

Establishment of a Cost-Effective Protocol for *In vitro* Propagation of *Fragaria vesca* L. and Study of its Genetic Stability through DNA Markers

Sushobhan Sen, Pulak Das, Moupriya Mondal, Moushumi Chatterjee, Sabyasachi Chatterjee, Indrani Chandra

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ABSTRACT

Fragaria vesca L., a member of the Rosaceae family is widely known for its pomocultural as well as medicinal importance. In the present study, a successful attempt was made for micropropagation of *Fragaria vesca* L. The basal media used was MS medium supplemented with 440 mg/l CaCl_2 and 0.2% phytigel. Different auxins (NAA/IBA) and cytokinins (BAP/KIN/TDZ) were used as PGRs in different combinations and concentrations. The highest number of shoot multiplication (i.e. 12.33 ± 0.75 shoots/explant)

was obtained in BAP(1.0mg/l) in combination with NAA(0.1mg/l). Here the shoots were smaller in size. Whereas, in KIN (2.0mg/l), shoot length was higher, but the number was comparatively smaller (10.00 ± 0.25 shoots/explant) than the BAP treatment. Although some roots were formed along with shoots. The highest number of roots (13.66 ± 0.25 roots/shoot) were formed in half-strength MS supplemented with 1.0mg/l IBA. IBA (1.0mg/l) in combination with BAP (0.1mg/l) resulted in branched roots (13.33 ± 0.45 roots/shoot). A successful and cost-effective acclimatisation process of in vitro grown plantlets was created, with 98% survival rate when transferred into a potting mixture of sand: soil (3:1v/v) with plastic support material to the stem and 50% UV-stabilized shade net. To make the procedure more cost-effective, some leftover plastic bottles, jars, and bowls were used as pots. The genetic stability of micropropagated *Fragaria vesca* L. plants was verified by uniform scorable bands generated by RAPD and ISSR DNA markers.

Sushobhan Sen¹, Pulak Das², Moupriya Mondal³, Moushumi Chatterjee⁴, Sabyasachi Chatterjee⁵, Indrani Chandra^{6*}

¹PhD Research Scholar, ^{2,3,4}PhD Research Scholar (Awarded), ^{5,6}Assistant Professor

^{1,2,3,4,6}Department of Biotechnology, University of Burdwan, Golapbag, West Bengal, India

⁵Department of Botany, Ramananda College, Bishnupur, West Bengal, India.

Email: ichandrabiotech@gmail.com

*Corresponding author

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INTRODUCTION

Strawberry is one of the most economically important horticultural plants. *Fragaria vesca* L. is a wild variety of strawberries belonging to the Rosaceae family also known as Alpine strawberry or Woodland

strawberry. It is native to the northern hemisphere. The plant is well known for its red edible fruits. Apart from its sweet taste, other vegetative parts of *Fragaria vesca* L. are also popular as a folk remedy for treating gastrointestinal, cardiovascular and urinary disorders (Liberal *et al.* 2014). In recent studies there are several reports of anticancer (Seeram *et al.* 2006, Somasagara *et al.* 2012, Baby *et al.* 2018), anti-influenza (Ma *et al.* 2020), anti-depressant (Naz *et al.* 2017), antidiabetic (Yella and Dass 2015, Takács *et al.* 2020) and anti-urolithiasis (Dhole and Yeligar 2020) activity of *Fragaria vesca* L. was reported earlier. Along with numerous phenolics, strawberry is a good source of micronutrients, such as vitamin C and minerals (Giampieri *et al.* 2012). Polyphenols from strawberries, ie- ellagitannins are the most abundant and show high antioxidant activity (Bueno *et al.* 2009). As these plants grow naturally in high altitudes where the temperature is very low, It is very difficult to cultivate them in temperate regions and plain lands. Micropropagation is a possible way to propagate and enhance important bioactive compounds by basal media manipulation (Liberal *et al.* 2014). But hardening of *in vitro* raised plant also has many challenges.

