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RESEARCH ARTICLE

Optimized *in vitro* regeneration and genetic fidelity assessment of strawberry (*Fragaria* × *ananassa* Duch. cv. Chandler)

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Abstract

This research developed an effective *in-vitro* method for regenerating strawberry plants, facilitating the large-scale production of planting materials. Various vegetative parts, such as runner tips, nodal segments, shoot apices, stems and leaves, were used for direct organogenesis. These explants were grown on MS media enriched with varying concentrations of plant growth regulators. The highest shoot multiplication rate has been found to be 89.8 % and the maximum number of shoots per explant (4.3) were achieved using runner tips in culture media supplemented with 1.5 mg/L BAP and 0.1 mg/L of NAA. The optimal conditions included pH of 5.8, 0.6 % agar, 3000 lux light intensity, 16-hr photoperiod and an incubation temperature of 25 ± 2 °C. The optimal percentage of rooting (90.8 %) for the regenerated shoots was achieved on ½ MS medium enriched with 1.0 mg/L IBA along with highest number of roots per culture. Subsequently. all regenerated plants were hardened in a greenhouse with 85 % relative humidity. To confirm the genetic fidelity of the *in vitro*-derived plants, 12 ISSR primers were used, revealing monomorphic bands. The standardized protocol developed in this study is crucial for large-scale multiplication and can also aid in identifying any clonal variations or adulteration.

Keywords: fidelity analysis; *in vitro* plant regeneration; micropropagation; runners' tip; strawberry

Introduction

Strawberry is a temperate fruit crop commonly grown in European countries, Northern Asia, the Americas and some regions of Africa. It finds its general propagation via runners, stolons and occasionally through seeds. Field cultivation of strawberries is marred with fungal and bacterial diseases, which significantly reduce the quality and quantity of fruits. Problematic diseases such as *Verticillium* wilt, *Fusarium* wilt, anthracnose, *Alternaria* leaf spot, leaf blight and powdery mildew plague conventional crop improvement pursuits. The polyploid nature of strawberries makes the cross-breeding-based selection tedious and unstable. Hence, in this context , *in vitro* strawberry micropropagation transpires as the most fruitful avenue for targeted genetic improvement in strawberries. Micropropagation offers quicker clonal multiplication, better plant establishments and secondary metabolite enhancements, all under aseptic management. Apart from *in vitro* approaches that assisted in protoplast isolation, its regeneration and transformation, germplasm conservation, creation of genetic variation (mutagenesis), etc., an array of cutting-edge biotechnological tools for genetic transformation and gene editing like CRISPR/Cas9, golden gate cloning and recombinant DNA engineering have also emerged as prospective options to administrate genetic enhancements in strawberry (1).

Worldwide, it is cultivated in 3.96 lakh hectares, producing 88.85 lakh metric tons of strawberries and an average yield of 22.4 t/ha (2). It is commercially grown in Canada, the USA, Italy, Germany, Spain, Japan, Thailand, Korea, Poland and many others (3). Traditionally, it has been propagated vegetatively through rooted runners; however, this method has proven unsuitable due to its labour-intensive nature, time consumption, susceptibility to viral diseases and environmental hazards, all of which contribute to the gradual degeneration of cultivar performance (4). Studies have shown that micro-propagated strawberry plants exhibit superior characteristics, such as larger crown size, more significant number of runners, earlier flowering and higher yield, compared to those propagated conventionally through runners (5). The high yield of micro-propagated plants can be attributed to the use of disease-free plant material, which prevent plant and soil-transmissible diseases (3, 6). The

protocol and procedure for strawberry micropropagation have been successfully standardized by several researchers (4, 5, 7) and have demonstrated their effectiveness as a superior alternative to traditional propagation methods (8).

In vitro plant production uses plant growth regulators to promote shoot and root development. However, auxins have been linked to plant genetic instability, a condition called soma clonal variation (9, 10). This variation typically occurs as a stress response to plants' conditions during *in vitro* culture. It is characterized by DNA methylation, chromosome rearrangements and point mutations (11). Somaclonal variation can serve as a source of genetic diversity for developing superior clones, posing a significant challenge in the plant tissue culture industry. This issue becomes particularly concerning when the objective is to produce uniform propagules of a specific variety, as soma clonal variation can result in undesirable traits or off-types (12, 13). The scalability of micropropagation protocols is frequently hindered by these variations, emphasizing the importance of conducting rigorous quality assessments to ensure genetic uniformity among the progeny. The efficiency of *in vitro* propagation is higher than that of runner production, as runner production depends upon the genetic makeup, training system, climatic condition, seasons and morphophysiological state of the plant. Auto regulatory systems of management control the aseptic condition, hormonal level, temperature, growing media and through proper regulatory updates on pest and disease infestation. Producing *in vitro* propagules of strawberries is a viable and stable method for mass-scale commercialization. A system capable of minimizing or eliminating genetic variations during tissue culture would be highly beneficial.

Historically, morphological characterization, physiological monitoring, biochemical evaluations and field trials were used to identify genetic variations. In recent years, molecular markers have emerged as powerful tools, complementing and enhancing these traditional approaches. Molecular markers provide a more reliable method for identifying certain kinds and assessing, monitoring the genetic consistency of plantlets grown from tissue culture. Studies that identify soma clonal differences in tissue culture-derived plants, such as strawberries, lilies , turmeric and Swertia chirayita (14-17). Among these techniques, ISSR markers are dependable, reproducible, cost-effective and versatile, offering the advantage of repeatable DNA sequence amplification using single primers. This study aimed to establish an efficient *in vitro* plant regeneration method for strawberries to support large-scale production of planting materials. Additionally, we assessed the genetic stability of these micro-propagated materials to ensure their suitability for reliable commercial cultivation.

