genotype	Treatment (compound and concentration (mgL ⁻¹) (mean)	Embryos per Petri dish (mean)	Embryo induction (%) (mean)	Embryos covered to plantlets (mean)
"Nirvin F1"	0.5% AC	3.6 ^c	36 ^c	2.8 ^{def}
	1% AC	2 ^d	20 ^{de}	1.5 ^{ij}
	2% AC	0.5 ^f	5 ^f	0.3 ^k
	3.33% NEAA	3.7 ^c	37 ^c	2.9 ^{cde}
	16.66% NEAA	4.5 ^b	45 ^b	3.7 ^b
	33.33% NEAA	2.5 ^d	25 ^d	2.3 ^{efg}
"LIAD F1"	0.5% AC	4.2 ^{bc}	42 ^{bc}	3.5 ^{bc}
	1% AC	2.6 ^d	26 ^d	2.1 ^{ghi}
	2% AC	0.4 ^f	4 ^f	0.2 ^k
	3.33% NEAA	3.5 ^c	35 ^c	3.1 ^{bcd}
	16.66% NEAA	5.6 ^a	56 ^a	4.5 ^a
	33.33% NEAA	2.4 ^d	24 ^e	2.2 ^{fgh}
"Dicapro F1"	0.5% AC	2.6 ^d	26 ^d	2.2 ^{fgh}
	1% AC	2.1 ^d	21 ^e	1.3 ^j
	2% AC	0.5 ^f	5 ^f	0.5 ^k
	3.33% NEAA	2.6 ^d	26 ^d	2.3 ^{efg}
	16.66% NEAA	3.7 ^c	37 ^c	3.5 ^{bc}
	33.33% NEAA	1.7 ^e	17 ^e	1.6 ^{hij}
different letters indicate significant differences among treatments according to LSD (p ≤ 0.05).				

Synthesis and Characterization of Nanobiochar and Nano-encapsulated ascorbic acid

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Flow Cytometric and Cytogenetic Assessment of Ploidy in Regenerated Plants

Flow cytometry combined with cytogenetic analysis was used to verify the ploidy status of regenerated *C. annuum* plants derived from the F1 hybrids (Nirvin, DiCaprio, and Liad). The flow cytometric profile of diploid samples (Fig. 4a) showed a distinct 2C peak, consistent with the diploid chromosome number ($2n = 24$) confirmed by root tip metaphase analysis (Fig. 4b). In contrast, histograms from mixed samples (Fig. 4c) displayed both 1C and 2C peaks, indicating the coexistence of haploid and diploid nuclei. Cytogenetic evaluation of root tips (Fig. 4d) further validated the haploid condition with a chromosome number of $2n = 12$. These results confirm the accuracy and complementarity of flow cytometry and cytogenetic approaches for reliable ploidy determination in *C. annuum*.

Figure 4 Ploidy analysis of regenerated *C. annuum* plants. Flow cytometry histograms showing (a) diploid 2C peak and (b) mixed 1C/2C peaks, complemented by cytogenetic analysis of root tips confirming (c) haploid ($2n = 12$) and (d) diploid ($2n = 24$) chromosome numbers

SSR Marker-Based Assessment of Ploidy Levels in Regenerated *C. annuum* Plants

SSR marker analysis was employed to determine the ploidy level of regenerated *C. annuum* plants. Electrophoresis on 3% Metaphor agarose gel revealed that haploids showed a faint single band, doubled haploids displayed a stronger single band due to homozygosity, and diploids frequently produced two allelic bands, reflecting heterozygosity (Fig. 5).

Discussion

DAPI staining confirmed that morphological markers effectively identify flower buds at the critical transition from VM YBP. Buds with 5–6 mm anthers and faint apical purple pigmentation on the distal side exhibited nuclear migration from the center toward the periphery, indicating active developmental processes and highlighting the reliability of these visual cues for selecting viable microspores and pollen (Parra-Vega et al 2013). These findings emphasize the coordination between morphological features and nuclear status, suggesting that observable traits can enhance the efficiency and accuracy of bud selection while minimizing destructive handling. This approach aligns with previous studies identifying

VM and YBP stages as key indicators for androgenesis, offering a practical framework to optimize microspore culture protocols (Parra-Vega et al 2013).

