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RESEARCH ARTICLE

Optimizing hardening substrates and homozygosity testing for anther culture derived doubled haploid sweet pepper (*Capsicum annuum* var. *grossum* L.) lines

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Abstract

Sweet pepper (*Capsicum annuum var. grossum* L.), a valuable vegetable crop, faces significant challenges in large-scale doubled haploid (DH) production due to high mortality rates during the hardening phase. This study aimed to optimize substrate conditions for successful DH hardening and to validate genetic uniformity using molecular markers. The research focused on four genotypes of sweet pepper: Asha, Sympathy, Nemalite and Indra. Various substrate combinations were evaluated, including coco peat, perlite, vermiculite, farmyard manure (FYM) and vermicompost. The results show that substrates containing coco peat, perlite, vermiculite and vermicompost consistently achieved high survival rates and superior growth parameters. Specifically, substrate T_6 (coco peat + perlite + vermiculite + vermicompost, (1:1:1:1)) achieved the highest survival rate, with the Asha genotype reaching 97.64%. This substrate also promoted optimal plant height and leaf production, with Asha reaching 11.75 cm and producing 14.5 leaves on average. Homozygosity testing using SSR markers (CAM-8, CAM-19, CAM-54, CAM-79) confirmed that 447 (97.0%) of the 461 DH plants were true homozygous lines, with minimal diploid escapees across all genotypes. Among the genotypes, Indra exhibited the highest efficiency, with 98.4% of plants identified as true DH lines. Chi-square analysis showed no significant deviation from expected Mendelian ratios, affirming genetic stability. These findings highlight the importance of substrate optimization in enhancing the acclimatization and survival of DH lines and demonstrate the effectiveness of SSR markers in ensuring genetic uniformity. This study provides valuable insights for improving DH production protocols in sweet pepper breeding programs.

Keywords

substrate; vermicompost; hardening; doubled haploid (DH); survival frequency

Introduction

Sweet pepper (*capsicum annuum var. grossum* L.)(Fig.1), a vegetable and spice crop with a chromosome count of 2n = 24, belongs to the Solanaceae family and is believed to have originated in South Central America, later being domesticated in Mexico(1). This crop was introduced to India by the Portuguese in the late 15th century and has been cultivated in the region for approximately 400 years (2). In India, the cultivated species include *Capsicum annuum*, bell peppers and chillies and *Capsicum frutescens*, known

Fig. 1. Sweet pepper (*capsicum annuum var. grossum* L.) with fruits.

for bird's eye chilli and tabasco varieties (3). Sweet pepper, scientifically known as *Capsicum annuum var. grossum*, is native to Mexico, Central America and northern South America (4). Appreciated globally for its large, bell-shaped, non-pungent fruits, it is a vital cash crop widely used in continental cuisine (5). The fruits, available in various colours such as red, yellow and orange when ripe and green, white and purple when unripe, are celebrated for their rich flavour and aroma. It is an excellent source of provitamin A, vitamin C and essential minerals (6–7). High demand for stress-tolerant and high-yielding sweet pepper cultivars has promoted the use of anther culture in breeding programs. This technique enables the rapid development of doubled haploid (DH) plants, achieving complete homozygosity in a single generation. Compared to traditional breeding, which requires six to seven generations of self-fertilization, anther culture significantly reduces the time and cost involved (8–9). Anther culture offers several advantages, including enhanced genetic uniformity, improved stress tolerance and the swift development of new cultivars with desirable traits such as better fruit quality and disease resistance (10). However, challenges persist. Sweet pepper is recalcitrant to androgenesis, often exhibiting low efficiency in generating haploid embryos. Other issues include abnormal embryo development, low regeneration frequency and poor survival rates of plantlets during hardening and acclimatization (11–13).

Hardening, the final tissue culture stage, is crucial for ensuring successful plant acclimatization (14). Acclimatization involves transitioning plantlets from *in vitro* to *in vivo* conditions, essential for their survival and growth in natural environments. However, the large-scale application of doubled haploid technology faces challenges, with high mortality during the transfer phase hinders successful acclimatization (15). *In vitro* plantlets are cultivated under specific conditions, such as sealed containers with high humidity, low irradiance and minimal air turbulence. These factors increase the leaf boundary layer, restrict CO_2 exchange and expose plantlets to risks such as contamination from saccharides in the medium and morphological abnormalities caused by high concentrations of growth regulators (16–20). The transfer from *in vitro* to *ex vitro* environments is particularly challenging due to stark differences between controlled conditions and natural greenhouse or field environments. Higher irradiance and lower air humidity in *ex vitro* conditions often lead to rapid wilting caused by excessive water loss (21). Also, roots' low hydraulic conductivity limits water uptake, exacerbating acclimatization difficulties. Gradual exposure to reduced humidity over several weeks enables plantlets to adjust to external conditions without experiencing water stress (22–25) in many plant species, including *Liquidambar styraciflua*, *Hedera helix*, *Brassica oleracea*, *Malus domestica*, *Rubus idaeus*, *Vaccinium corymbosum* and *Nicotiana tabacum*, leaves formed in vitroitro exhibit limited development when transferred to *ex vitro* conditions (21). Stomatal characteristics undergo significant changes during acclimatization, such as reduced stomatal density and alterations in stomatal size and morphology. These changes and increased leaf thickness and mesophyll differentiation contribute to water potential stabilization (22, 26–28). The growth medium plays a crucial role in the success of hardening. Optimal media should provide essential characteristics for root development and overall plantlet health.

However, plantlet roots from tissue culture often exhibit underdeveloped root systems with limited root hairs, reducing their efficiency upon transplantation. Compatibility between plantlet type and medium is vital, as various substrates, including rice husk charcoal, cocopeat, sphagnum moss, wood charcoal, zeolite, cadaka root and coconut husk, have been reported to influence plantlet growth differently (22, 29–31). For instance, zeolite rock media supports the development of *Vanda sanderiana* orchids, while *Anthurium sp.* thrives in mixtures of rice husk charcoal, cocopeat and fern (32, 33). Acclimatization is often associated with high mortality rates, as observed in crops like *Anthurium sp.*, *Dendrobium sp.*, *Carica papaya L.*, black orchids and mango (29, 34–35). However, acclimatization has been achieved using peat moss for chilli pepper plantlets (36) and a perlite-soil mixture for *Capsicum chinense Jacq.* Plants (37). Connect this sentence with molecular part which is written in new new paragraph starting from - Molecular markers.