Materials and Methods

In vitro propagation

The strawberry cultivar *Chandler* was selected for this study due to its adaptability to subtropical plains. Strawberry plants were grown in pots of 12 inches in size at the experimental garden of the Department of Fruit Science and Technology, College of Agriculture, Odisha University of Agriculture and Technology, Odisha, for providing suitable explants viz., runner tips, nodal explants, shoot apices, stems and leaves for *in-vitro* direct regeneration. The explants were carefully cleaned for 30 min under running water to guarantee cleanliness. The explants were treated with 0.5 % carbendazim (Bavistin) for 15 min and 0.1 % mercuric chloride ($HgCl_2$) for 7 min for surface sterilization, followed by rinsing five times with double - distilled water to remove any residual mercuric chloride. Subsequently, they were transferred to a basal culture medium containing 0.6 % (w/v) agar and the macro - and micronutrients based on the MS formulation (18). The culture medium was sterilized through autoclaving at 121 °C for 15 min under 15 psi pressure. The cultures were then incubated under specific conditions to optimize growth and regeneration. Growth regulators were added to the medium before autoclaving and different concentrations of shoot and root-inducing phytohormones were added to promote shoot and root development. Runner tips were added as primary explant due to their rapid and proliferous growth rate.

Explants were cultivated on MS media supplemented with different concentrations of BAP (0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0 mg/L), Kinetin 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0 mg/L) and Ads (0, 50 mg/L) to ascertain the ideal cytokinin concentration for shoot proliferation. Similarly, explants were cultivated on MS media with varying concentrations of NAA (0, 0.1, 0.25, 0.5, 1.0 mg/L), IAA (0, 0.1, 0.25, 0.5, 1.0 mg/L) and IBA (0, 0.1, 0.25, 0.5 mg/L) to determine the optimal auxin concentration for shoot proliferation. Additionally, explants were cultured on MS media supplemented with combinations of cytokinin and auxin to identify the most suitable medium for shoot proliferation. Once the optimal growth regulator combination for shoot multiplication was determined, separate experiments were conducted to identify the best culture conditions for micropropagation. Cultures containing multiplied shoots from the standardized MS basal media (supplemented with 1.5 mg/L BAP and 0.1 mg/L NAA) were exposed to varying light intensities (1000, 2000, 3000, 4000, 5000 and 6000 lux). Based on the results from the light intensity experiments, the effects of different photoperiods (0, 8, 12, 16, 24 and 28 hr) on shoot growth and multiplication were examined at 3000 lux. Additionally, the effect of different incubation temperatures (18 ± 2°C, 20 ± 2°C, 22 ± 2°C, 25 ± 2°C, 28 ± 2°C and 30 ± 2°C) on shoot multiplication was investigated. The pH of the medium was a critical factor in shoot multiplication, as it influences nutrient availability. Therefore, the pH of the media was adjusted to various levels (5.0, 5.5, 5.8, 6.0, 6.2 and 6.5) using NaOH or HCl before autoclaving. To investigate the impact of nutrient concentrations on shoot multiplication, media containing full-strength MS nutrients were compared with media having different nutrient strengths (½, ¼, 1/8, 2 and 2.5 times the standard concentration) while maintaining the standard concentrations of BAP (1.5 mg/L), NAA (0.1 mg/L) and sucrose (30 g/L). The percentage of explants producing multiple shoots varied significantly across the different concentrations of basal MS media.

To determine the most suitable carbohydrate source for strawberry regeneration, the medium was supplemented with various carbohydrates: D-glucose, sucrose, fructose, maltose, mannitol and commercial sugar. Additionally, MS medium was prepared at half strength, one-fourth strength, one-eighth strength, double strength and 2.5 times the normal concentration, adjusting all salts to half or quarter strength, except for iron, glycine and vitamins, to evaluate the best nutrient medium. Among the carbohydrate sources tested, sucrose and commercial sugar outperformed D-glucose, fructose and maltose. Based on these results, different concentrations of sucrose (0, 1, 2, 3, 4 and 5 %) were tested for shoot multiplication. The effect of varying agar concentrations (0.4 %, 0.5 %, 0.6 %, 0.7 %, 0.8 % and 1.0 %) on shoot multiplication was also studied. Initiating root, development and elongation requires auxin (19). Newly grown shoots of 1-2 cm were separated from the clumps and transferred to ½ MS basal media with and without growth regulators. After root development, micro propagules were thoroughly washed and planted in black polythene pots containing a mixture of sand, soil and FYM (1:1:1 w/v). For acclimatization, these pots were subsequently housed in a greenhouse with temperatures between 25 ºC - 28 ºC and relative humidity above 85 %. The Post-Hoc Multiple Comparison Test was used to statistically analyze the mean percentage of cultures exhibiting response, the number of shoots per culture, the mean rate of rooting and the number of roots per shoot. After being transferred, almost 90 % of the plantlets were successfully established in the greenhouse after 1-2 weeks. In just four weeks, the plants had grown to a height of 6 to 8 inches. The early survival of the potted plantlets was significantly impacted by the temperature and humidity levels that prevailed during the transplanting season (19). After acclimatization in the growth chamber, the rooted plants were transferred to the field for hardening.

Genetic fidelity test

The CTAB method was followed to extract genomic DNA from the mother and tissue cultured plantlets to evaluate the genetic fidelity. The purity of the isolated uncut lambda - DNA was checked by loading about 2 µL of the DNA aliquot onto a 0.78 % (w/v) agarose gel. DNA concentrations were around 200 to 800 ng of DNA per gram of leaf tissue. Twelve specially designed ISSR primers (M/s Bangalore Genei, Bangalore) were chosen for the current study due to their high polymorphic nature. About 20 ng of DNA template, 100 µM of each dNTPs, 1.5 mM of $MgCl_2$, 20 ng of primer, 1× Taq buffer and 0.5 U of Taq DNA polymerase were used in the Polymerase Chain Reaction (PCR) and the final reaction was carried out in a final reaction volume of 25.0 µL. A thermal cycler (MJ Research, Watertown, USA) was used for amplification. The program included an initial denaturation at 94 °C (2 min), denaturation at 94 °C (20 s), annealing at the temperature designated for each ISSR primer (for 30 s) and extension at 72 °C (for 10 min) (approximately 40 cycles of last three steps). The amplicons were separated by gel electrophoresis (1.8 % agarose gel, alongside a 1.0 kb plus Ladder, pre-stained with ethidium bromide and were further examined under UV. Gel images were captured using a Gel Documentation System (Bio-Rad, California, USA) and the sizes of the amplicons were analyzed using Quantity One software (Bio-Rad, California, USA). Repeated PCR reactions were conducted to ensure the reliability of the results.