The present study demonstrates that carbon-based materials and their concentrations significantly influenced embryo induction and plantlet regeneration in bell pepper (Table 1). Across all genotypes, the application of AC and biochar enhanced the number of embryos, embryogenesis percentage, and regenerated plantlets. These findings align with previous studies; for instance, Cheng et al (2013) reported that AC can improve embryogenesis in pepper microspore culture by adsorbing inhibitory metabolites and modulating endogenous hormone balance. In the case of NBC, the most pronounced effects were observed at a 1% concentration, after which embryo induction and plantlet regeneration declined. The highest embryo number (2), embryogenesis percentage (20%), and regenerated plantlets (1.66) were recorded at 1% NBC, while the lowest values were observed in the control. These results suggest that NBC may act as a novel elicitor in plant tissue culture, potentially through enhanced availability of active carbon and its effects on endogenous phytohormones and cellular carbohydrate levels (Wiszniewska et al 2024; Chaubey et al 2024). Notably, the highest plantlet regeneration percentage was obtained with 1% AC (96.66%), highlighting the positive role of metabolite adsorption and improved physical properties of the culture medium (Kim et al 2008). These findings are also consistent with reports indicating that nanoparticles and NBC s can serve as innovative elicitors in tissue culture, enhancing embryogenesis and regeneration by modulating physiological and hormonal responses (Singh et al 2023). The lack of a significant three-way interaction among genotype, carbon source, and concentration suggests a relatively uniform effect of carbon amendments across genotypes. This observation is valuable for optimizing haploid induction protocols and doubled haploid production in bell pepper. Overall, the study demonstrates that both NBC and AC can serve as effective tools for improving microspore culture efficiency and generating robust plantlets, in agreement with findings from diverse plant species.

NBC with NEAA significantly enhanced embryo induction and plantlet regeneration in bell pepper (Tables 2 and 3). The highest embryo numbers (5–5.6), embryogenesis percentages (50–56%), and regenerated plantlets (4.2–4.5) were observed in the treatment combining NBC with NEAA at 16.66 mg L⁻¹. Moreover, the highest plantlet regeneration percentage was achieved with 33.33 mg L⁻¹ NEAA combined with NBC. This trend indicates that nano-encapsulation enables ascorbic acid to exert positive effects on embryogenesis and plantlet regeneration at lower concentrations while mitigating inhibitory effects observed at higher concentrations of free AA (Heidari-Zefreh et al 2019). The scientific rationale for using NEAA includes enhanced stability, reduced oxidation, controlled release, and sustained activity throughout the culture period (Sampedro-Guerrero et al 2023). Free AA is prone to rapid oxidation and can exert inhibitory effects on cell division and embryogenesis at high concentrations. In contrast, nano-encapsulation protects AA molecules from oxidation, improves cellular uptake, and maintains bioactivity within the culture medium. NBC also played a crucial role in enhancing embryogenesis and plantlet regeneration. Its high surface area, adsorption capacity, and ability to modulate nutrient and hormone availability have been shown to improve growth and morphogenesis in tissue culture systems (Sani et al 2023). These results align with previous studies indicating that combining carbon-based elicitors with

controlled antioxidants can substantially improve embryogenesis and regeneration efficiency in bell pepper (Olszewska et al 2021). Additionally, the effects of NBC and NEAA were relatively consistent across different genotypes, which is valuable for optimizing haploid induction protocols and doubled haploid production in bell pepper. Overall, the simultaneous application of NBC and NEAA represents an effective strategy to enhance microspore culture efficiency and generate robust, healthy plantlets. These findings highlight the potential of integrating nanotechnology-based delivery systems with carbon-based culture media to improve plant tissue culture outcomes (Singh et al 2023).

