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MICROPROPAGATION AND ANALYSIS OF SOMACLONAL VARIATION IN "MY DA" STRAWBERRY (*Fragaria ananassa* Duch.) USING RAPD MARKER

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ABSTRACT

In vitro propagation of strawberry presents many challenges that must be addressed to enhance survival rates during *ex vitro* acclimation and to ensure the production of high-quality seedlings. This study aimed to develop an optimized protocol for the disinfection, multiplication, rooting, and acclimatization of "My Da" cultivar. Results showed that treating runner segments of "My Da" cultivar with 1% NaClO was effective. For multiplication, Murashige and Skoog (MS) medium supplemented with 0.5 mg. L^{-1} BAP with 0.3 mg. L^{-1} kinetin for "My Da" yielded the highest shoot proliferation, leaf count, and plant height. Rooting was most effective on half-strength MS medium supplemented with 0.3 mg. L^{-1} BAP provided optimal rooting results. In the acclimatization stage, seedlings grown in organic compost was an improved survival rate. RAPD analysis revealed cultivar-dependent somaclonal variation, with the highest polymorphism in the OPK03 marker. The genotype-specific protocol supports efficient large-scale propagation and highlights the need for genetic monitoring in commercial systems.

Keywords: Coconut coir, meristem culture, multiplication, plant regulators, root formation, strawberry.

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INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is globally recognized as one of the most economically important fruits, prized not only for its delightful taste and fragrance but also for its exceptional nutritional profile and health benefits. Rich in vitamins, minerals, and antioxidants, strawberries contribute to reducing cholesterol, lowering blood pressure, and preventing cancer, as well as regulating blood sugar levels, making them a vital component of a balanced diet (Giampieri et al., 2014; Giampieri et al., 2016; Meyers et al., 2003; Hannum, 2004; Basu et al., 2014).

In Vietnam, the "My Da" variety is widely grown due to its heat tolerance, ease of cultivation, high economic value, and consistent fruiting throughout the year. In addition, "My Da" strawberry is particularly valued for its firm flesh and mildly sweet taste, making it suitable for long-distance transport. However, virus infections have drastically reduced the yield and quality of these cultivars, leading to a significant reduction in their cultivation area (Tzanetakis & Martin 2013). The lack of sufficient virus-free plants exacerbates this decline, underscoring the urgent need for effective propagation techniques. Meristem culture has been widely recognized for its potential to generate virus-free plants from infected stocks, ensuring the production of healthy seedlings (Munri et al., 2015; Naing et al., 2019).

Previous studies suggested that the concentrations of auxin in culture media, culture technique and number of subcultures are important factors for the induction of somaclonal variation in an *in vitro* system (Gaafar & Saker, 2006). Somaclonal variation is caused by damaging to chromosomes (insertions, deletions, translocations, mutations, etc), changing in chromosome number (polyploidy or aneuploidy) or changing in methylation of chromatin (Evans et al., 1984; Kaeppler & Phillips, 1993; Peschke & Phillips, 1992). Previous studies indicated that somaclonal variation was found in micropropagated bananas (Sahijram et al., 2003), in apple (Chevreau et al., 1998), peach (Hammerschlag & Oginianov, 1990) and blackberries (McPheeters & Skirvin, 1989). Strawberries too are amenable to *in vitro* somaclonal variation (Battistini & Rosati, 1991).

The aim of this study was to develop a new protocol for micropropagation through the meristem culture and evaluate somaclonal variation in "My Da" variety. In this study, mersitem explants were applied to induce somaclonal variation and Random Amplified Polymorphic DNA (RAPD) was applied to investigate the genetic diversity of "My Da" strawberry.

MATERIALS AND METHODS

In vitro shoot regeneration via strawberry meristem culture

The strawberry runner (10−15 cm) of the "My Da" cultivar was collected in June 2021 from mature plants at a commercial strawberry farm in Da Lat, Vietnam. The runners were harvested from one-year-old plants from 8: 00 am to 3: 00 pm. After cutting, the strawberry runners were wrapped in moist paper towels, placed in plastic bags, stored in a cooler at 12 ^{o}C, and transported to the laboratory at Tra Vinh University, Vietnam for further treatments.

At the laboratory, the collected strawberry runner segments (2−3 cm) were thoroughly washed under a strong stream of tap water for 1–2 min, then shaken in a soap solution for 2–3 min. They were rinsed under running tap water to remove the soap and then washed three times with distilled water. Next, the samples were shaken in 70% alcohol for 1–2 min, rinsed once with sterile distilled water, and then shaken in a NaClO solution at various concentrations (1, 2, and 3%) for 20 min. The sterilized runner segments were rinsed three times with sterile distilled water and used as explant materials for meristem culture.

After sterilization, the base of the runners of three strawberry cultivars that were directly contacted with the disinfectant solution was removed. The runner tips were then placed under a stereomicroscope, and the outer leaf

layers were removed using a scalpel and tweezers until the meristem tissue (dome-shaped shoot tip) was exposed. The meristems (0.3 mm) were dissected from the sterilized runners and then cut and placed in sterilized Petri dishes containing Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) with the addition of 0.3 $mg.L^{-1}$ 6-benzylaminopurine (BAP), 30 $g.L^{-1}$ sucrose, and 9 g. L^{-1} agar, and the pH of 5.8. The experiment was performed in a completely randomized design with one factor and five replicates, with each repetition containing 1 dish, and each dish containing 4 meristems. The meristem cultures were placed in a culture room at 22−25 ^{o}C, under a light/dark cycle of 16 h/8 h, and light intensity of 36–48 $\mu mol.m^{-2}.s^{-1}$. After 8 weeks of culture, the percentage of contamination, viability, and death of meristems were recorded and calculated.