Molecular markers, particularly PCR-based techniques, are increasingly employed for genetic analysis and cultivar differentiation of DH plants (39–41). PCR analysis has revealed significant genetic diversity among DH-R2 plants, emphasizing the importance of these techniques in identifying meiotic recombinants with altered genome constitutions for developing new genotypes in crops like pepper (41).SSR markers, such as Hpms1-117, have been instrumental in identifying homozygous DH plants, con-

firming genetic uniformity and distinguishing between different lines (42). Ensuring homozygosity in DH plants is crucial for consistent trait expression and reliable performance in breeding programs (38). The origin of each plant, confirmed as diploid (2n) by flow cytometry, must be investigated using markers to rule out the possibility of somatic embryo occurrence, which, although rare, can compromise the creation of true DH lines. Molecular marker technology is widely employed in plant breeding to select individual plants based on genotype, with PCR-based markers being the most commonly used (44). Codominant markers are preferred over dominant ones due to their ability to distinguish clearly between homozygous and heterozygous individuals. High-throughput analysis favours robust PCR markers with high reproducibility.

This study aims to evaluate the effectiveness of specific substrates in enhancing the hardening success rate of doubled haploid lines of sweet pepper generated through anther culture. Additionally, it assesses the application of molecular markers to ensure genetic uniformity in these lines.

Materials and Methods

Materials and instrumentation

The substrates utilized during the hardening phase included sand, coco peat, perlite, vermiculite (all sourced from Keltech Energies Limited), farmyard manure (FYM) and vermicompost from local suppliers. A 1/10 dilution of Hoagland solution (HiMedia) and N:P:K fertilizer (19:19:19) (Shriram) were also used. The entire process was conducted in a greenhouse under controlled conditions, facilitating a successful transition from *in vitro* to *ex vitro* environments.

Chemicals used for molecular studies comprised CTAB (Sigma), NaCl (HiMedia), EDTA (Sigma), Tris-HCl (SRL), 2-mercaptoethanol (Qualigens), chloroform, isoamyl alcohol (Qualigens), PVP (HiMedia) and boric acid (HiMedia). Molecular work also incorporated primers (Eurofins), dNTPs (Sigma-Aldrich), Taq polymerase and $MgCl_2$ (Kapa Biosystems PCR Reagents), along with agarose (HiMedia) and TBA buffer (Thermo Fisher). Ethidium bromide (Sigma-Aldrich) was used for DNA visualization. The instruments used in the study included a tissue lyser (Qiagen), water bath (Remi), centrifuge (Eppendorf 5810R), thermal cycler (BIO-RAD T100), gel electrophoresis equipment (Thermo Scientific) and a BIO-RAD Geldoc Go gel imaging system with Image Lab Touch software. Additional equipment included a vortex mixer (Neuation) and a deep freezer (-20°C) (Blue Star). These instruments provided the precision and reliability required for the experiments.

Plant material

The current study was conducted at the Temperate Breeding and Research Station under the supervision of ACSEN Agriscience Pvt. Ltd., located in Kullu, Himachal Pradesh, India. Doubled haploid (DH) lines of sweet pepper genotypes, Asha, Sympathy, Nemalite and Indra, were generated in our previous research (45). These lines now serve as the current experiments' source material, as listed in Table 1.

Table 1. Number of doubled haploid (DH) obtained.

Genotype	DH
Asha	129
Sympathy	32
Nemalite	114
Indra	186
Total	461

DH-doubled haploid.

Hardening

The DH population was cloned to ensure uniformity, with plants possessing 3-4 leaves selected for standardization. The genetic uniformity facilitated consistent treatment applications during subsequent experimental stages. The hardening process was designed to transition the DH lines from the controlled tissue culture environment to the variable conditions necessary for successful adaptation and plantlet survival. Fig. 2 depicts the entire hardening process. During hardening, *in vitro*-rooted plants from the DH lines were delicately removed from tissue culture boxes. Roots were trimmed uniformly to a standardized length of 10-11 mm, followed by gentle washing to eliminate residual agar. The DH lines were then transplanted into potting mixtures comprising different substrate combinations, as shown in Table 2. Fertigation practices were consistently applied, using a nutrient solution diluted to 1/10 strength Hoagland solution. The hardening environment was optimized with initial humidity levels set at 90%, gradually reduced to 60% over seven days by creating openings in transparent glass covers. Temperature and light conditions were maintained at 25±2°C and 4000–5000 lux, respectively. The nutrient application involved administering an N:P: K (19:19:19) mix at a concentration of 1 gram per liter every ten days to all hardened plants. This standardized regimen supported robust growth and acclimatization of the DH lines. After one month of hardening, the efficacy of different substrates was evaluated based on the average survival rate in both replicates. Key morphological indicators of plant health, including plant height and the number of leaves, were meticulously measured.

Homozygosity testing

461 anther culture-derived sweet pepper plantlets were used for SSR marker identification, including autodiploidized and colchicine/triflurin-induced doubled haploids (DHs). Over 200 SSR markers derived from the pepper genome (Sol Genom network) were tested and CAM-8, CAM-19, CAM-54 and CAM-79 were selected based on their explicit polymorphism. Fresh leaves were collected in the early morning for DNA isolation, following the method described with modifications (46). The leaves were washed, surface-sterilized and stored at -20°C before grinding with a genomic DNA extraction buffer containing CTAB, NaCl, EDTA, Tris-HCl and 2-mercaptoethanol. After incubation and centrifugation, DNA was purified using chloroform-isoamyl alcohol, precipitated with isopropyl alcohol,