Results and Discussion

In vitro micropropagation has been widely applied to strawberry cultivars, offering benefits such as reduced time to produce planting stock, maintenance of genetic uniformity and the production of disease-free plants (20). Also, micro-propagated plants often yield more fruit, resulting in earlier and greater production (21). The current study standardized an *invitro* protocol for regenerating true-to-type strawberry saplings. To determine the best source of cytokinin for shoot multiplication, we tested three cytokinins: 6-benzylaminopurine (BAP), kinetin (Kn) and adenine sulphate (Ads). Among these, BAP and kinetin promoted the highest rate of shoot proliferation, while no positive response was observed without cytokinin. Moderate concentrations of BAP (1.0 mg/L), combined with 0.25 mg/L kinetin and 50 mg/L adenine sulphate, resulted in the highest response. Lower concentrations of BAP were ineffective for shoot induction. For *Fragaria* × *ananassa* Duch. cv. Chandler, the maximum percentage of shoot multiplication (89.8 %) has occurred in MS media supplemented with 1.0 mg/L BAP, 0.25 mg/L kinetin and 50 mg/L adenine sulfate (Table 1). Increasing the BAP concentration from 0.25 to 3.0 mg/L improved the response percentage and the number of multiple shoots per culture, with shoot numbers ranging from 1.1 to 3.7 and an average of 1.8 shoots per culture. We also tested three auxins (IAA, IBA and NAA) for shoot multiplication from runner tips of *Fragaria* × *ananassa* Duch. cv. Chandler. Among these, NAA resulted in the highest rate of shoot proliferation. Without auxins, no positive response was observed (Table 2). The combination of NAA and IAA showed the best shoot growth and multiplication results, mainly when using a full-strength MS medium. Lower concentrations of NAA (0.1 mg/L) combined with 0.25 mg/L IAA provided the highest response (Table 3). Higher concentrations of NAA were ineffective for shoot induction. The highest shoot proliferation percentage (85.8 %) from runner tips was recorded on MS medium supplemented with 1.5 mg/L BAP, 0.1 mg/L NAA and 3 % sucrose after 4 weeks of culture (Fig. 1 & 2). The average number of shoots per culture varied from 1.1 to 4.3, depending on the treatment. The lowest shoot multiplication rate was observed when only IAA (0.1 mg/L) was used. Thus, the results highlight the importance of a combination of auxins and cytokinin for effective organogenesis in *Fragaria* × *ananassa* Duch. cv. Chandler. The combination of cytokinins and auxins also positively impacted shoot proliferation and multiplication (22). Similarly, regeneration of plantlets of Honeoye strawberry from auxiliary buds cultured on MS medium supplemented with BAP (2.5 mg/L) and thiazuron (5.5 mg/L) for 4 weeks, demonstrating successful shoot proliferation (23).

Regenerated shoots of *Fragaria* × *ananassa* Duch. cv. Chandler, measuring less than 4 cm in length, initiated root formation within 20 days of inoculation when cultured on 1/2 MS medium with different concentrations of NAA and IBA

Table 1. Effect of cytokinin on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS + Growth regulator (mg/L)			Percentage of plants developed multiple shoots (Mean ± S.E.) *	Number of shoots/cultures (Mean ± S.E.)*
BAP	Kn	Ads		
0	0	0	0	0
0.25	0	0	13.6 ± 0.3a	1.1 ± 0.1a
0.5	0	0	21.5 ± 0.9b	1.2 ± 0.03a
1.0	0	0	65.7 ± 0.6h	1.3 ± 0.04a
1.5	0	0	68.9 ± 1.1i	1.6 ± 0.1a
2.0	0	0	77.1 ± 1.3j	2.6 ± 0.2b
3.0	0	0	78.2 ± 1.3j	2.4 ± 0.1b
0	0.25	0	21.5 ± 0.9b	1.8 ± 0.1a
0	0.5	0	39.9 ± 0.8c	2.3 ± 0.2b
0	1.0	0	41.6 ± 0.7c	2.2 ± 0.2b
0	1.5	0	50.7 ± 1.0d	2.4 ± 0.2b
0	2.0	0	52.5 ± 0.6e	1.5 ± 0.1a
0	3.0	50	61.3 ± 0.6g	1.8 ± 0.2a
1.0	0.25	50	89.8 ± 0.8 k	3.7 ± 0.2c
2.0	0.25	50	78.2 ± 1.3 j	2.8 ± 0.2b
3.0	0.25	50	77.1 ± 1.3j	1.4 ± 0.1a
1.0	0.5	50	68.9 ± 1.1 i	2.6 ± 0.2b
1.5	0.5	50	76.1 ± 0.7 j	3.1 ± 0.2c
2.0	0.5	50	65.7 ± 0.6 h	2.2 ± 0.2b
3.0	0.5	50	54.5 ± 0.7 f	1.6 ± 0.1a
1.0	0.5	50	60.8 ± 0.8 g	1.4 ± 0.1a
1.5	1.0	50	55.4 ± 0.7 f	1.9 ± 0.2a
2.0	1.0	50	42.3 ± 0.7 c	1.3 ± 0.04a
3.0	1.0	50	41.1 ± 0.5 c	1.1 ± 0.04a
3.0	1.5	50	43.7 ± 0.8 c	1.1 ± 0.04a

*Thrice replication, one treatment consists of 20 cultures. At the 5 % level, the mean followed by different letters are significantly different (post-hoc multiplication comparison test, p= 0.05) a