Effect of kinetin and BAP on shoot multiplication from meristem-derived plantlets

After 12 weeks of culture, the regenerated shoots (0.5 cm) from meristems were separated and placed in MS medium supplemented with 30 $g.L^{-1}$ sucrose, 9 $g.L^{-1}$ agar, BAP (0.1, 0.3, and 0.5 $mg.L^{-1}$), and Kinetin (0.1, 0.2, 0.3 $mg.L^{-1}$) either in combination or individually, with a pH of 5.8. A phytohormone-free medium was used as a control. The experiment was performed with a completely randomized design with 3 factors, 3 replicates, 3 bags per replicate, and 4 samples per bag. The subcultures were incubated in a culture room at 22−25 ^{o}C, under a light/dark cycle of 16h/8h, and light intensity of 40 ± 5 $\mu mol.m^{-2}.s^{-1}$. After 6 weeks of the subculture, the number of shoots showing proliferation, leaf number, and shoot height were recorded (Ai et al., 2023).

Evaluation of root induction and plant growth in strawberry cultivars

The same size of regenerated shoots with 5 to 6 leaves were separated and placed in a root-inducing medium consisting of 1/2 MS supplemented with 30 $g.L^{-1}$ sucrose, 9 $g.L^{-1}$ agar, α-naphthaleneacetic acid (NAA) (0.1, 0.3, 0.5, 0.7, 1.0 $mg.L^{-1}$), and BAP (0.1, 0.3, 0.5 $mg.L^{-1}$), either in combination or individually, with a pH of 5.8. A phytohormone-free medium was used as a control. The experiment was conducted with a completely randomized design with 3 factors, three replicates, 3 bags per replicate, and 4 samples per bag. The regenerated shoots were incubated in a culture room at 22−25 ^{o}C, under a light/dark cycle of 16 h/8 h, and light intensity of 36−48 $\mu mol.m^{-2}.s^{-1}$. The root number per shoot, root length, and seedling height were recorded and evaluated after 6 weeks of the subculture (Ai et al., 2023).

***Ex vitro* acclimatization of micro-propagated strawberry plants**

Six-week-old rooted shoots were removed from the root-induced medium, washed to remove agar traces, and then transferred to the tissue-cultured plant bags. The tissue-cultured plant bags containing strawberry shoots were kept at room temperature for 7 days before planting in the growing medium. When the strawberry plants had around 10 roots, an average height of 4−5 cm, and about 10 true leaves, they were moved to a greenhouse to acclimate to outdoor conditions for 3−4 days. The plants were then planted in an organic fertilizer substrate (manure composted with *Trichoderma* fungus and coconut coir in a 1:1:1 ratio), coconut coir pellets, or organic fertilizer and coconut coir in the ratio of 1:1. The experiment was conducted completely randomly with 9 replicates with 20 plants per replicate. The survival rate (percentage of the surviving plants per total plants), average plant height (measured from the root collar to the tip of the highest leaf of the plant using a ruler), average number of leaves (count the total number of leaves on the stem from the first true leaf to the topmost green leaf), and other plant growth characteristics were observed and recorded after 6 weeks.

Molecular assay for selected somaclonal variants

The leaves of the acclimated variety were used as the samples in the population. The DNA from 6 shoots was isolated using DNeasy® Plant Pro Kit (Qiagen).

Subsequently, they were amplified by the Polymerase Chain Reaction (PCR) machine. Each reaction mixture contained 4,5 µL Master mix one tag (New England Biolabs), 1 µL primer as listed in Table 1 at 10 ng.µL^{-1}; 2,5 µL distilled water and 2 µL of 50 ng.L^{-1} genomic DNA. The PCR consisted of pre-denaturation at 94 ^{o}C for 5 minutes, 40 cycles at 94 ^{o}C for 15 seconds (denaturation), 32 ^{o}C for 60 seconds (annealing) and 15 seconds at 72 ^{o}C (extension), post-extension at 72 ^{o}C for 5 minutes. The PCR products were resolved on 2% agarose and electrophoresed at a constant voltage of 100 V for 70 mins and then the bands were stained with 6X GelRed Loading Buffer with Tricolor and visualized under a UV transiluminator. A 1 kb DNA ladder was used as a molecular standard. The samples were scored based on the presence (coded as 1) or absence (coded as 0) of the same size bands. The primers that showed polymorphic bands and/or scorable bands were selected.

Table 1. The list of primers and their sequences used in the RAPD assay (Morales et al., 2011)

Number	Name primer	Primer sequencing (5'-3')	Annealing temperature
1	OPA16	AGCCAGCGAA	32 ^{o}C
2	OPA17	GACCGCTTGT	32 ^{o}C
3	OPA20	GTTGCGATCC	32 ^{o}C
4	OPG09	CTGACGTCAC	32 ^{o}C
5	OPG11	TGCCCGTCGT	32 ^{o}C
6	OPG14	GGATGAGACC	32 ^{o}C
7	OPG18	GGCTCATGTG	32 ^{o}C
8	OPK01	TGC CGA GCT G	34 ^{o}C
9	OPK02	GTG AGG CGT C	34 ^{o}C
10	OPK03	CCC TAC CGA C	34 ^{o}C

Statistical analysis

Data were entered using Microsoft Excel and statistically analyzed using Stagraphics 19 software. All data were shown as mean ± standard deviation (SD).

RESULTS

Shoot regeneration from meristems of various strawberry cultivars

Meristems excised from "My Da" cultivar' was cultured on MS medium supplemented with 0.3 mg.L^{-1} BAP to induce shoot regeneration. After 8 weeks of culture, the meristems exhibited noticeable swelling, and by 12 weeks, shoot primordia were clearly visible. The response to shoot regeneration is highest at 1−2% NaOCl (80%) (Fig. 1). By the 16th week, the shoot primordia had developed into complete plantlets, which were subsequently utilized as explants for further shoot multiplication experiments.