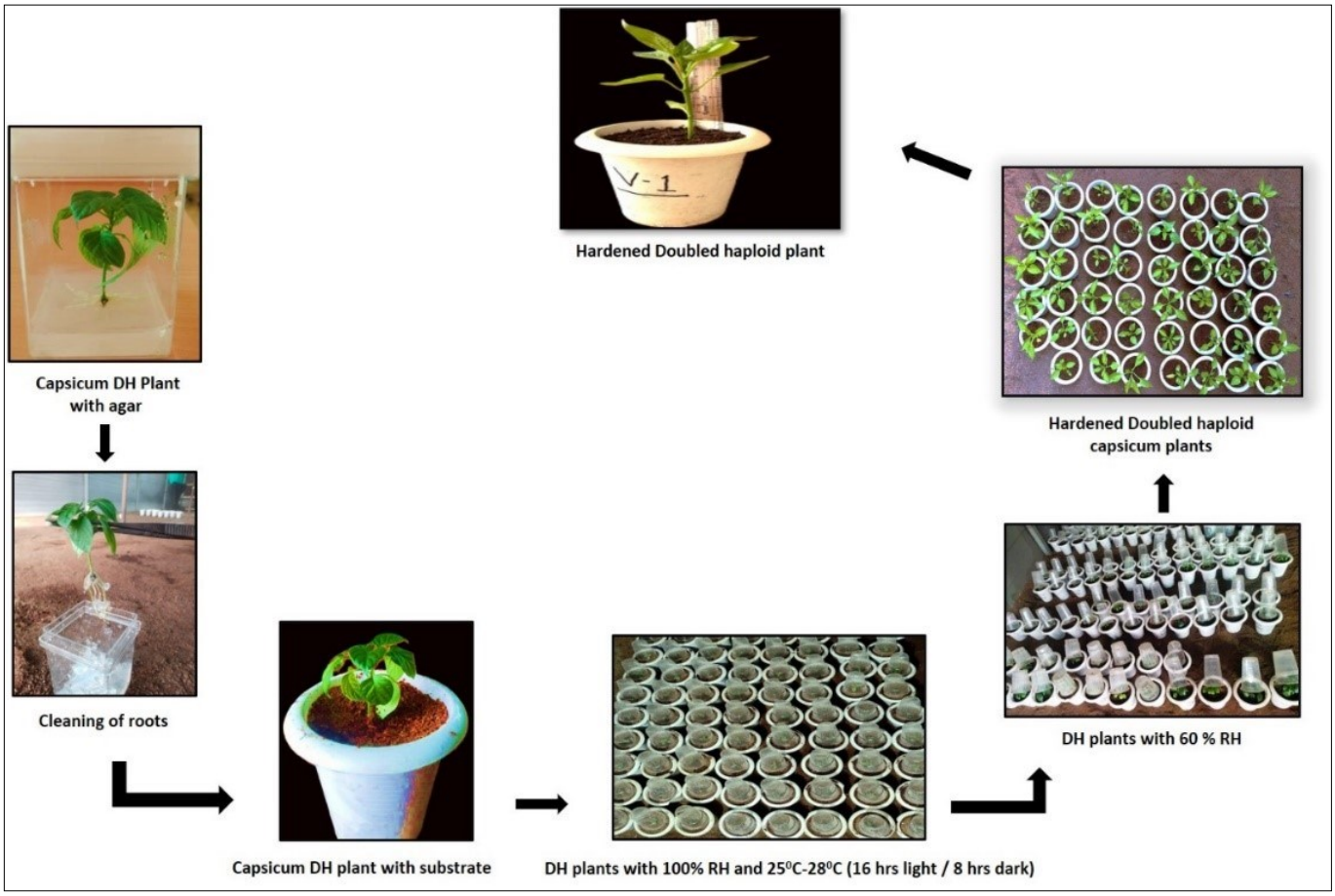

Fig. 2. Flowchart of hardening of capsicum doubled haploid plants.

Table 2. Substrate compositions for hardening sweet pepper DH lines.

S. No	Treatment	Substrate	Ratio
1	T_1	Sand	100
2	T_2	Coco peat	100
3	T_3	Coco peat + Perlite	1:1
4	T_4	Coco peat + Vermiculite	1:1
5	T_5	Cocopeat + Perlite + Vermiculite + FYM	1:1:1:1
6	T_6	Cocopeat + Perlite + Vermiculite +Vermicompost	1:1:1:1

washed and air-dried. DNA quality was assessed via gel electrophoresis, followed by PCR amplification. The PCR reaction mixture consisted of 2µl of 50 ng/µl genomic DNA, 1µl of 50mM $MgCl_2$, 0.8µl of 10mM deoxyribonucleotide triphosphate mix, 1U/ µl of TaqDNA polymerase, 17.7µl of 1x PCR buffer, 1.5µl each forward and reverse primers (10µmol/L). It was performed using a BIO-Rad T-100 Master cycler under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 10 minutes. The amplified products were separated on 2.5% agarose gels and visualized using the BIO-Rad gel doc go gel imaging system, with image lab touch software for analysis.

Statistical analysis

The experimental data was analyzed using analysis of variance (ANOVA) with the OPSTAT software, followed by Duncan's multiple range tests performed in the SPSS-16 program for Windows. A chi-square test was also performed to assess the distribution of doubled haploid (DH) lines among the four genotypes.

Results

Hardening

The study examined the performance of doubled haploids from four distinct genotypes, namely Asha, Sympathy, Nemalite and Indra, across six different substrates, each presenting varying compositions. Fig. 3 illustrates the initial hardening and growth parameters, including survival rates, average plant height and leaf production. Table 3 provides a detailed analysis of the impact of different substrates on each genotype's survival rates, average plant height and leaf production. The table highlights the best-performing substrates for each genotype based on these growth and development parameters, offering insights into the most effective substrate compositions for optimal plant performance.

Effect of different substrates on survival frequency (%)

Survival frequency varied significantly across different substrates and genotypes. The Asha genotype's survival frequencies ranged from 18.50% with T_1 (sand) to 97.64% with T_6 (cocopeat, perlite, vermiculite, vermicompost). The Sympathy genotype exhibited a survival rate ranging from 12.08% with T_1 to 62.36% with T_6. Similarly, Nemalite's survival frequencies varied from 7.65% with T_1 to 74.49% with T_6. Indra showed survival frequencies ranging from