Table 2. Effect of auxin on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS+ Growth regulator (mg/L)			Percentage of plants developed multiple shoots (Mean ± S.E.) *	Number of shoots/ cultures (Mean ± S.E.) *
IAA	NAA	IBA		
0	0	0	0	0
0.1	0	0	10.2 ± 0.06a	1.1 ± 0.1a
0.25	0	0	25.4 ± 0.7b	1.1 ± 0.1a
0.5	0	0	25.4 ± 0.7b	1.4 ± 0.1a
1.0	0	0	25.6 ± 0.8b	1.3 ± 0.04a
0	0.1	0	50.8 ± 0.9c	1.3 ± 0.04a
0	0.25	0	53.7 ± 0.9d	1.4 ± 0.1a
0	0.5	0	61.6 ± 0.8f	1.5 ± 0.1a
0	1.0	0	66.8 ± 0.5g	1.2 ± 0.03a
0	0.25	0.25	71.1 ± 1.3h	1.2 ± 0.03a
0.25	0.25	0.25	77.4 ± 1.2i	1.3 ± 0.04a
0.25	0.1	0	80.7 ± 0.6j	2.8 ± 0.2b
0.5	0.1	0	74.9 ± 1.1i	2.4 ± 0.2b
1.0	0.1	0	76.4 ± 0.8i	2.5 ± 0.2b
0.25	0.25	0	70.1 ± 0.9h	2.2 ± 0.2b
0.25	0.5	0	75.2 ± 0.6i	2.4 ± 0.2b
0.25	0.5	0.25	74.8 ± 0.9i	1.9 ± 0.2a
0.5	0.5	0	76.1 ± 0.8i	2.3 ± 0.2b
0.5	0.5	0.5	77.6 ± 0.7i	1.3 ± 0.04a
1.0	0.5	0	78.3 ± 0.9i	1.7 ± 0.2a
0.25	1.0	0.25	79 ± 1.0i	1.1 ± 0.05a
0.25	1.0	0	75.3 ± 0.6i	1.2 ± 0.03a
0.5	0.1	0.1	58.4 ± 0.9e	1.6 ± 0.1a
0.5	0.1	0.5	61.6 ± 0.8f	1.1 ± 0.05a
1	1	0	66.8 ± 0.5g	1.1 ± 0.05a

*Thrice replication, one treatment consists of 20 cultures. At the 5 % level, the mean followed by different letters are significantly different (post-hoc multiplication comparison test, p= 0.05)

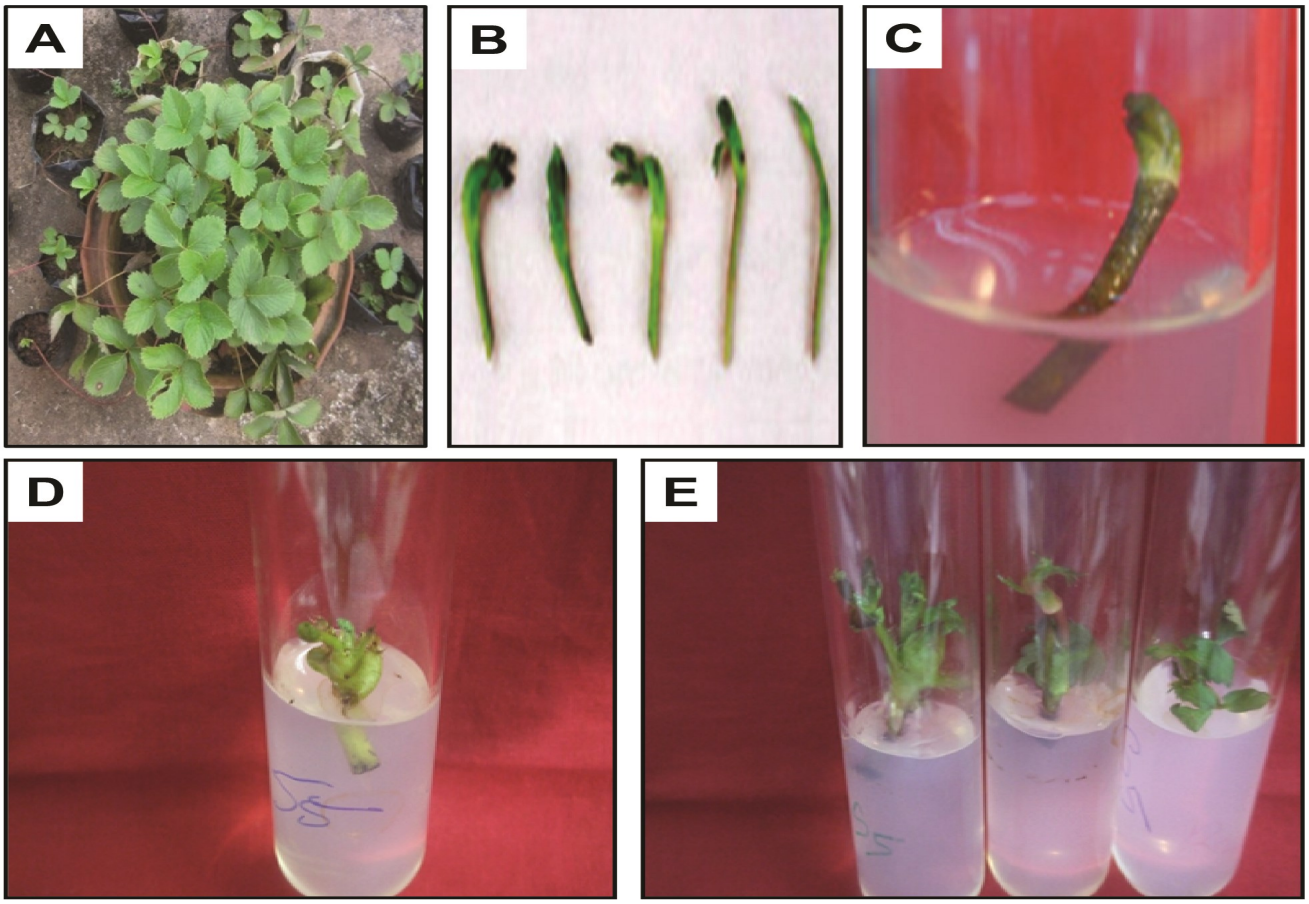

Fig. 1. Adventitious shoot induction in strawberry cultivar Chandler. A) Explant source; B) Runner tips used as explants; C) Inoculation of runner tips; D) Shoot bud proliferation; E) Shoot multiplication on MS medium supplemented with 1.5 mg/L BAP, 0.1 mg/L NAA and 3 % sucrose.