The rough and porous texture of the Nanobiochar reflects structural disruption and defect formation induced by high-energy milling, while slight agglomeration results from van der Waals interactions typical of carbon materials. The high specific surface area and microporous architecture suggest favorable properties for adsorption and redox applications (Naghdi et al 2017). The predominantly smooth and uniform morphology of NEAAs indicates successful internal encapsulation of ascorbic acid within the silica framework rather than surface adsorption. The mesoporous network, partially occupied by encapsulated AA molecules, confirms the formation of a stable encapsulated structure, which may facilitate controlled release of the active compound (Weisany et al 2024).

Flow Cytometric and Cytogenetic Assessment of Ploidy in Regenerated Plants: Flow cytometry combined with cytogenetic analysis was used to verify the ploidy status of regenerated *C. annuum* plants derived from the F1 hybrids (Nirvin, Dicaprio, and LIAD). The flow cytometric profile of diploid samples (Fig. 4a) showed a distinct 2C peak, consistent with the diploid chromosome number ($2n = 24$) confirmed by root tip metaphase analysis (Fig. 4d). In contrast, histograms from mixed samples (Fig. 4b) displayed both 1C and 2C peaks, indicating the coexistence of haploid and diploid nuclei. Cytogenetic evaluation of root tips (Fig. 4c) further validated the haploid condition with a chromosome number of $2n = 12$. These results confirm the accuracy and complementarity of flow cytometry and cytogenetic approaches for reliable ploidy determination in *C. annuum*. SSR marker analysis was employed to determine the ploidy level of regenerated *C. annuum* plants. Electrophoresis on 3% Metaphor agarose gel revealed that haploids showed a faint single band, doubled haploids displayed a stronger single band due to homozygosity, and diploids frequently produced two allelic bands, reflecting heterozygosity (Fig. 5). The ploidy status of regenerated *C. annuum* plants was accurately determined by integrating flow cytometry, cytogenetic observations, and molecular marker analysis. Flow cytometric data revealed distinct nuclear DNA peaks at 1C and 2C, allowing clear discrimination between haploid and diploid individuals. Complementary cytological analysis of mitotic chromosomes confirmed chromosome numbers of $2n = 12$ in haploids and $2n = 24$ in diploids, verifying the ploidy assignments. Instances of spontaneous genome doubling were observed among regenerated lines, consistent with earlier studies (Park et al 2024), suggesting a natural alternative to chemical chromosome-doubling agents such as colchicine. Although flow cytometry provides rapid DNA content estimates, it cannot reliably differentiate homozygous doubled haploids from heterozygous diploids when both share the same ploidy level. To overcome this limitation, codominant SSR markers were employed as a complementary tool. In particular, the HPMS1-117 marker consistently exhibited single-band patterns in electrophoresis, confirming the homozygosity of selected doubled haploid lines. This molecular verification reinforces the

accuracy of ploidy determination and aligns with current strategies reported in the literature (Kaushik et al 2025). Moreover, combining multiple molecular marker systems, such as SSR, RAPD, and ISSR, has been shown to be effective for evaluating genetic uniformity and purity in doubled haploid lines across plant species (Gemesne et al 2001; Keleş et al 2015). The integration of nuclear DNA quantification, chromosomal assessment, and marker-based genotyping provides a robust and comprehensive approach for doubled haploid identification, supporting accelerated breeding programs and ensuring genetic stability in regenerated populations (Keleş et al 2015; Pareeth 2015).

Limitations

Although the combined application of Nanobiochar and Nano-encapsulated ascorbic acid enhanced androgenesis, the exact molecular and physiological mechanisms behind this synergy remain unclear. Future studies employing transcriptomic, proteomic, or metabolomic analyses are necessary to elucidate how these nanomaterials influence cellular pathways during microspore culture. Additionally, potential cytotoxic and phytotoxic effects of nanoparticles, including Nanobiochar and NEAA, must be carefully evaluated, as higher concentrations were observed to diminish embryogenic responses, possibly by disrupting hormonal balance or triggering oxidative stress. The long-term consequences of nanoparticle exposure on genome integrity, epigenetic regulation, and overall plant physiology are also not yet understood and warrant systematic investigation. Finally, spontaneous chromosome doubling occurred irregularly among genotypes, limiting the predictability and reproducibility of doubled haploid recovery, and emphasizing the need for more controlled and consistent strategies for chromosome doubling in breeding programs.