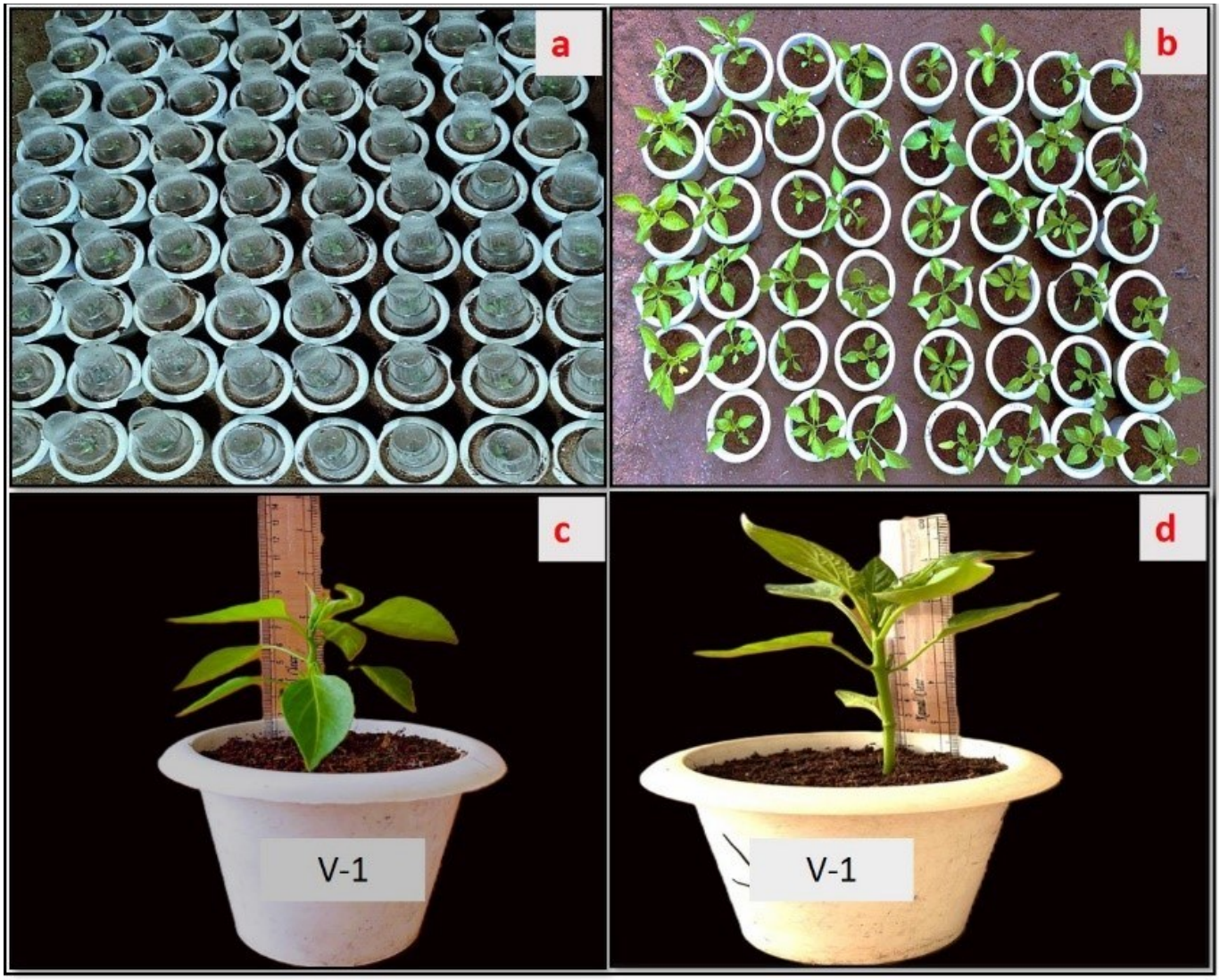

Fig. 3. Process of hardening. (**a**) The beginning phase on day 1 (**b**) the development of hardened plantlets after 15 days. The subsequent examination of different growth parameters (**c**) plant height (**d**) number of leaves.

13.46% with T_1 to 82.69% with T_6. Across all genotypes, the

Table 3. Hardening of DH tissue cultured plantlets of sweet pepper using various substrates

Genotype	Substrate	Survival frequency (%)	Plant height (Cm)	No. of leaves
Asha	T_1	18.50 ±1.18^{l}	3.05±0.45^{i}	4.0±0.00^{jkl}
	T_2	34.65±1.57^{j}	4.00±0.50^{hi}	4.5±0.50^{ijkl}
	T_3	46.85±0.39^{h}	4.05±0.05^{hi}	6.5±0.50e^{fghi}
	T_4	53.13±0.39f^{g}	6.00±0.00^{efgh}	7.0±0.00^{efgh}
	T_5	89.37±0.39^{b}	9.50±0.50^{b}	11.5±0.50^{b}
	T_6	97.64±0.79^{a}	11.75±0.75^{a}	14.5±0.50^{a}
Sympathy	T_1	12.08±0.28^{m}	3.00±0.00^{i}	3.0±0.00^{l}
	T_2	20.51±0.84^{l}	4.50±0.50^{ghi}	3.5±0.50^{kl}
	T_3	39.89±1.12^{i}	5.00±0.00^{fghi}	5.0±0.00^{hijkl}
	T_4	42.7±0.56^{i}	6.50±0.50^{defg}	5.0±0.00^{hijkl}
	T_5	51.4±0.28^{g}	8.50±0.50^{bcd}	8.0±0.00^{def}
	T_6	62.36±0.56^{e}	9.50±0.50^{b}	10.0±1.00^{bc}
Nemalite	T_1	7.65±0.51^{n}	4.00±1.00^{hi}	4.5±1.50^{ijkl}
	T_2	28.06±0.51^{k}	5.00±1.00^{fghi}	5.0±0.00^{hijkl}
	T_3	53.57±0.51^{fg}	6.00±0.00^{efgh}	6.5±0.50^{efghi}
	T_4	56.12±1.02^{f}	6.00±0.00^{efgh}	7.5±0.50^{efg}
	T_5	61.73±0.51^{e}	7.00±1.00^{cdef}	10.0±1.00^{bc}
	T_6	74.49±1.02^{d}	9.00±1.00^{bc}	9.5±0.50^{cd}

Indra	T_1	13.46±1.92^{m}	4.25±0.25^{hi}	4.0±0.00^{jkl}
	T_2	32.69±1.92^{j}	5.75±0.75^{fgh}	5.5±0.50^{ghijk}
	T_3	51.92±1.92^{g}	8.00±0.00^{bcde}	6.0±0.00^{fghij}
	T_4	55.77±1.92^{f}	8.50±0.50^{bcd}	6.0±1.00^{fghij}
	T_5	80.77±0.00^{c}	8.50±1.50^{bcd}	7.0±1.00^{efgh}
	T_6	82.69±1.92^{c}	10.00±1.00^{ab}	8.5±0.50^{cde}

Values are mean ± sd. Different letters (a-n) within the same column indicate significant differences by duncans' multiple range test (dmrt), p ≤ 0.05.

T_6 substrate consistently resulted in the highest survival frequencies, underscoring its effectiveness in enhancing plant survival.

Effect of different substrates on plant height (cm)

Plant height was also significantly influenced by substrate type. For the Asha genotype, plant heights ranged from 3.05 cm with T_1 (sand) to 11.75 cm with T_6 (cocopeat, perlite, vermiculite and vermicompost). Sympathy plants exhibited heights from 3.00 cm with T_1 to 9.50 cm with T_6, while Nemalite showed heights varying from 4.00 cm with T_1 to 9.00 cm with T_6. Indra plants recorded heights ranging from 4.25 cm with T_1 to 10.00 cm with T_6. The T_6 substrate combination consistently resulted in the tallest plants across all genotypes, highlighting its effectiveness in promoting plant growth.