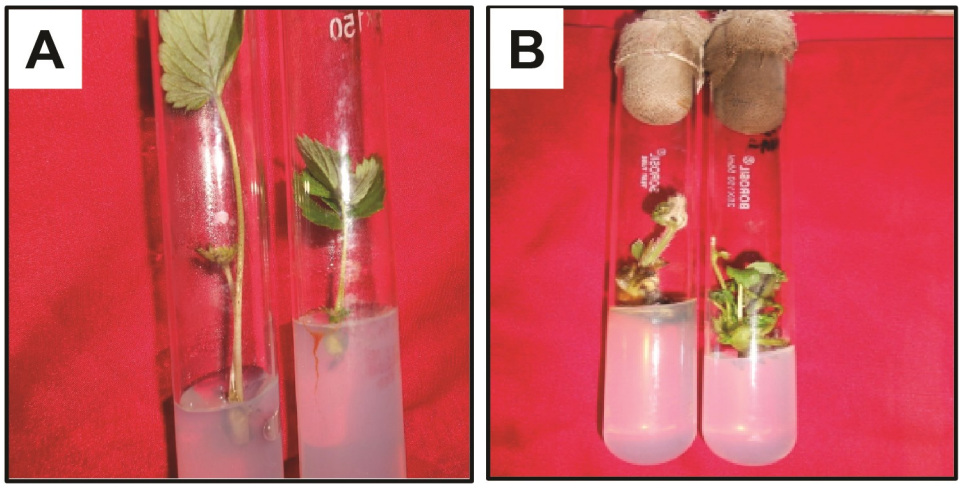

Fig. 2. *In vitro* shoot multiplication of strawberry cultivar Chandler. A) Growth of multiple shoots on MS medium after 2 weeks of culture; B) Growth of multiple shoots on MS medium shoots on MS medium after 4 weeks of culture.

separately (Table 4). The highest rooting percentage (90.8 %) was observed in ½ MS medium supplemented with 1.0 mg/L IBA and 3 % sucrose (Fig. 3). Although NAA also promoted root initiation, IBA was more effective in enhancing rooting. Additionally, the number of roots per shoot was higher (5.4) in the IBA-supplemented medium. These findings align with the earlier results where the genotype AOG of strawberry showed the highest number of roots per shoot and rooting frequency (90 %) in a medium fortified with 1.0 mg/L IBA. In this study, MS medium supplemented with 3 % sucrose, 6 % agar and varying concentrations of hormones, maintained at a pH of 5.8, was optimal for plant regeneration (24).

In the investigation of optimal conditions for plant regeneration and growth, it was found that the highest shoot multiplication rate (84.3 %) occurred at a light intensity of 3000 lux. Lower light intensities were less effective in promoting shoot multiplication. Among various photoperiod cycles, combining a 16 hr photoperiod at 3000 lux proved to be the most favourable for both shoot multiplication and growth in *Fragaria* × *ananassa* Duch cv. Chandler (Fig. 4). These findings align with previous studies on light effects on *in vitro* morphogenesis in other species (25, 26). The optimal conditions for shoot multiplication also included an incubation temperature of 25 ± 2 °C and a pH of 5.8. These results were consistent with that earlier report (27). The

Table 3. Effect of auxin and cytokinin on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS + Growth regulator (mg/L)			Percentage of plants developed multiple shoots (Mean ± S.E.) *	Number of shoots/cultures (Mean ± S.E.) *
BAP	IAA	NAA		
0	0	0	0	0
1.0	0.1	0	65.7 ± 0.6c	1.1 ± 0.05a
1.25	0.1	0	68.9 ± 1.1c	1.2 ± 0.03a
1.5	0.25	0	76.1 ± 0.7d	1.3 ± 0.04a
2.0	0.25	0	76.1 ± 0.7d	1.9 ± 0.2a
3.0	0.5	0	77.4 ± 1.0d	1.3 ± 0.04a
1.0	0	0.1	78.2 ± 1.3d	1.7 ± 0.2a
1.25	0	0.1	78.3 ± 0.9d	1.9 ± 0.2a
1.5	0	0.25	80.7 ± 0.6d	2.9 ± 0.2b
2.0	0	0.25	80.1 ± 0.7d	2.8 ± 0.2b
3.0	0	0.5	79 ± 0.9d	2.4 ± 0.2b
0.25	1.5	0.2	13.6 ± 0.3a	2.9 ± 0.2b
0.5	1.5	0.2	52.5 ± 0.6b	3.6 ± 0.2c
1.0	1.5	0.2	61.6 ± 0.8c	2.6 ± 0.2b
2.0	0.5	0.2	70.1 ± 0.9d	2.3 ± 0.2b
1.5	2.0	0.2	75.3 ± 0.6d	2.4 ± 0.2b
2.0	2.0	0.2	76.1 ± 0.7d	3.2 ± 0.2c
2.0	2.0	0.2	78.3 ± 0.9d	3.6 ± 0.2c
1.5	0	0.1	85.8 ± 0.9e	4.3 ± 0.4d
2.0	0.1	0.1	76.1 ± 0.7d	1.4 ± 0.2a
2.0	0	0.1	74.8 ± 0.9d	1.1 ± 0.05a
3.0	0	0.5	65.7 ± 0.6c	1.4 ± 0.1a
3.0	0.1	0.5	68.9 ± 1.1c	1.4 ± 0.1a

*Thrice replication, one treatment consists of 20 cultures. At the 5 % level, the mean followed by different letters are significantly different (post-hoc multiplication comparison test, p= 0.05)

Table 4. Effect of Auxins on rooting of *Fragaria* × *ananassa* Duch. cv. Chandler

½ MS + Growth regulators (mg/l)		Percentage of plants developed root (Mean ± S.E)*	Number of roots/cultures (Mean ± S.E.) *	Days to rooting
NAA	IBA			
0	0	0	0	0
0.25	0	20.7 ± 0.7 a	1.5 ± 0.1a	20
0.50	0	24.7 ± 0.5 b	2.5 ± 0.2b	18
1.0	0	38.4 ± 0.6 c	3.1 ± 0.2 c	17
0	0.1	78.7 ± 0.2g	3.5 ± 0.2c	12-14
0	0.25	72.3 ± 0.2e	4.1 ± 0.1d	10-14
0	0.5	81.4 ± 0.2h	4.1 ± 0.1d	10-14
0	1.0	90.8 ± 0.2i	5.4 ± 0.3e	8-10
0	1.5	74.3 ± 0.2f	1.8 ± 0.1a	8-10
0.25	0.25	60.5 ± 0.8d	4.03 ± 0.2 d	10-12