Effect of kinetin and BAP on shoot multiplication from meristem-derived plantlets

Plantlets derived from meristems in the previous experiment were cultured on MS medium supplemented with either Kinetin (Kn) or BAP, individually or in combination, to evaluate their effects on shoot multiplication. As presented in Tables 2 and Figure 2, the addition of BAP significantly enhanced the shoot multiplication rate compared to both the control (0 mg.L^{-1}) and Kn treatments.

Interestingly, when plantlets were cultured on media containing various concentrations of Kinetin (Kn) and BAP in combination, a significant improvement in shoot multiplication was observed across all cultivars. However, the number of shoots produced per plantlet varied depending on the specific concentrations and combinations of Kn and BAP.

The combinations of Kn (0.2 mg/L) with BAP (0.3 mg/L) and Kn (0.3 mg/L) with BAP (0.5 mg/L) produced the highest shoot multiplication rates, with an appropriate number of leaves per shoot and adequate shoot length. Notably, the shoot multiplication rate was higher with the combination of Kn (0.3 mg/L) and BAP (0.5 mg/L) compared to Kn (0.2 mg/L) and BAP (0.3 mg/L). These results highlight that the optimal concentrations and combinations of Kn and BAP for shoot multiplication, with the best outcomes determined by both shoot number and overall plantlet quality.

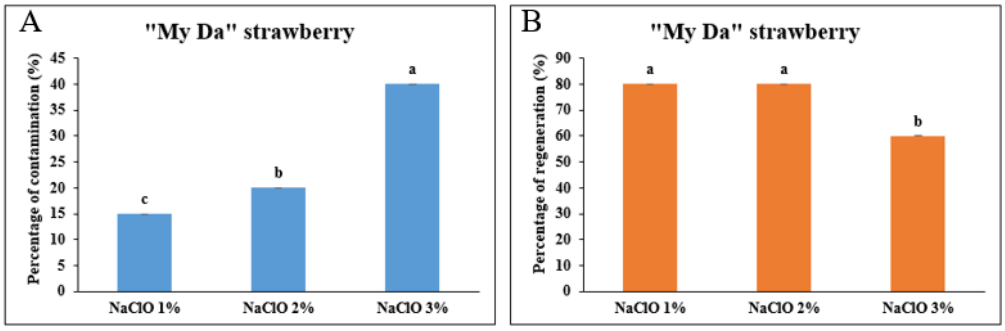

Figure 1. Effect of different concentrations of NaClO on "My Da" strawberry meristem viability during *in vitro* establishment. Different letters (a-c) in the column indicate the significant differences among treatments (Duncan, $p = 0.05$). Data were presented as mean ± SD

Table 2. Effect of various concentrations and combinations of BAP and kinetin on multiplication of "My Da" strawberries

Kinetin (mg. L^{-1})	BAP (mg. L^{-1})	Number of shoots per plant (shoots)	Number of leaves per shoot (leaves)	Shoot height (cm)
0	0	1.7 ± 1.1 f	10.1 ± 3.9 a	4.3^{a} ± 1.1a
	0.1	6.0 ± 3.4 cdef	5.9 ± 1.6 ef	2.8 ± 0.6 cdef
	0.3	6.2 ± 2.2 cdef	4.4 ± 2.7 f	2.1 ± 0.4 fg
	0.5	5.1 ± 1.9 def	4.6 ± 1.4 ef	1.5 ± 0.4 g
0.1		2.2 ± 1.7 f	8.2 ± 1.9 bc	3.4 ± 0.8 abcd
0.2		2.7 ± 2.3 ef	7.8 ± 1.6 cd	3.3 ± 0.7 bcde
0.3		2.3 ± 1.1 f	9.9 ± 1.9 ab	3.7 ± 0.5 ab
0.1	0.1	7.9 ± 10.3 bcde	4.4 ± 1.2 f	3.7 ± 1.4 abc
	0.3	10.1 ± 7.9 abcd	5.4 ± 2.1 ef	3.0 ± 1.0 bcdef
	0.5	9.4 ± 3.3 abcd	5.3 ± 1.8 ef	2.6 ± 0.8 def
0.2	0.1	8.8 ± 7.5 bcd	6.1 ± 1.9 def	3.3 ± 1.2 bcde
	0.3	11.9 ± 10.8 ab	5.6 ± 1.9 ef	3.2 ± 1.1 bcde
	0.5	10.8 ± 7.2 abc	5.0 ± 2.0 ef	2.5 ± 0.8 ef
0.3	0.1	9.4 ± 6.7 abcd	6.3 ± 2.2 de	3.2 ± 1.5 bcde
	0.3	9.1 ± 5.1 bcd	5.0 ± 1.9 ef	3.0 ± 1.0 bcdef
	0.5	14.8 ± 11.7 a	5.4 ± 2.1 ef	2.7 ± 0.7 def
Average		7.4 ± 41.4	6.2 ± 4.5	3.0 ± 0.8
CV%		86.9	33.9	30.5

Note: Different letters in the column indicate the significant differences among treatments (Duncan, p=0.05). Data were presented as mean ± SD.