Effect of different substrates on number of leaves

The substrate type significantly influenced the number of leaves. For the Asha genotype, the leaf count ranged from 4.0 in T_1 (sand) to 14.5 in T_6 (cocopeat, perlite, vermiculite, vermicompost). Sympathy exhibited leaf numbers ranging from 3.0 with T_1 to 10.0 with T_6. Similarly, Nemalites showed leaf count varying from 4.5 in T_1 to 9.5 with T_6, while Indra recorded leaf numbers ranging from 4.0 with T_1 to 8.5 in T_6. Among all genotypes, the substrate combination T_6 consistently yielded the highest leaf count, demonstrating its effectiveness in promoting foliar growth across different varieties.

Homozygosity testing

In this study, 461 plantlets, comprising both autodiploidized and colchicine/trifluralin-induced doubled haploids (DHs) from four varieties, Asha, Sympathy, Nemalite and Indra, were validated for homozygosity using codominant SSR markers. Four SSR markers—CAM-8, CAM-19, CAM-54 and CAM-79, were identified as polymorphic in each donor hybrid variety, with one marker applied across the entire DH population. The molecular validation process distinguished doubled haploids from diploids based on banding patterns observed in gel electrophoresis. Haploids and doubled haploids, being homozygous, exhibited a single band corresponding to either allele, while diploid plants,

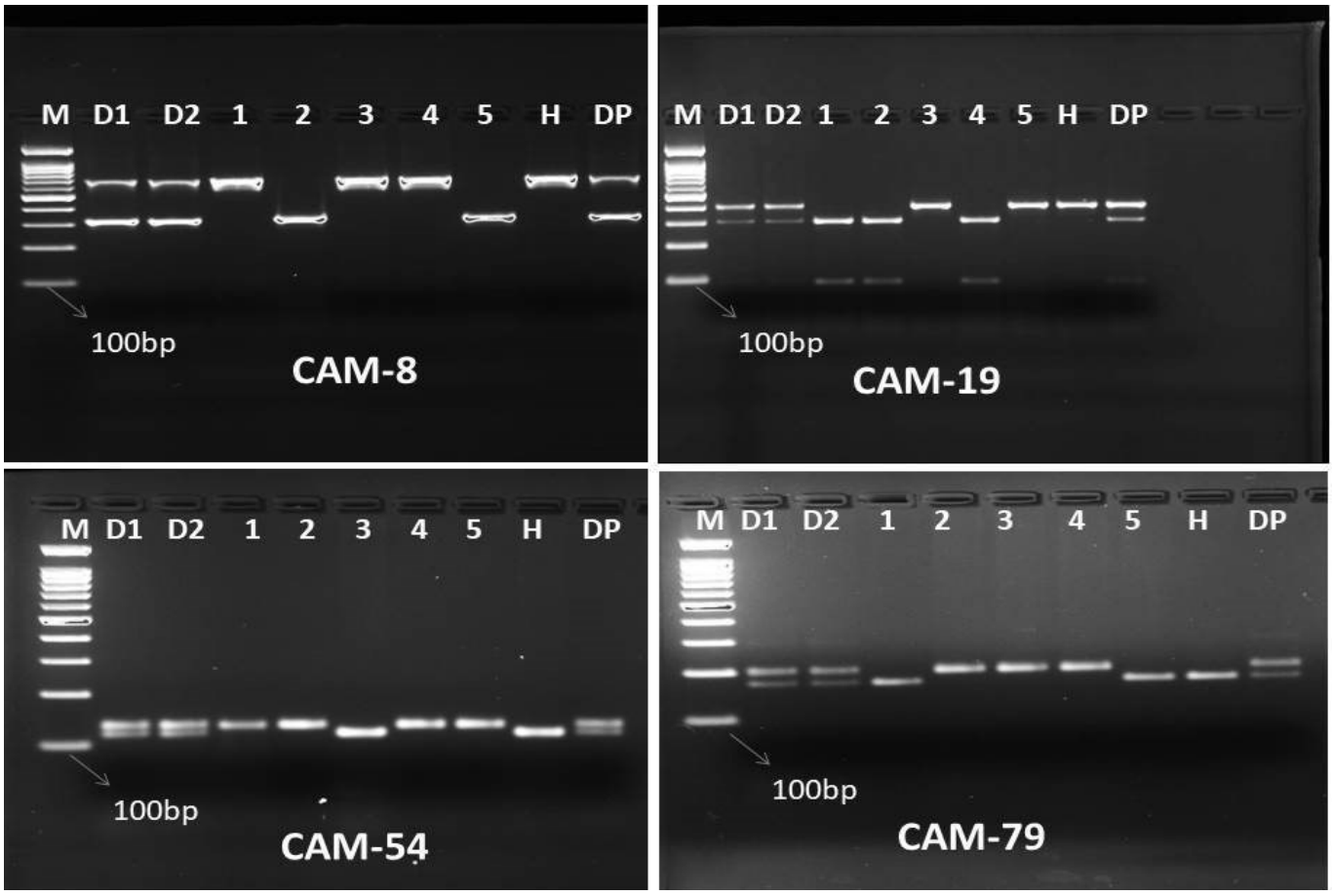

Fig. 4. The banding pattern of polymorphic SSR markers (CAM-8, CAM-19, CAM-54 and CAM79) in the DH line and their progeny (**M**: Marker; **D1**, **D2**: Control donor ; **1**, **2**, **3**, **4**, **5**: DH lines; **H**: Haploid line; **DP**: Diploid line.)

Table 4. Summary of doubled haploid (DH) production and allele distribution across genotypes.

Genotype	Total DH	Allele 1	Allele 2	Diploid	True DH	True DH %	Diploid %
Asha	129	64	59	6	123	95.3	4.7
Sympathy	32	12	19	1	31	96.9	3.1
Nemalite	114	48	62	4	110	96.5	3.5
Indra	186	100	83	3	183	98.4	1.6
	461	224	223	14	447	97.0	3.0

%-percentage, **cm**-centimetre.

being heterozygous, displayed double bands (Fig. 4). Table 4 outlines the DH production data for the four genotypes, comparing total DH lines allele counts, diploid occurrences and the percentages of actual DH plants and diploids. The Indra variety demonstrated superior consistency and efficiency, producing 183 true DHs out of 186 plants, with a notably low diploid percentage of 1.6%. Conversely, the Asha variety yielded the lowest number of true DHs, with 123 out of 129 plants and recorded the highest diploid percentage of 4.7%.

The total DH population across all varieties was 461, with 447 validated as true DHs, resulting in an overall diploid percentage of 3.0%. These findings highlight the effectiveness of the DH production protocols, particularly for the Indra variety, which exhibited the highest efficiency and the lowest diploid occurrence. However, the relatively lower success rate in the Asha variety highlights potential areas for improvement in DH production processes.