Conclusion

The findings of this study demonstrate that the integration of NBC with NEAA effectively enhances embryo induction and plantlet regeneration in bell pepper, facilitating the production of homozygous doubled haploids. However, the precise molecular and physiological mechanisms underlying these effects remain unclear, and higher concentrations may reduce embryogenic efficiency or induce oxidative stress. Additionally, spontaneous chromosome doubling occurred inconsistently across genotypes, highlighting the need for controlled strategies to improve predictability and reproducibility in doubled haploid production. Overall, this study provides a reliable approach to accelerate breeding programs and develop genetically uniform lines in bell pepper, while future research is needed to elucidate cellular mechanisms and assess the long-term effects of nanoparticle exposure.

Abbreviations

AC
Activated Charcoal
AA
Ascorbic Acid

NEAA
Nano–Encapsulated Ascorbic Acid
BC
Biochar
CRD
Completely Randomized Design
DH
Doubled Haploid
DAPI
4',6–Diamidino–2–Phenylindole
F1
First Filial Generation
LSD
Least Significant Difference
MS
Murashige and Skoog Medium
NBC
Nanobiochar
PCR
Polymerase Chain Reaction
R
Regeneration Medium
SE
Standard Error
SSR
Simple Sequence Repeat
VM
Vacuolated Microspore
YBP
Young Binucleate Pollen

Declarations

Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Ethics declaration

not applicable.

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Authors' contributions:

JP and MES conceived of the presented idea and planned the experiments. MHS developed the theory, conducted the research experiments, and wrote the manuscript with the support of all co-authors. MES prepared the project proposal and managed the budgeting. JP and MES supervised the project, with the support of AMA and MH. 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.

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Figures

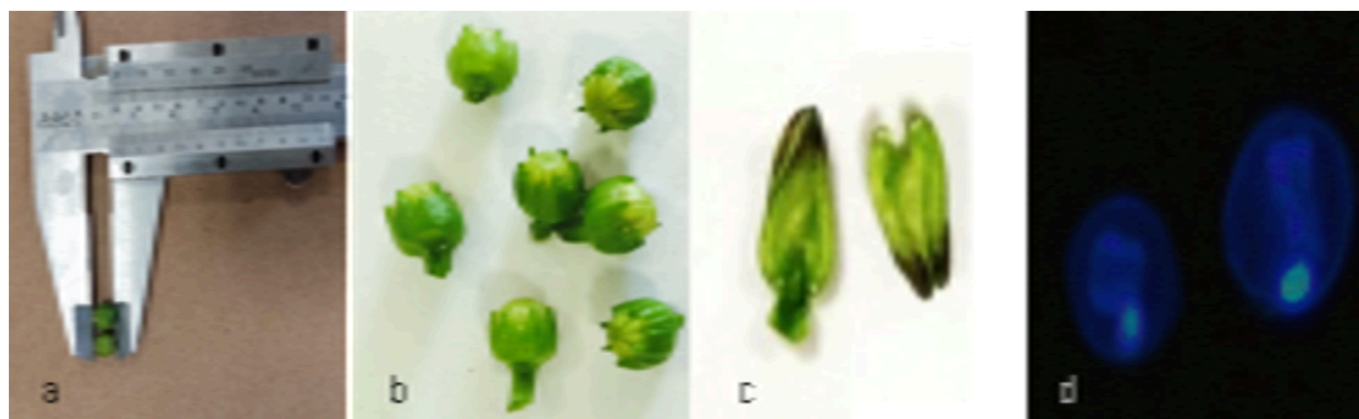


Figure 1

Morphological features of bell pepper buds and anthers at the optimal stage for androgenesis. Legend: (a) Flower buds with sepals slightly shorter than petals. (b) Anther length: 5–6 mm. (c) Faint purple pigmentation at anther apex. (d) Vacuolated microspores (VMs) transitioning to young binucleate pollen (YBP), with nuclei migrating toward the cell periphery