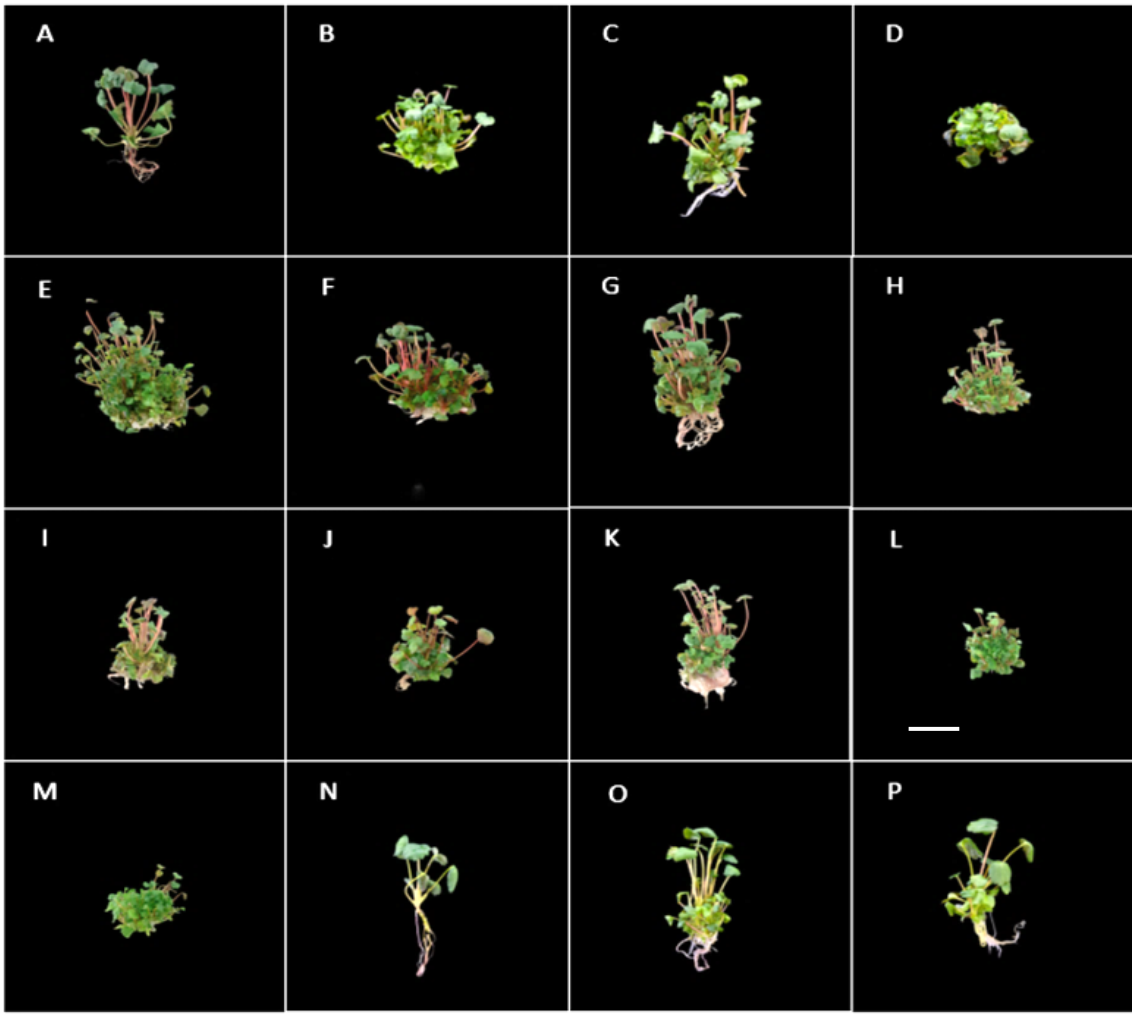

Figure 2. Effects of various concentrations and combinations of BAP and kinetin on shoot proliferation of "My Da" strawberries after 6 weeks of subculture. A, Control; B, 0.1 mg.L^{-1} BAP; C, 0.3 mg.L^{-1} BAP; D, 0.5 mg.L^{-1} BAP; E, 0.1 mg.L^{-1} BAP + 0.1 mg.L^{-1} Kinetin; F, 0.1 mg. L^{-1} BAP + 0.2 mg. L^{-1}Kinetin; G, 0.1 mg. L^{-1} BAP + 0.3 mg. L^{-1} Kinetin; H, 0.3 mg.L^{-1} BAP + 0.1 mg.L^{-1} Kinetin; I, 0.3 mg.L^{-1} BA + 0.2 mg.L^{-1} Kinetin; J, 0.3 mg.L^{-1} BAP + 0.3 mg.L^{-1} Kinetin; K, 0.5 mg.L^{-1} BAP + 0.1 mg.L^{-1} Kinetin; L, 0.5 mg.L^{-1} BAP + 0.2 mg.L^{-1} Kinetin; M, 0.5 mg.L^{-1} BAP + 0.3 mg.L^{-1} Kinetin; N, 0.1 mg.L^{-1} Kinetin; O, 0.2 mg.L^{-1} Kinetin; and P, 0.3 mg. L^{-1} Kinetin. The scale bar is 1 cm for all photos

Evaluation of root induction and plant growth in strawberry cultivars

Uniformly sized shoots were selected from the previous experiment and cultured on MS medium supplemented with varying concentrations of BAP or NAA, individually or in combination, to evaluate root induction and overall plant growth (Table 3 and Fig. 3). The results indicated that BAP positively influenced rooting and plant growth, although it did not affect the number of leaves per plant. Regarding the effect of NAA on root formation, the data showed that NAA enhanced root induction but resulted in a reduction in root length and overall plant growth at higher concentrations.

When shoots were cultured on media containing combinations of NAA and BAP, differential responses in rooting and plant growth were observed in the "My Da" cultivar. Notably, combinations where the concentration of either NAA or BAP exceeded 0.1 mg/L had a detrimental effect on rooting, plant growth, or both. Therefore, the combination of NAA (0.1 mg/L) and BAP (0.1 mg/L) was identified as optimal, promoting significant improvements in both rooting and plant growth. These findings demonstrate that the effects of NAA and BAP on rooting and growth are strongly influenced by the specific concentrations, with certain combinations proving more effective in enhancing plant development.