The chi-square analysis of the doubled haploid (DH) lines of Asha, Sympathy, Nemalite and Indra indicated no significant deviations from the expected Mendelian allele frequencies, as shown in Table 5. The calculated chi-square values for all genotypes and alleles were small, with the highest value recorded being 0.890909 for Nemalite for Allele 1 and Allele 2, respectively. Similarly, the combined chi-square values for each genotype remained below the significance threshold, with the highest combined value observed at 1.781818 for Nemalite.

Table 5. Chi-square analysis of doubled haploid (DH) lines.

Genotype	Category	Observed	Expected	Chi-Square	Chi-Square combined	P value
Asha	Allele 1	64	61.5	0.101626	0.203252	0.652109
	Allele 2	59	61.5	0.101626		
Sympathy	Allele 1	12	15.5	0.7903226	1.580645	0.208668
	Allele 2	19	15.5	0.7903226		
Nemalite	Allele 1	48	55	0.8909091	1.781818	0.181926
	Allele 2	62	55	0.8909091		
Indra	Allele 1	100	91.5	0.7896175	1.579235	0.208871
	Allele 2	83	91.5	0.7896175		

A p-value of 0.05 was used as the significance level for this analysis.

Corresponding p-values for all genotypes exceeded 0.05, with the lowest p-value being 0.181926 for Nemalite. These results indicate that the observed allele distributions align with the expected Mendelian ratios (1:1), providing robust evidence of the genetic stability and homogeneity of the DH lines.

Discussion

The results of this study demonstrate the significant impact of substrate composition on the survival, growth and overall performance of the four plant genotypes tested. Coco peat-based substrates (T_2, T_3, T_4) consistently outperformed plain sand (T_1), aligning with previous research highlighting the favourable properties of coco peat. Its superior water-holding capacity, aeration and moderate nutrient content create an optimal root environment compared to sand (47,48). This likely accounts for the improved survival rates, increased height and enhanced leaf production observed across all genotypes grown in coco peat mixtures. Adding FYM (T_5) and, more notably, vermicompost (T_6) further enhanced plant performance. FYM is a well-established source of essential macro- and micronutrients for plant growth (49,50). Vermicompost, however, offers benefits beyond nutrient provision; its rich microbial communities enhance nutrient cycling, exhibit hormone-like activity and potentially suppress plant pathogens (51). The marked increases in survival, plant height and leaf production observed with vermicompost (T_6) highlight its value as a comprehensive soil amendment. These results align with previous studies demonstrating the benefits of various organic amendments for acclimating and cultivating pepper plants. For instance, improved acclimation was observed in chilli pepper plantlets using peat moss (15), while a perlite and soil mixture yielded similar success for *Capsicum chinense Jacq.* Plants (36).

Genotypic variations were observed, with the Asha and Indra varieties outperforming Sympathy and Nemalite across substrates. This variation suggests differences in stress tolerance and nutrient use efficiency. However, even the less robust genotypes benefited significantly from enriched substrates, emphasizing the universal importance of substrate optimization in plant cultivation. Hardening

plants with appropriate substrates provides farmers with numerous advantages. It enhances plantlet resilience, improving survival rates post-transplantation and reducing losses due to environmental stresses. This, in turn, increases yield potential and produces better-quality crops, contributing to greater profitability. Additionally, healthier plants require fewer inputs and crops such as fertilizers and pesticides reduce the need for inputs, promoting sustainable farming practices. Farmers can achieve more consistent and reliable harvests by ensuring robust plant establishment, supporting economic stability and food security.

In plant breeding, doubled haploids (DHs) significantly speed up the development of homozygous lines. However, distinguishing true DHs from diploids derived from somatic tissues poses a challenge. This study used codominant markers to verify the homozygosity of DH plants, as flow cytometry alone cannot differentiate these types. Gel electrophoresis using the markers CAM-8, CAM-19, CAM-54 and CAM-79 confirmed that 96.96% of DH plants were homozygous, as indicated by a single band per gel lane. This approach aligns with previous research; for example, SSR markers were used by (40) to confirm DH homozygosity in maize, aligning with our results. Additionally, the effectiveness of RAPD, SSR and ISSR-PCR markers in assessing DH purity has been demonstrated by (41,51), further supporting the findings of our study.

Statistical analysis yielded p-values ranging from 0.3452 to 0.7499, suggesting that observed deviations could be attributed to random variation rather than genetic bias. These high p-values affirm the reliability of the DH production process, ensuring that the genetic stability of the lines is not compromised by unintended selection or experimental error. Thus, the DH lines generated in this study are genetically representative of the parent populations, making them suitable for genetic studies and breeding programs. Androgenic DHs in *Capsicum annuum* were confirmed using CAPS markers, while SSR markers validated their gametophytic origin, further reinforcing their reliability (52-53). Our findings, including a low diploid rate of 1.6% in the Indra variety, support these results. This study's codominant marker-based validation method is reliable for confirming DH homozygosity and distinguishing true DHs from somatic tissue-derived diploids. This precision enhances breeding efficiency by reducing time, labour and resource expenditure, thereby accelerating the development of stable, uniform lines with desirable traits. In sweet pepper breeding, using true DH lines enables the development of high-quality, locally bred varieties, reducing reliance on imported seeds. This approach improves crop quality and resilience, supports local agriculture and contributes to economic stability and food security.

Conclusion

The study highlights the critical role of substrate composition in enhancing the survival and growth of sweet pepper plants during the hardening process. Coco peat-based substrates, especially those enriched with vermicompost, demonstrated superior performance in promoting plant health and vigour. This agricultural advantage improves crop yields and profitability, as healthier plants are more resilient and productive. Additionally, using doubled haploid (DH) lines, which are genetically true homozygous, ensures consistent performance and simplifies breeding efforts, allowing the development of superior sweet pepper varieties. These findings underscore the importance of considering substrate quality and genotype-specific responses for optimizing plant growth in agricultural and horticultural practices.

Molecular analysis using codominant markers, capable of distinguishing between homozygous and heterozygous lines, confirmed that 97.0 % of the doubled haploid (DH) plants were true homozygous, ensuring high genetic uniformity and minimal diploid contamination. Although some minor variation was observed among the lines, it was negligible and unlikely to impact the value of the anther culture-derived DH population for sweet pepper improvement.

Consequently, we recommend employing these DH lines in future breeding and genomics research, particularly those identified as homozygous. Further investigation is warranted to explore the long-term effects of these substrates and potential synergies among organic amendments to improve plant productivity and sustainability during the hardening process.