*Thrice replication, one treatment consists of 20 cultures. At the 5 % level, the mean followed by different letters are significantly different (post-hoc multiplication comparison test, p= 0.05)

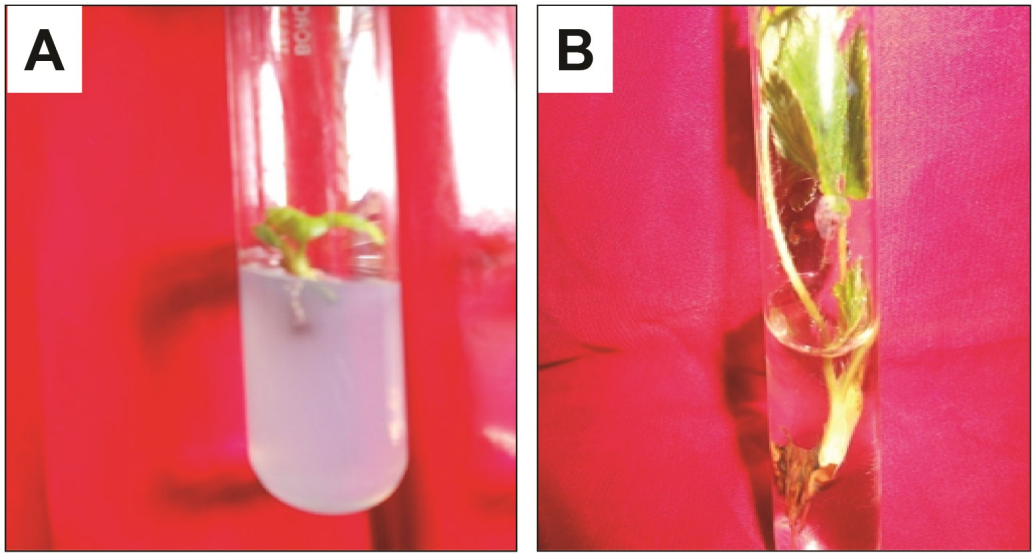

Fig. 3. Rooting of Micro shoots. A) Initiation of rooting from micro shoot after 1 week of transfer to ½ MS medium supplemented with 1.0 mg/ IBA and 3 % sucrose; B) Root growth after 2 weeks of which is ready to transfer to ex-agar medium.