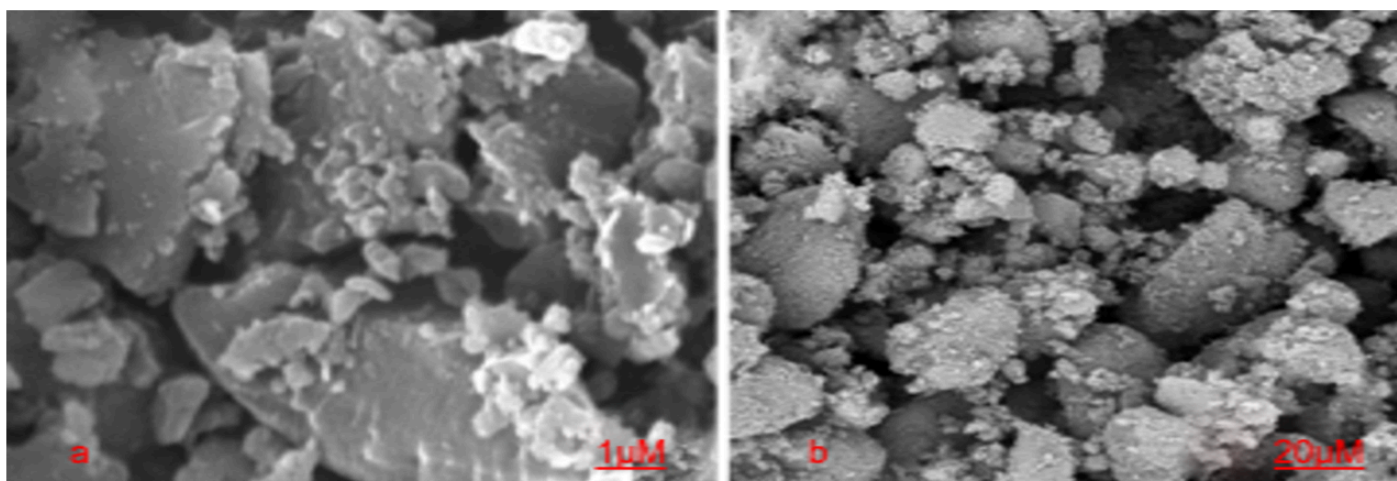


Figure 2

Title: SEM images of synthesized nanomaterials. Caption: (A) NBC nanoparticles exhibiting irregular, angular morphology (80–300 nm) with occasional aggregates up to 500 nm. The rough, porous surface reflects structural disruption from high-energy milling. (B) NEAAs nanoparticles showing predominantly spherical to slightly elongated shapes (90–160 nm) with smooth surfaces, indicating successful internal encapsulation of AA within the silica framework