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Authors' contributions

KJ, PKC and AA did conceptualization. KJ, PKC and AA also designed the experiments. KJ and AA contributed experimental materials. KJ executed the field/lab experiments and collected the data. KJ, PKC and AA performed data analysis and interpretation. KJ prepared the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

References

1. Pruthi JS. Capsicums or chillies. In: Pruthi JS, editor. Spices and condiments. New Delhi: National Book Trust; 1976. p. 40-49
2. Krishna De A, editor. *Capsicum*: The genus *Capsicum*. 1st ed. CRC Press; 2003 https://doi.org/10.1201/9780203381151
3. Bhalabhai JG, Rajhans S, Pandya H, Mankad A. A comprehensive review on *Capsicum* spp. Inter J Res Anal Rev. 2021;8(4):581-99.

4. Bharti H, Singh MC, Singh PK, Sahana KP, Mishra IM. Hi-Tech cultivation of *Capsicum*. Indian Horticulture. 2021;66(2). https://epubs.icar.org.in/index.php/IndHort/article/view/114654
5. Sood S, Kumar N. Heterosis for fruit yield and related horticultural traits in bell pepper. Inter J Veg Sci. 2010;16(4):361-73. https://doi.org/10.1080/19315260.2010.490576
6. Agarwal A, Gupta S, Ahmed Z. Influence of plant densities on productivity of bell pepper (*Capsicum annuum* L.) under greenhouse in high altitude cold desert of Ladakh. Acta Hortic. 2007;756:309-14. https://doi.org/10.17660/ActaHortic.2007.756.32
7. Chassy AW, Bui L, Renaud EN, Van-Horn M, Mitchell AE. Three-year comparison of the content of antioxidant microconstituents and several quality characteristics in organic and conventionally managed tomatoes and bell peppers. J Agric Food Chem. 2006;54(21):8244-52. https://doi.org/10.1021/jf060950p
8. Seguí-Simarro JM. Androgenesis revisited. Botanical Review. 2010;76(3):377-404. https://doi.org/10.1007/s12229-010-9056-6
9. Comlekcioglu N, Ellialtioglu S. Review on the research carried out on *in vitro* androgenesis of peppers (*Capsicum annuum* L.). Res J Biotech. 2018;6:75-84.
10. Palacios MA, Seguí-Simarro JM. Anther culture in sweet pepper (*Capsicum annuum* L.). Methods Mol Biol. 2021;2288:279-91. https://doi.org/10.1007/978-1-0716-1335-1_17
11. Seguí-Simarro JM. Androgenesis in Solanaceae. In: Germana MA, Lambardi M, editors. *In vitro* embryogenesis in higher plants. New York: Humana Press; 2016. p. 209-44
12. Çiner DÖ, Tipirdamaz R. The effects of cold treatment and charcoal on the *in vitro* androgenesis of pepper (*Capsicum annuum* L.). Turk J Bot. 2001;26(3):131-39.
13. Nowaczyk P, Kisiała A. Effect of selected factors on the effectiveness of *Capsicum annuum* L. anther culture. J Appl Genet. 2006;47(2):113-17.
14. Hazarika BN. Acclimatization of tissue-cultured plants. Curr Sci. 2003;85(12):1704-12. http://www.jstor.org/stable/24109975
15. Valadez-Bustos MG, Aguado-Santacruz GA, Carrillo-Castaneda G, Aguilar-Rincon VH, Espitia-Rangel E, Montes-Hernandez S, et al. *In vitro* propagation and agronomic performance of regenerated chili pepper (*Capsicum* spp.) plants from commercially important genotypes. *In Vitro* Cell Dev Biol Plant. 2009;45(5):650-58. https://doi.org/10.1007/s11627-009-9193-y
16. Kozai T. Micropropagation under photoautotrophic conditions. In: Debergh PC, Zimmerman RH, editors. Micropropagation technology and application. Dordrecht: Kluwer Academic Publishers; 1991. p. 447-69 https://doi.org/10.1007/978-94-009-2075-0_26
17. Buddendorf-Joosten JMC, Woltering EJ. Components of the gaseous environment and their effects on plant growth and development *in vitro*. Plant Growth Regul. 1994;15(1):1-16. https://doi.org/10.1007/BF00024671
18. Pospisilova J, Solarova J, Catsky J. Photosynthetic responses to stresses during *in vitro* cultivation. Photosynthetica. 1992;26(1):3-18.
19. Kozai T, Smith MAL. Environmental control in plant tissue culture - general introduction and overview. In: Aitken-Christie J, Kozai T, Smith ML, editors. Automation and environmental control in plant tissue culture. Dordrecht: Kluwer Academic Publishers; 1995. p. 301-18 https://doi.org/10.1007/978-94-015-8461-6_1
20. Desjardins Y. Photosynthesis *in vitro* - on the factors regulating $_{CO2}$ assimilation in micropropagation systems. Acta Hortic. 1995;393:45-61. https://doi.org/10.17660/ActaHortic.1995.393.5
21. Pospíšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š. Acclimatization of micropropagated plants to *ex vitro* conditions. Biologia Plantarum. 1999;42(4):481-97.
22. Preece JE, Sutter EG. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh PC, Zimmerman RH, editors. Micropropagation technology and application. Dordrecht: Kluwer Academic Publishers; 1991. p. 71-93. https://doi.org/10.1007/978-94-009-2075-0_5
23. Kadlecek P. Effect of pretreatment by irradiance and exogenous saccharose under *in vitro* conditions on photosynthesis and growth of tobacco (*Nicotiana tabacum* L.) plants during acclimatization after transfer to soil [Diploma Thesis]. Charles University, Department of Plant Physiology; 1997
24. Bolar JP, Norelli JL, Aldwinckle HS. An efficient method for rooting and acclimation of micropropagated apple cultivars. Hort Science. 1998;33(7):1251-52. https://doi.org/10.21273/HORTSCI.33.7.1251
25. Fila G, Ghashghaie J, Hoarau J, Cornic G. Photosynthesis, leaf conductance and water relations of *in vitro* cultured grapevine rootstock in relation to acclimatization. Physiol Plant. 1998;102(3):411-18. https://doi.org/10.1034/j.1399-3054.1998.1020309.x
26. Diettrich B, Mertinat H, Luckner M. Reduction of water loss during *ex vitro* acclimatization of micropropagated *Digitalis lanata* clone plants. Biochem Physiol Pflanz. 1992;188:23-31. https://doi.org/10.1016/S0015-3796(11)80255-6
27. Marin JA, Gella R, Herrero M. Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. Ann Bot. 1988;62(6):663-70. http://www.jstor.org/stable/42765013
28. Pospisilova J, Synkova H, Haisel D, Catsky J, Wilhelmova N, Sramek F. Effect of elevated CO_2 concentration on acclimation of tobacco plantlets to *ex vitro* conditions. J Exp Bot. 1999;50(330):119. https://doi.org/10.1093/jxb/50.330.119
29. Adi NKAP, Astarini IA, Astiti NPA. Acclimatization black orchid (*Coelogyne pandurata lindl.*) propagated *in vitro* on different media. Jurnal Simbiosis. 2014;2:203-14.
30. Andriani V, Pramushinta IAK. Pengaruh media tanam terhadap respon fisiologi aklimatisasi anggrek Cattleya. STIGMA: Jurnal Matematika dan Ilmu Pengetahuan Alam Unipa. 2017;10(1):17-19. https://doi.org/10.36456/stigma.vol10.no1.a1469
31. Kurniasih W, Nabiila A, Karimah SN, Fauzan MF, Riyanto A, Putra RR. Utilization of zeolite as a media of acclimatization to optimize growth of *Phalaenopsis* sp. hybrid. Bioma: Jurnal Ilmiah Biologi; 2017. https://doi.org/10.26877/bioma.v6i2.1713
32. Julhendri J, Gultom H, Fathurrahman F. Acclimatization of anthurium (*A thurium* sp.) with various growth media and grow quick fertilizer. Dinamika Pertanian. 2013;28(2):103-12.
33. Rachmawati F, Pramanik D, Winarto B. Adventitious shoots derived from leaf explants in *in vitro* mass propagation of indonesian selected *Anthurium* clones. Int J Agric For Life Sci. 2020;4(2):48-55.
34. Malabadi RB, Kumar SV, Mulgund GS, Nataraja K. Induction of somatic embryogenesis in papaya (*Carica papaya* L.). Res Biotechnol. 2011;2(1):40-55.
35. Nasution LZ, Hasibuan M, Manurung ED. Adaptability of tissue-cultured *Dendrobium* orchid plantlets on planting media and its position during acclimatization process. EandES. 2020;454(1):012166. https://doi.org/10.1088/1755-1315/454/1/012166
36. Barrales-Lopez A, Robledo-Paz A, Trejo C, Espitia-Rangel E, Rodriguez-De La O JL. Improved *in vitro* rooting and acclimatization of *Capsicum chinense* Jacq. plantlets. *In Vitro* Cell Dev Biol Plant. 2015;51(3):274-83. https://doi.org/10.1007/s11627-015-9671-3
37. Murovec J, Bohanec B. Haploids and doubled haploids in plant breeding. IntechOpen; 2012. https://doi.org/10.5772/29982
38. Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. 1987;155:335-50. https://doi.org/10.1016/0076-6879(87)55023-6