Table 3. Effects of various concentrations and combinations of NAA and BAP on root formation and development of "My Da" strawberries

NAA ($mg.L^{-1}$)	BAP ($mg.L^{-1}$)	Number of roots per plant (roots)	Root length (cm)	Number of leaves per plant (leaves)	Plant height (cm)	Callus formation
0	0	7.2 ± 2.0 b	5.7 ± 1.6 bcd	14.0 ± 2.4 ab	4.2 ± 0.5 abcd	-
	0.1	9.9 ± 3.4 ab	5.7 ± 1.4 bcd	10.4 ± 2.7 bc	5.2 ± 1.2 a	-
	0.3	9.2 ± 2.3 ab	6.0 ± 2.0 bcd	10.1 ± 1.8 bc	5.1 ± 1.2 a	-
	0.5	10.3 ± 2.5 ab	6.8 ± 1.2 abc	10.0 ± 1.3 bc	5.0 ± 0.8 a	-
0.1	0	8.4 ± 2.1 ab	5.4 ± 3.8 bcd	13.1 ± 2.5 ab	4.9 ± 0.8 ab	-
	0.1	12.4 ± 3.3 ab	9.3 ± 5.7 ab	15.0 ± 4.4 a	5.0 ± 0.8 a	-
	0.3	14.4 ± 3.0 a	10.8 ± 6.8 a	13.1 ± 3.8 ab	4.3 ± 0.7 abc	-
	0.5	10.4 ± 4.6 ab	5.7 ± 3.7 bcd	11.9 ± 3.0 abc	4.5 ± 0.4 abc	-
0.3	0	13.6 ± 4.8 ab	5.0 ± 2.9 bcd	10.2 ± 1.7 bc	4.4 ± 0.9 abc	++
	0.1	12.6 ± 3.5 ab	4.2 ± 3.3 cd	8.3 ± 2.5 c	4.5 ± 0.7 abc	++
	0.3	9.0 ± 3.2 ab	2.9 ± 1.3 cd	8.0 ± 1.6 c	4.0 ± 0.8 abcd	++
	0.5	9.8 ± 3.3 ab	2.9 ± 1.4 cd	8.2 ± 2.6 c	3.4 ± 1.0 cd	+
0.5	0	14.6 ± 5.3 a	2.9 ± 1.5 cd	11.4 ± 1.7 abc	3.6 ± 0.5 bcd	+
	0.1	10.4 ± 2.9 ab	3.4 ± 1.6 cd	10.0 ± 1.1 bc	3.9 ± 0.8 abcd	+
	0.3	13.0 ± 3.1 ab	3.2 ± 1.2 cd	12.1 ± 1.7 abc	4.2 ± 0.6 abcd	+
	0.5	9.3 ± 2.6 ab	2.2 ± 0.5 d	11.6 ± 2.1 abc	4.0 ± 0.9 abcd	+
0.7	0	9.7 ± 4.0 ab	2.3 ± 0.8 d	11.2 ± 1.0 abc	4.3 ± 0.5 abc	+
	0.1	9.7 ± 3.1 ab	2.0 ± 0.6 d	12. 2 ± 3.3 abc	3.4 ±0.6 cd	++
	0.3	8.7 ± 3.2 ab	2.8 ± 1.0 cd	12.2 ± 2.8 abc	4.3 ± 0.9 abc	+
	0.5	10.8 ± 4.8 ab	3.1 ± 1.3 cd	11.0 ± 1.3 abc	4.9 ± 0.4 ab	+
1.0	0	9.3 ± 4.4 ab	2.3 ± 0.6 d	10.7 ± 1.8 bc	4.7 ± 0.5 abc	++
	0.1	9.6 ± 4.3 ab	2.4 ± 0.5 cd	11.3 ± 2.4 abc	4.4 ± 0.7 abc	+
	0.3	8.7 ± 3.4 ab	1.7 ± 0.6 d	9.8 ± 1.1 bc	2.9 ± 0.6 d	+
	0.5	9.7 ± 2.9 ab	2.5 ± 1.9 cd	10.4 ± 2.2 bc	3.6 ± 0.3 bcd	++
Average		10.5 ± 12.4	4.2 ± 6.4	11.1 ± 5.6	4.3 ± 0.6	
CV%		33.7	60.1	21.3	17.5	

Note: Different letters in the column indicate the significant differences among treatments (Duncan, $p = 0.05$). Data were presented as mean ± SD. -, no callus; +, small callus; and ++, large callus.