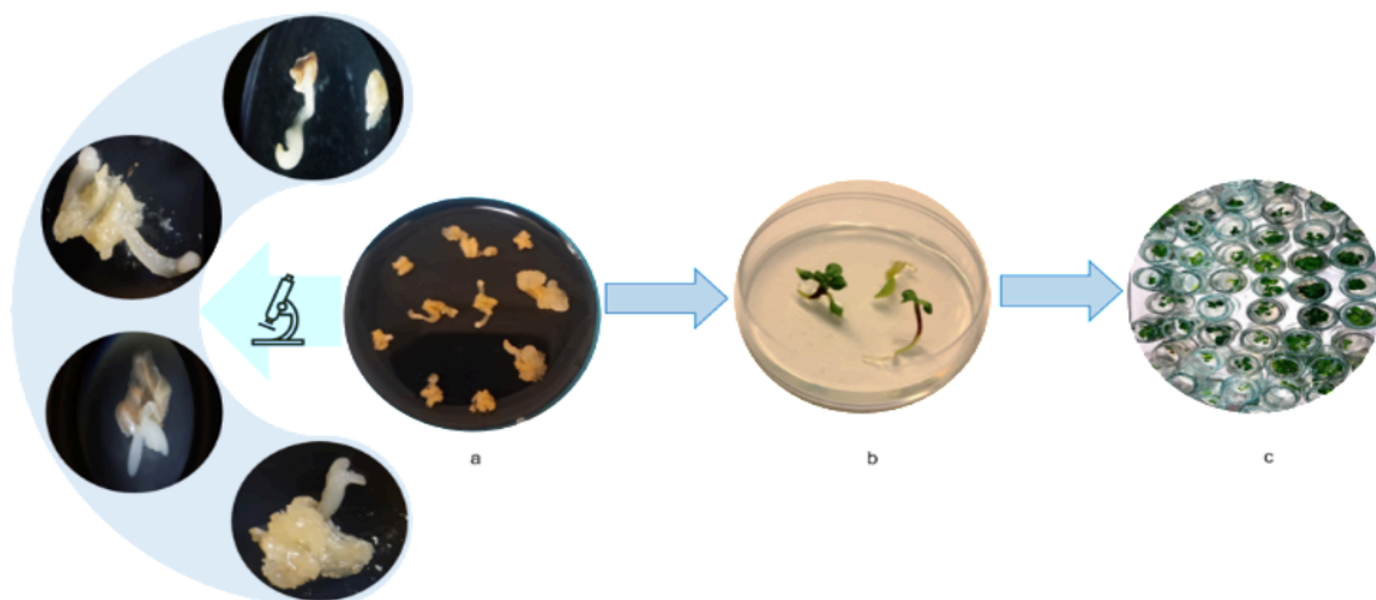
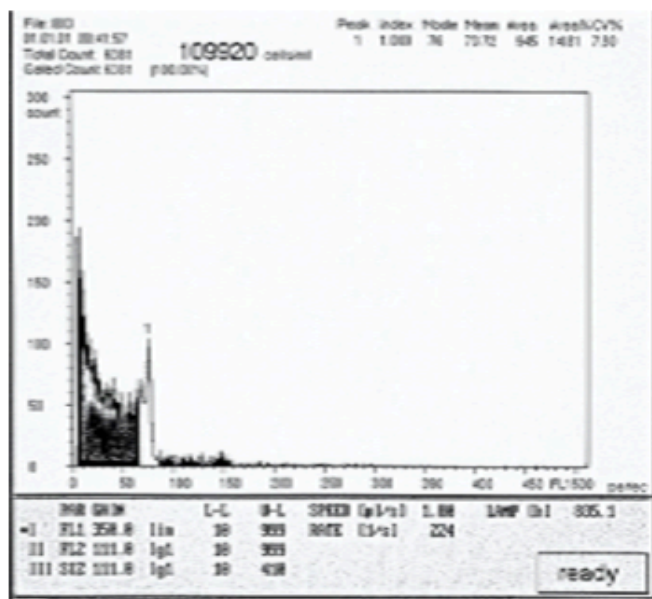
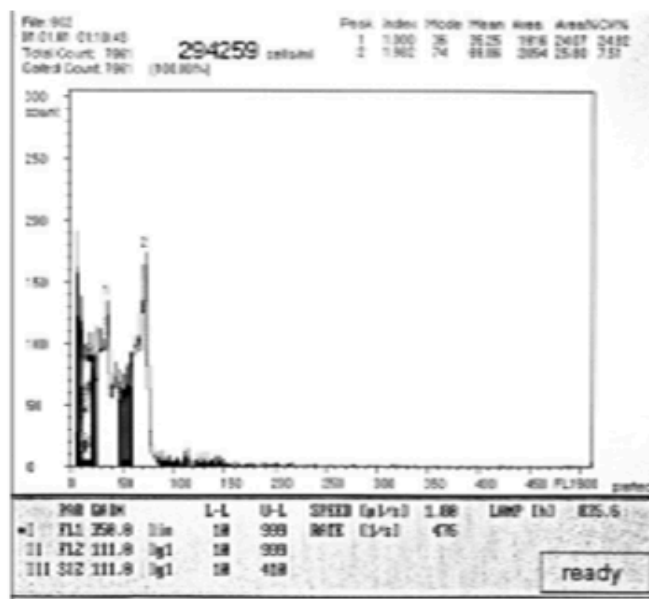