39. Prince JP, Lackney VK, Angeles C, Blauth JR, Kyle MM. A survey of DNA polymorphism within the genus *Capsicum* and the fingerprinting of pepper cultivars. Genome. 1995;38(2):224-31. https://doi.org/10.1139/g95-027

40. Gyulai G, Gemesne JA, Sagi ZS, Venczel G, Pinter P, Kristof Z, et al. Doubled haploid development and PCR-analysis of F1 hybrid derived DH-R2 paprika (*Capsicum annuum* L.) lines. J Plant Physiol. 2000;156(2):168-74. https://doi.org/10.1016/S0176-1617(00)80302-8

41. Keleş D, Pınar H, Ata A, Taşkın H, Yıldız S, Büyükalaca S. Effect of pepper types on obtaining spontaneous doubled haploid plants via anther culture. Turk J Agric For. 2022;46(1):31-42. https://doi.org/10.3906/tar-2107-73

42. Segui-Simarro JM, Jacquier NM, Widiez T. Overview of *in vitro* and *in vivo* doubled haploid technologies. Methods Mol Biol. 2021;2287:3-22. https://doi.org/10.1007/978-1-0716-1315-3_1

43. Nadeem MA, Nawaz MA, Shahid MQ, Doğan Y, Comertpay G, Yıldız M, et al. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnol Equip. 2017;32(2):261-85. https://doi.org/10.1080/13102818.2017.1400401

44. Jha K, Choudhary PK, Agarwal A. Optimizing androgenic embryo regeneration and chromosome doubling of haploid plants for sweet pepper (*Capsicum annuum var. grossum* L.). J Coast Life Med. 2023;11(2):1326-34.

45. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990;12(1):13-15.

46. Prasad M. Physical, chemical and biological properties of coir dust. Acta Hortic. 1997;450:21-30. https://doi.org/10.17660/ActaHortic.1997.450.1

47. Abad M, Noguera P, Puchades R, Maquieira A, Noguera V. Physico-chemical and chemical properties of some coconut coir dusts for use as a peat substitute for containerized ornamental plants. Bioresour Technol. 2002;82(3):241-45. https://doi.org/10.1016/S0960-8524(01)00189-4

48. Ahmed S, Sharma A, Bhushan B, Wali VK, Bakshi P, Singh AK. Studies on hardening and acclimatization of micropropagated plantlets of banana cv. *Grand Naine*. The Bioscan. 2013;9(3):965-67. https://thebioscan.com/index.php/pub/article/view/772

49. Parkhe S, Dahale M, Ningot E, Gawande S. Study of different potting mixture on hardening of banana tissue culture plantlets its field performance. Int J Curr Microbiol App Sci. 2018;6:1941-47.

50. Hassan SA, Taha RA, Zaied NS, Essa EM, Maek. Effect of vermicompost on vegetative growth and nutrient status of acclimatized *Grand Naine* banana plants. Heliyon. 2022;8(10). https://doi.org/10.1016/j.heliyon.2022.e10914

51. Gemesne JA, Petus M, Venczel G, Zatyko L, Gyulai G, Cseplo M. Genetic variability of anther donor versus spontaneous doubled haploid descendents and colchicine induced doubled haploid sweet pepper (*Capsicum annuum* L.) lines. Acta Hortic. 2001;560:149-52. https://doi.org/10.17660/ActaHortic.2001.560.24

52. Grozeva S, Todorova V, Nankar AN. Creation of pepper doubled haploids and morphological characterization of androgenic plants. Euphytica. 2021;217(6):1-16. https://doi.org/10.1007/s10681-021-02840-w

53. Parra-Vega V, Renau-Morata B, Sifres A, Seguí-Simarro JM. Stress treatments and *in vitro* culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult. 2013;112(3):353-60. https://doi.org/10.1007/s11240-012-0242-6