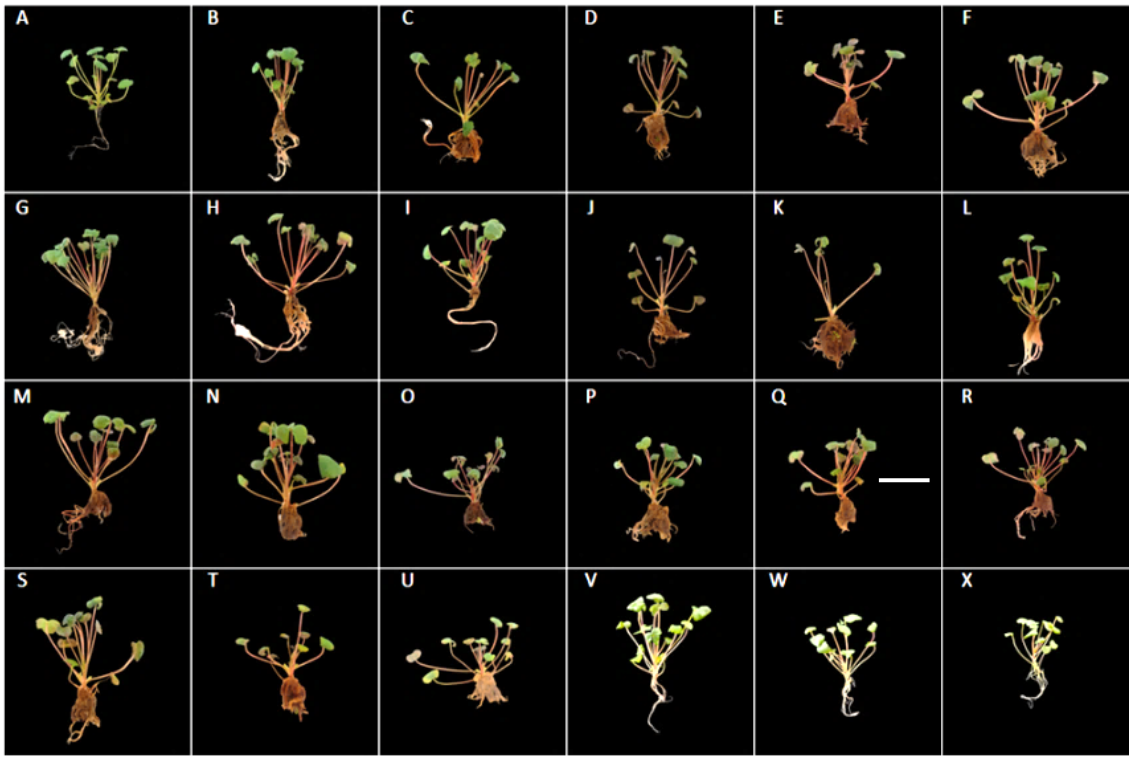

Figure 3. Effects of various concentrations and combinations of NAA and BAP on root information and development of "My Da" strawberries after 6 weeks of subculture. A, Control; B, 0.1 mg.L^{-1} NAA; C, 0.3 mg.L^{-1} NAA; D, 0.5 mg.L^{-1} NAA; E, 0.7 mg.L^{-1} NAA; F, 1 mg.L^{-1} NAA; G, 0.1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP; H, 0.1 mg.L^{-1} NAA + 0.3 mg.L^{-1} BAP; I, 0.1 mg.L^{-1} NAA + 0.5 mg. L^{-1} BAP; J, 0.3 mg. L^{-1} NAA + 0.1 mg. L^{-1} BAP; K, 0.3 mg.L^{-1} NAA + 0.3 mg.L^{-1} BAP; L, 0.3 mg.L^{-1} NAA + 0.5 mg.L^{-1} BAP; M, 0.5 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP; N, 0.5 mg. L^{-1} NAA + 0.3 mg. L^{-1} BAP; O, 0.5 mg. L^{-1} NAA + 0.5 mg.L^{-1} BAP; P, 0.7 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP; Q, 0.7 mg.L^{-1} NAA + 0.3 mg.L^{-1} BAP; R, 0.7 mg.L^{-1} NAA + 0.5 mg.L^{-1} BAP; S, 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP; T, 1 mg.L^{-1} NAA + 0.3 mg.L^{-1} BAP; U, 1 mg.L^{-1} NAA + 0.5 mg.L^{-1} BAP; V, 0.1 mg.L^{-1} BAP; W, 0.3 mg.L^{-1} BAP; and X, 0.5 mg.L^{-1} BAP. The scale bar is 1 cm for all photos

Ex vitro acclimatization of micro-propagated strawberry plants

Growing seedlings in *ex vitro* conditions is often challenging, with a high mortality rate due to the plants being succulent and easily infected. Therefore, the use of an appropriate cultivation substrate is an important factor that determines the survival and development of the seedlings. After root formation, strawberry plants were transferred to different substrates to evaluate the survival rate and growth characteristics of micro-propagated strawberries.

In "My Da" strawberries, the plants showed optimal survival rates (62.9%) and the highest plant height (8.5 cm) on organic compost substrate (S2). Coconut coir combined with organic compost (S3) achieved a survival rate of 54.8%, with no statistically significant difference compared to the coconut coir pellet substrate. The average number of leaves across the three substrates was similar and ranged from 11.3 to 12.6 leaves (Figs. 4–5).

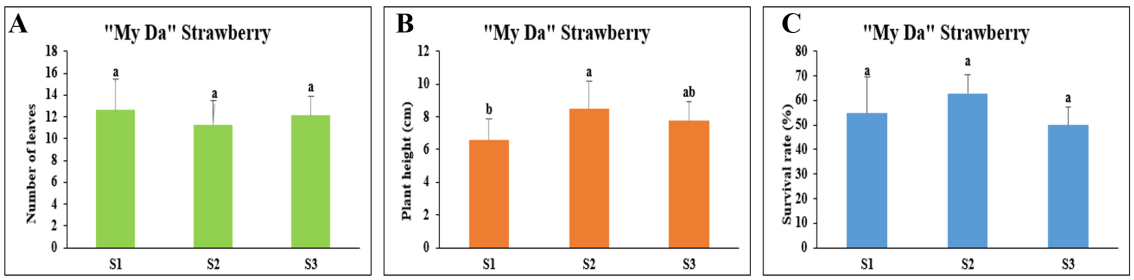

Figure 4. Effect of different substrates on the survival ability and plant development of micro-propagated strawberries during acclimatization. S1, coconut coir pellets; S2, organic fertilizer; and S3, coconut coir pellets+organic fertilizer (1:1). Data were presented as mean ± SD. Different letters in the column indicate the significant differences among treatments (Duncan, $p = 0.05$)