Figure 3

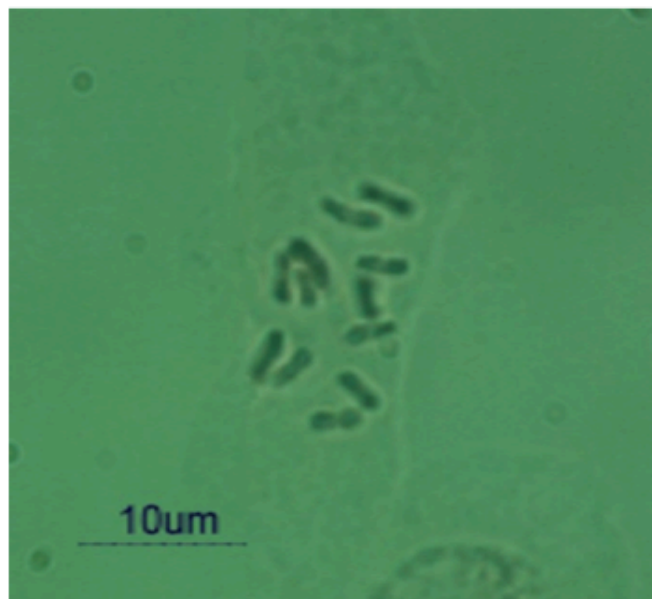
Embryos and regenerated plantlets in bell pepper: a. Embryos from anthers transferred from medium C with NBC and NEAA to medium R containing 0.1 mg L⁻¹ kinetin. b. Embryo germination on MS medium without growth regulators. c. Regenerated plantlets on MS medium without growth regulators



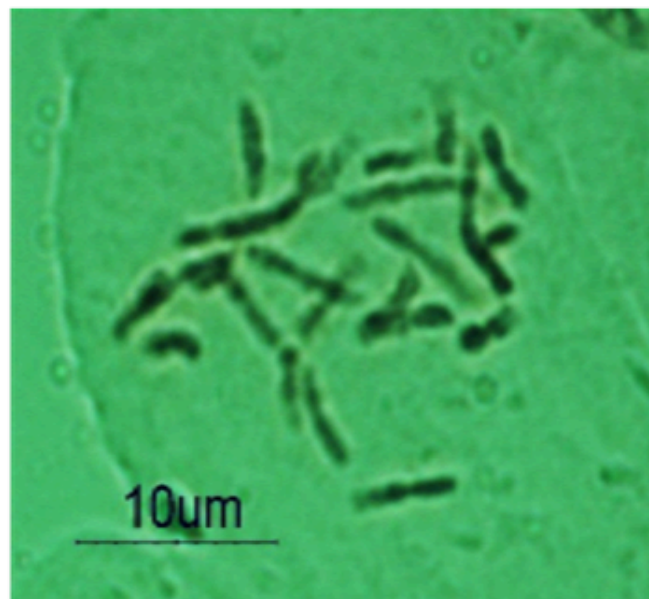
a



b



c



d

Figure 4

Ploidy analysis of regenerated *C. annuum* plants. Flow cytometry histograms showing (a) diploid 2C peak and (b) mixed 1C/2C peaks, complemented by cytogenetic analysis of root tips confirming (c) haploid ($2n = 12$) and (d) diploid ($2n = 24$) chromosome numbers