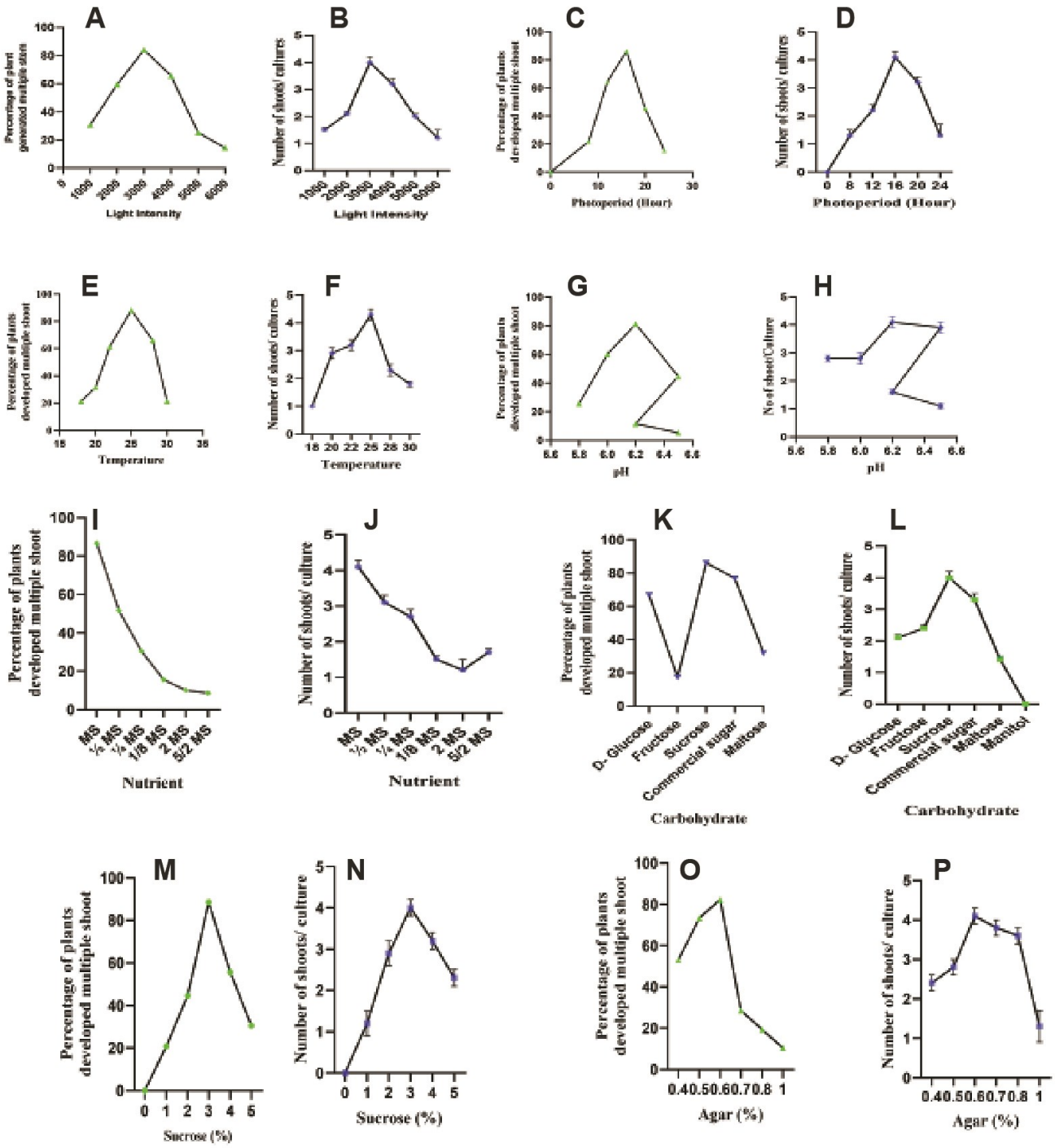

Fig. 4. A-B) Optimum light intensity for the maximum percentage of multiple shoots and number of shoots; C-D) Optimum photoperiod for the maximum percentage of multiple shoots and number of shoots; E-F) Optimum temperature for the maximum percentage of multiple shoots and number of the shoot; G-H) Optimum pH for the maximum percentage of multiple shoot and number of the shoot; I-J) Optimum nutrient for the maximum percentage of multiple shoot and number of the shoot; K-L) Optimum carbohydrate for the maximum percentage of multiple shoot and number of shoot; M-N) Optimum sucrose (%) for the maximum percentage of multiple shoot and number of shoot; O-P) Optimum agar (%) for the maximum percentage of multiple shoot and number of shoot.

highest shoot multiplication rate (86.6 %) and the most significant number of multiple shoots (4.1) were achieved using full-strength MS medium supplemented with 1.5 mg/L BAP and 0.1 mg/L NAA. Additionally, the highest shoot multiplication rate (86.2 %) and the most significant number of multiple shoots (4.0) were observed with a 3 % sucrose concentration, followed by commercial sugar (76.9 %). In *Fragaria* × *ananassa* Duch. cv. Chandler, the best shoot multiplication (82.4 %) and the highest number of shoots per culture (4.1) were obtained using a medium containing 0.6 % agar. The healthy, well-elongated rooted plantlets were gently washed with sterile distilled water to remove any traces of MS medium in preparation for hardening (Fig. 5). After one day, these rooted plants were pre-hardened in black polythene pots containing a 1:1:1 autoclaved mixture of soil, sand and FYM. Plants were placed in the greenhouse, maintaining 85 % humidity for acclimatization. Within 1-2 weeks of transfer, approximately 90 % of the plantlets were successfully established in the greenhouse. Research indicates similar results (26). For further hardening, the rooted plants were shifted to a nursery (27).

To assess the quality of *in vitro*-derived regenerates, twelve ISSR primers were used for screening, which showed monomorphic bands across all plantlets (Fig. 6). The monomorphism in plantlets shows the absence of genetic variability caused by *in vitro* multiplication. In the DNA profiles, no polymorphism was noticed. Although some weak bands were absent in specific regenerates, these were found to be infrequent and non-reproducible upon repetition. The ISSR banding patterns of the 19 *in vitro*-raised plantlets were identical to that of the mother plant (*Fragaria* × *ananassa* Duch. cv. Chandler). This study aligns with a similar report of genetic uniformity between the mother plant and its tissue-cultured progenies using the ISSR marker (28). Their study generated monomorphic bands across all the 12 UBC di-nucleotide motif primers. These findings indicate that the tissue-cultured strawberry plantlets are genetically identical and clonally uniform.

Conclusion

The current investigation aimed to optimize the method for large-scale production of disease-free strawberry propagules (*Fragaria* × *ananassa* Duch.) cv. Chandler through *in-vitro* culture. We established an efficient *in-vitro* plant regeneration protocol for strawberries, facilitating the mass production of planting material. After evaluating various growth hormone combinations, we determined that the highest percentage of shoot proliferation and the most significant number of multiple shoots per culture from runner tips were achieved on MS medium supplemented with 1.5mg/L BAP, 0.1 mg/L NAA and 3 % sucrose within 4 weeks of culture. The rooting was optimal on ½ MS medium