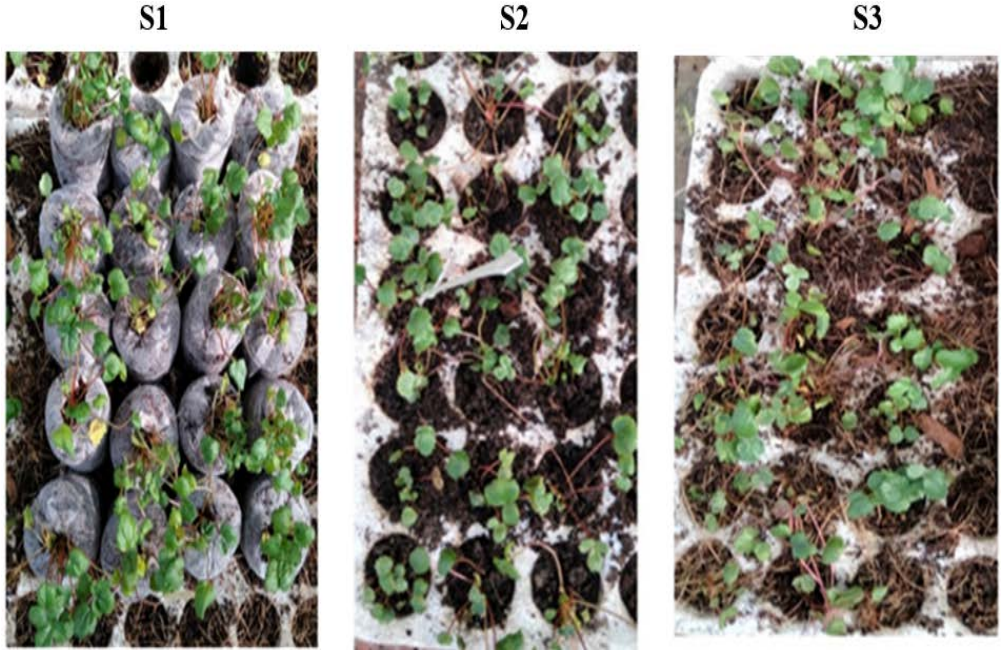

Figure 5. Strawberry seedlings of "My Da" after 2 months of growth on different substrates. S1, coconut coir pellets; S2, organic fertilizer; and S3, coconut coir and organic fertilizer (1:1). The scale bar is 4 cm for all photos

Molecular assay for selected somaclonal variant

In order to determine the source of phenotypic variantions that occurred in the micropropagated cultures and plants, molecular analysis using RAPD markers was conducted. Plants with normal phenotype and exhibited several phenotypic variations (thicker stem, darker purple stem) were chosen for RAPD analysis using 10 primers. However, primer OPA11 did not give amplified bands. The results showed that primer OPG09 generated 6 scorable bands without polymorphic bands. (Table 4 and Fig. 6).

Table 4 showed that a total 82 scorable bands were amplified with an average 9.11 bands per primer. The number of polymorphic bands ware recorded 29/82 scorable bands. The OPG18 showed the lowest percentage of polymorphic bands was 23.07%, compared to the OPK03 primer (77.78%) variety (Fig. 6).

Table 4. The number of amplified products and polymorphic bands in the population using 9 RAPD primers for "My Da" strawberry

Primer	Numer of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	PIC	Rp	PCR amplified size (bp)
OPA16	12	6	50	0.44	6	390–1900
OPA17	10	3	30	0.39	3	400–1850
OPA20	7	2	28.57	0.47	2.33	410–2170
OPG09	6	0	0	0	0	500–1600
OPG14	6	2	33.33	0.36	1.67	500–1850
OPG18	13	3	23.07	0.35	4.33	230–1700
OPK01	8	3	37.5	0.33	4	300–1800
OPK02	11	3	27.27	0.28	3.67	220–1200
OPK03	9	7	77.78	0.28	11.67	200−1400
Total	82	29	-	-	-	-
Average	9.11	3.22	31.17	0.32	4.07	-

Note: *PIC: polymorphic Information Content; Rp: Resolving Power.

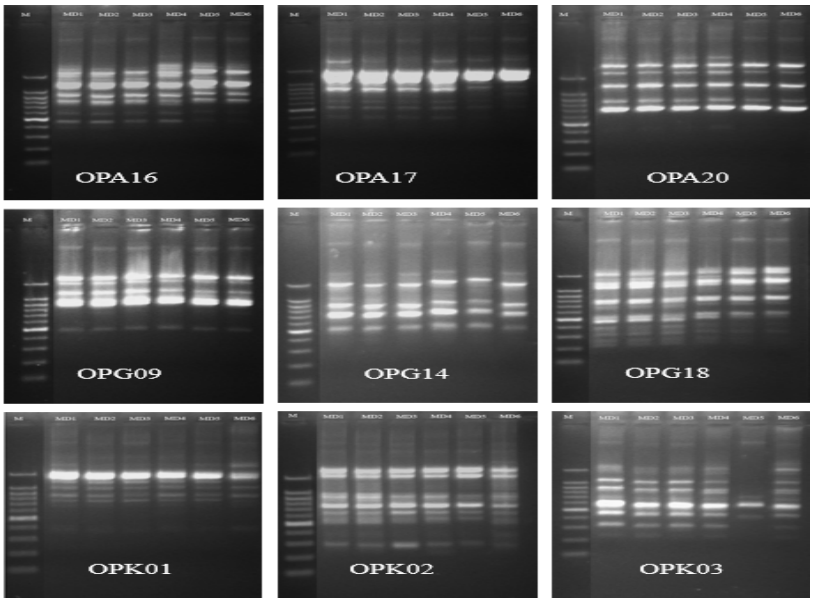

Figure 6. RAPD markers My Da. M: DNA ladder (100bp). MD: My Da; MD1: mother plant; MD 2-MD6: somaclonal variation plants

DISCUSSION

Micropropagation techniques using various phytohormones and types of explants have been developed and improved for the reproduction of strawberries (Borkowska, 2001; Debnath, 2005; Biswas et al., 2009; Munir et al., 2015). However, no studies have established micropropagation systems for "My Da" strawberry. To achieve numerous of disease-free outcomes, the first step is to determine the appropriate sterilization method. The initial sample treatment is crucial as it determines the success of the micropropagation process; creating a sterile sample source is a key step in providing materials for the subsequent stages of the culture process. Therefore, in this study, we established an efficient micropropagation

protocol for “My Da” strawberry cultivar from the meristem to obtain good-quality and disease-free regenerated plants for the local farmers. NaClO is a commonly used disinfectant in plant cell tissue culture to eliminate microorganisms that contaminate samples through oxidative processes (creating disulfide bonds that disrupt the metabolic functions of bacterial cells) and also by affecting bacterial DNA (Yildiz & Er, 2002; Yıldız et al., 2012; Jin et al., 2020; Zhang et al., 2021). However, during the disinfection process, concentration and exposure time are closely related to the viability of the plant tissues. Direct exposure of tissues to high concentrations of disinfectant can severely affect the viability and regeneration ability of the samples, and the development of plantlets in tissue culture. Therefore, during the sample sterilisation process, it is advisable to use the lowest effective concentration of disinfectant that yields high efficacy while minimising the impact on plant tissues.