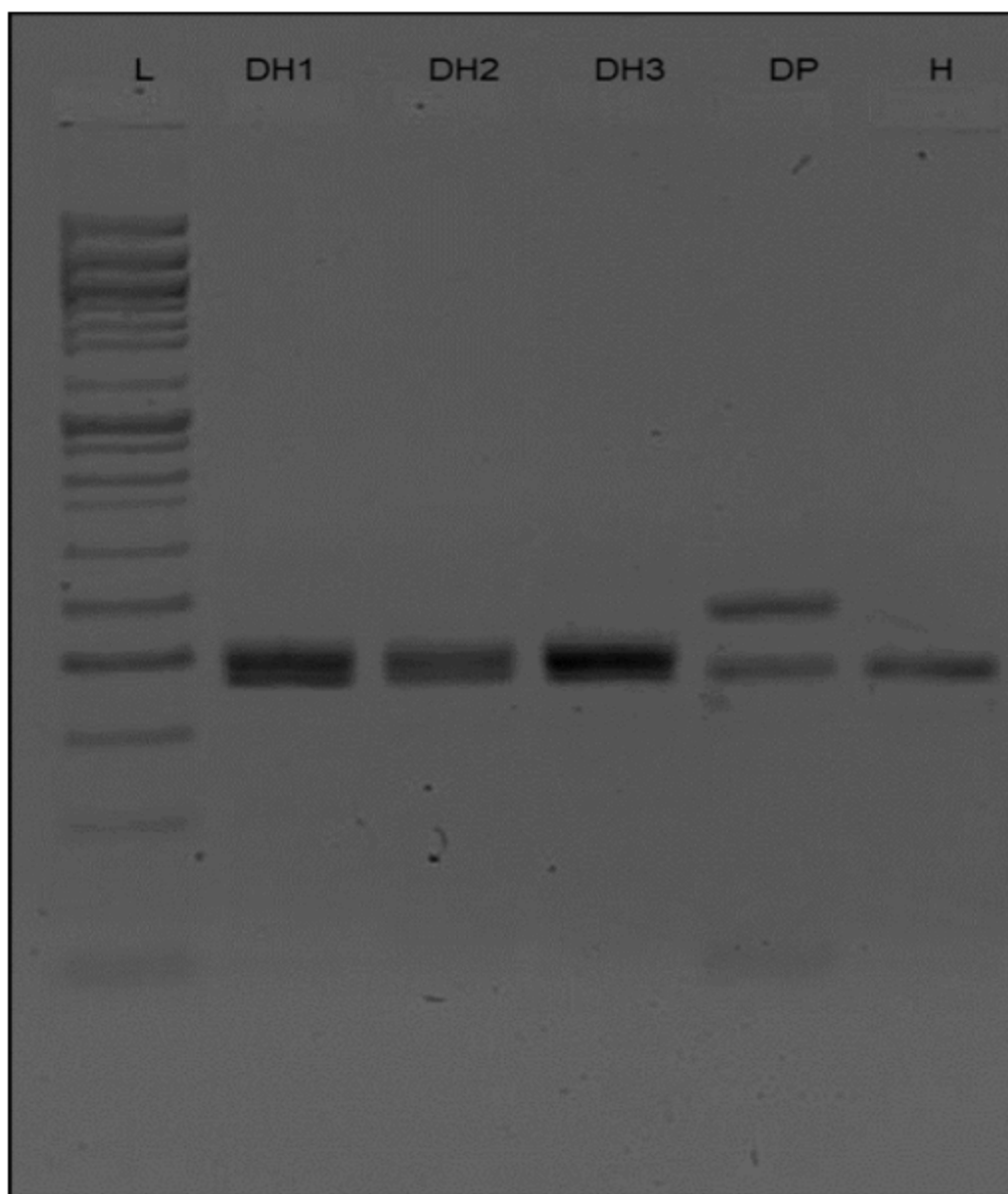


Figure 5

SSR marker profiles on a 3% Metaphor agarose gel for ploidy level identification in *C. annuum* . Lane L: DNA ladder (50 bp); Lanes DH1–DH3: doubled haploids; Lane DP: diploid; Lane H: haploid