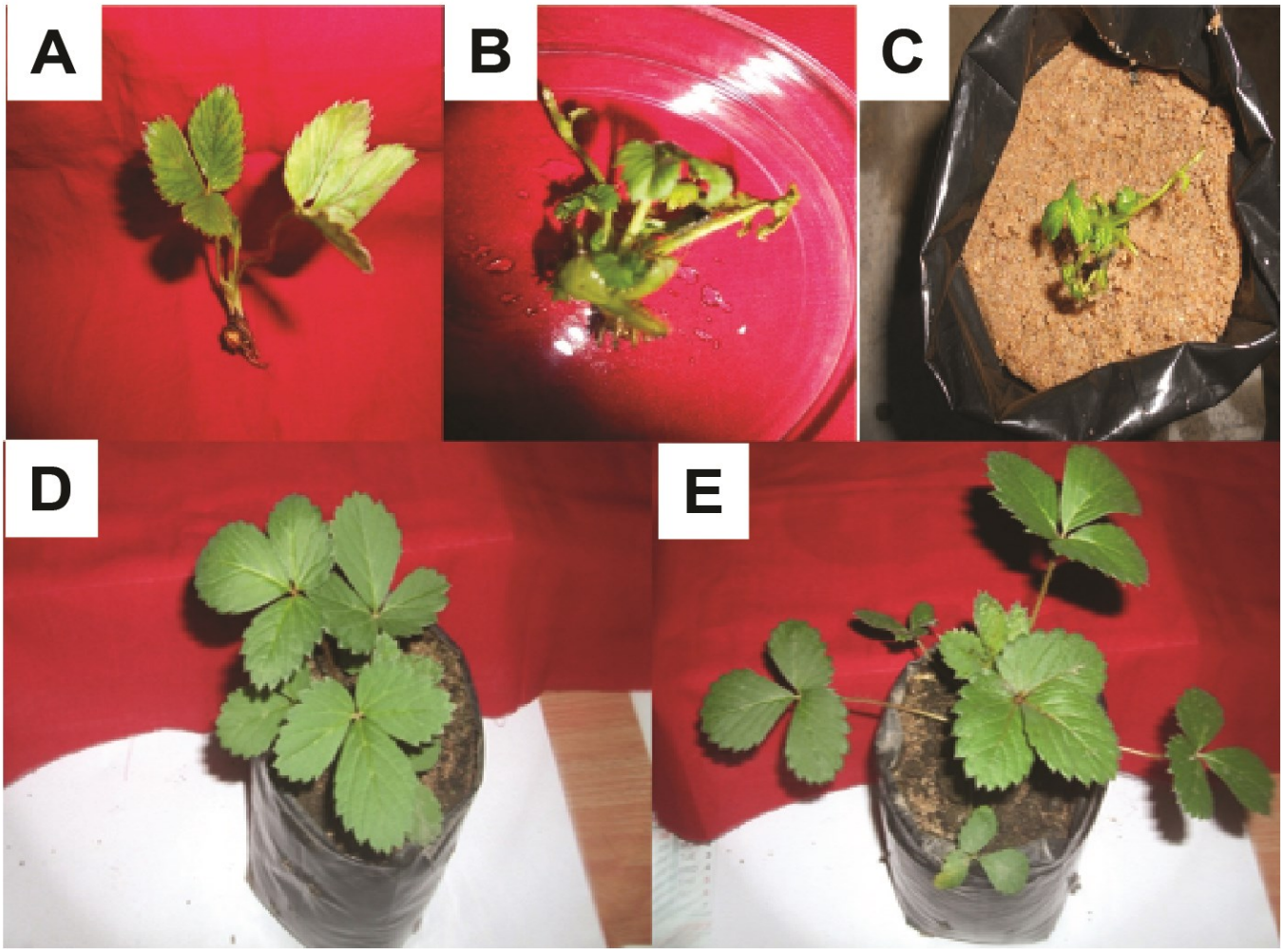

Fig. 5. Successful *in vitro* regeneration of strawberry cultivar Chandler: A and B) Agar-based tissue culture-raised plantlets; C) Plantlet grown in sterile sand; D and E) *In vitro*-raised strawberry plantlet transplanted into a hardening medium composed of soil, sand and FYM in 1:1:1 ratio.

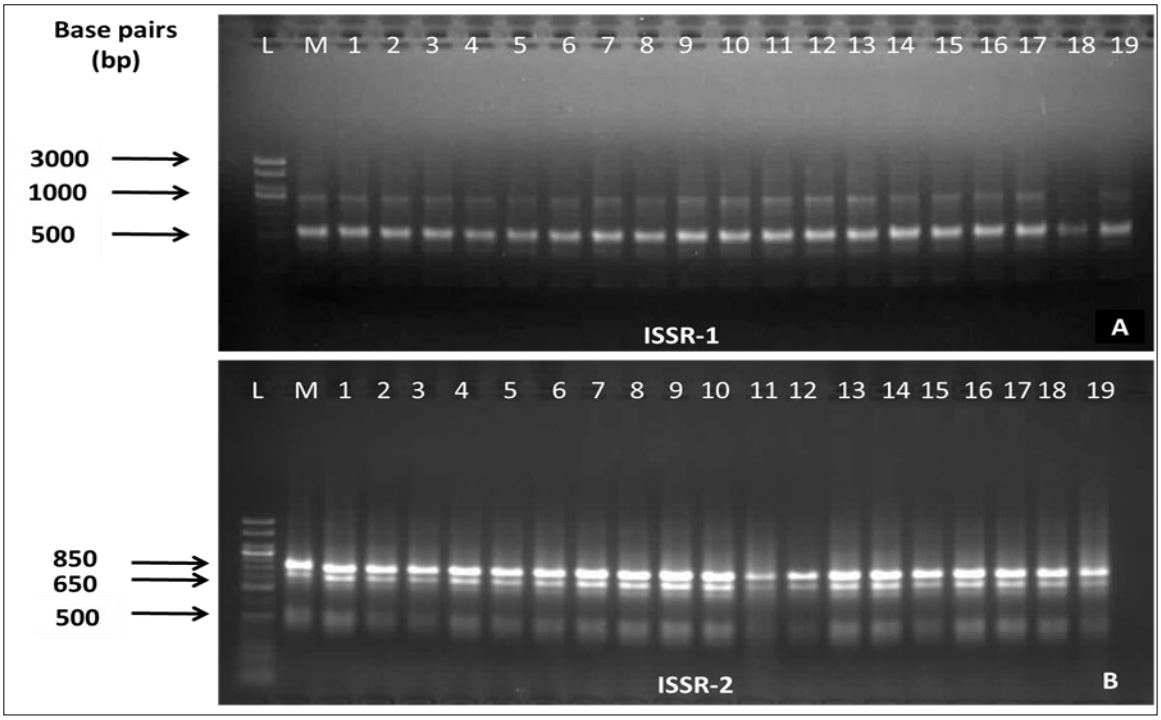

Fig. 6. Genetic fidelity analysis of *in vitro* propagated strawberry plants (var. Chandler) using ISSR markers. A) UBC-864* and B) UBC-825**.

* UBC-864 : ATGATGATGATGATGATG

** UBC-825: ACACACACACACACAC

supplemented with 1.0 mg/L IBA and 3 % sucrose. Genetic uniformity study of in-vitro raised plants with mother plants using molecular markers ensure true-to-type. These findings provide valuable insights into the *in vitro* propagation of strawberries for commercial cultivation. Additionally, careful optimization of protocols for each cultivar is crucial, as it enables the potential for automation through bioreactor systems, which can significantly reduce micropropagation costs. The *in vitro* interventions hold prospective opportunities for biotechnological crop improvement in strawberries. Optimized *in vitro* methodologies have been developed in direct and indirect organogenic experiments across the scientific community, which could be efficaciously utilized to propel genetic transformation and genetic engineering exploits in strawberries. As strawberries are rich in bioactive compounds and secondary metabolites, an optimized choice of explant source and regeneration protocol will help develop large-scale automated aseptic microenvironments with controlled culture parameters and an optimized culture substrate with minimal manual handling.

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Authors' contributions

SP, GR, DK and AK helped in conceptualization. SP and GR helped in the methodology. SP, GR, MK and MSK helped prepare the original draft and MK, DS, SR, AM and SJ helped review and edit the manuscript. All authors read and agreed to final version of the manuscript.

Compliance with ethical standards

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

Ethical issues: None

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