In plant tissue culture, plant growth regulators are crucial components of the medium that determine the developmental pathway of plant cells (Pasternak & Steinmacher, 2024). The success of micropropagation depends on various factors such as genetic type, explant type, medium composition, growth regulators, and environmental conditions (Abdalla et al., 2022). Cytokinins play a pivotal role in plant development, shoot regulation, shoot bud formation, and promoting cell division and growth (Li et al., 2021). In this study, the supplementation of BAP or Kinetin individually did not significantly affect shoot formation, leaf number, and shoot height in the “My Da” cultivar. A previous study illustrated that a Kinetin-containing culture medium inhibits shoot development in *Albizzia lebbeck* (Perveen et al., 2011). BAP-enriched medium has been deemed effective in increasing shoot numbers compared to Kinetin-enriched medium. However, our results revealed that higher concentrations of BAP lead to reduced shoot formation in ‘My Da” strawberries. This result is consistent with previous findings demonstrating that high cytokinin concentrations reduce shoot formation (Müller & Leyser, 2011; Kurepa & Smalle, 2022). A previous study showed that shoot proliferation of *Philodendron erubescens* on MS medium supplemented with 1.0 $mg.L^{-1}$ BAP yielded the best results with an average of 11.2 shoots/explant and 4.7 leaves/explant (Klanrit et al., 2023). The medium supplemented with 0.7 $mg.L^{-1}$ BAP inhibited shoot regeneration of strawberries unless combined with Kinetin. The efficacy of BAP compared to other cytokines has also been reported in various plant species (Muhammad et al., 2007; Khatri et al., 2019; Sessou et al., 2020; Ptak et al., 2023). In this study, BAP combined with Kinetin resulted in the highest number of new shoots; BAP increased shoot numbers, while Kinetin promoted leaf formation. Similarly, the combined BAP and Kinetin strongly stimulated shoot information and development in bananas (Ali et al., 2011).

In this study, the effects of BAP and NAA concentrations on root development were evaluated based on indicators such as the number of roots, root length, number of leaves, and plant height. The results indicate that different concentrations of BAP or NAA, and the interaction between these two growth regulators, significantly influenced the monitored indicators. The 1/2 MS medium supplement with 0.1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP and 0.1 $mg.L^{-1}$ NAA + 0.3 $mg.L^{-1}$ BAP strongly promoted root formation and development in the “My Da” strawberry. A previous study showed that a higher concentration of auxin compared to cytokinin stimulated root formation (Aloni et al., 2006; Sosnowski et al., 2023). Our results are consistent with the previous finding showing that high concentrations of auxin (NAA) alone or in combination with high cytokinin (BAP) inhibit root formation, resulting in poor root development and increased callus formation (Dar et al., 2021). Media containing both BAP and NAA tend to produce callus, which can inhibit root elongation because the callus covers the shoots and prevents root formation. This suggests the need for an adequate balance

of NAA and BAP, as those have opposite effects on root formation and development; a higher concentration of auxins promotes the formation of roots and a higher concentration of cytokinin may inhibit it. The optimal proportion of auxins and cytokinins depends on the plant species used for micropropagation.

During *ex vitro* acclimatization, seedlings need to possess a well-developed root system (Dewir et al., 2015). This allows the seedlings to effectively absorb water and nutrients and ensures they are securely anchored to the substrates. In this study, the organic fertilizer substrate (S2) was suitable for acclimatizing seedlings of "My Da" strawberry, with a higher survival rate than those of other substrates, while coconut coir pellets (S1) and coconut coir pellets+organic fertilizer (S3) were a negative effect of survival rate.

RAPD markers as a molecular tool for analysis of *in-vitro* regenerated plants have been well documented (Palombi & Damiano, 2002). RAPD markers have been used in the detection of somaclonal variation in garlic (Al-Zahim et al., 1999), tomato (Soniya et al., 2001), sugarcane (Devarumath et al., 2007). The use of RAPDs to detect somaclonal variation have been reported in *Hibiscus sabdariffa* (Govinden-Soulange et al., 2010). Polymorphism obtained with RAPD primers could be explained by genotype or explant source, or *in-vitro* culture time, or three-way interactions between initial explants, the culture conditions and the genotype of mother plants (Rani & Raina, 2000; Vencatachalam et al., 2007). In this study, the polymorphism was detected 31.17% in "My Da" strawberry. The results obtained from these plantlets of investigation are promising and suggest that RAPD markers can be utilized as a simple molecular tool to assess the genetic integrity of plants derived *in-vitro* on a commercial scale or integrated in a crop improvement program.

CONCLUSION

The findings achieved in the present study are an optimal protocol for micropropagation and *ex vitro* acclimatization of the "My Da" cultivar. Our results revealed that treatment with 1% NaClO for 20 min showed high efficiency in decreasing contamination and elevating meristem viability after the disinfection in "My Da" cultivar. The MS medium supplemented with 0.5 $mg.L^{-1}$ BAP + 0.3 $mg.L^{-1}$ Kinetin were the optimal media for the shoot proliferation and growth (the highest number of shoots and leaves, and greater plant height). The 1/2 MS medium supplemented with 0.1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP was suitable for root formation. During *ex vitro* acclimatization, organic compost substrate was optimal for "My Da" strawberry. Moreover, using a molecular marker helps evaluate the genetic variation of meristem-derived and conventionally propagated strawberry plants was conducted in these strawberry cultivars.

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