Acclimatization of *in vitro* regenerated plants requires many precautionary measures. There are several issues-including excess transpiration, microbial infection, inability to use soil nutrients directly. (Seon *et al.* 2000, Bag *et al.* 2019), which must be countered to obtain a hardened plant. Initially, higher moisture and low light intensity are required for newly transplanted plantlets to avoid excess transpiration and to maintain water content inside the plant system. Then microbial infection is another threat to hardening. To prevent fungal contamination, fungicides are used. Even after taking these measures sometimes the survival rate is lower than expected. Some other protocols allow *in vitro* grown plantlets to harden directly in modified natural environments to shorten the period of hardening. But after all of those higher expenses is a serious issue for plant tissue culture and hardening.

On the other hand, long-term *in vitro* propagation may induce somaclonal variation due to different concentrations of plant growth regulators and other

environmental factors. So it is important to ensure genetic homogeneity by using various genetic markers like RAPD, ISSR, AFLP. (Butiuc-Keul *et al.* 2016, Amiri *et al.* 2020).

As *Fragaria vesca* L. grows in the alpine region, the present aim of this study is to propagate the plants in plain lands. Therefore standardization of a micropropagation is necessary that must be cost-effective. Another objective is to assess genetic uniformity by using different DNA markers for confirmation of true clones.

Abbreviations

PGR: Plant Growth Regulator
 NAA: 1-Naphthaleneacetic Acid
 IBA: Indole-3-Butyric Acid
 BAP: 6-Benzylaminopurine
 KIN: Kinetin
 TDZ: Thidiazuron
 mg/L: Milligram/Litre
 RAPD: Random Amplified Polymorphic DNA
 ISSR: Inter Simple Sequence Repeat
 ANOVA: Analysis of variance

MATERIALS AND METHODS

Seed collection and surface sterilization

Strawberry seeds were purchased from New Rama Seed Corporation, New Delhi. Seeds were then surface sterilized using 70% alcohol for 30 seconds followed by Tween 20 for 5 mins and 0.1% HgCl₂ solution for 1 min consequently. After each step, the seeds were rinsed thoroughly with sterilized distilled water thrice. The micropropagation process was carried out at the Plant Tissue Culture Laboratory, Department of Biotechnology, The University of Burdwan (latitude and longitude of 23.2324° N, 87.8615° E).

In vitro seed germination and authentication of seed variety

Axenic culture medium was prepared by adding 34.1gm/l MS (Murashige and Skoog) basal medium (Murashige and Skoog 1962) supplemented with 440

mg/l Calcium chloride and 0.2% Phytigel in distilled water at pH 5.8. Surface sterilized seeds were inoculated into conical flasks containing sterilized axenic culture medium under laminar airflow by maintaining aseptic condition and incubated at $25^{\circ}\pm 2^{\circ}\text{C}$ temperature along with 2000lux light intensity, 60% humidity and with a 16 hour photoperiod.

After 28 days of incubation DNA sequencing was performed using plantlets germinated from seed culture and sequence data was submitted to the NCBI database (Gen Bank: MT-657322.1).

Shoot multiplication

For standardization of shoot multiplication, the basal medium different combinations and concentrations were used plant growth regulators (PGRs) along with MS culture media. Shoot apexes from *in vitro* germinated seedlings were used as explants. Various combinations of PGRs were used *viz.* BAP (1.0-4.0 mg/l), BAP/NAA (1.0-4.0 mg/l; 0.1 mg/l), BAP/IBA (1.0-4.0 mg/l; 0.1 mg/l), BAP/TDZ (1.0-4.0 mg/l; 1.0 mg/l), Kin (1.0-4.0 mg/l), Kin/NAA (1.0-4.0 mg/l; 0.1 mg/l), Kin/IBA (1.0-4.0 mg/l; 0.1 mg/l), Kin/TDZ (1.0-4.0 mg/l; 1.0 mg/l), TDZ (1.0-4.0 mg/l), TDZ/NAA (1.0-4.0 mg/l; 0.1 mg/l).

Root induction

For root induction, full strength and 1/2 strength MS media were used along with 0.2% Phytigel (Sigma Aldrich) instead of agar powder. Various concentrations and combinations of PGRs were used NAA (0.5-2.0 mg/l), IBA (0.5-2.0 mg/l), IBA (0.5-2.0 mg/l) + BAP (0.1mg/l), NAA (0.5-2.0 mg/l) + BAP (0.1mg/l).

Hardening

In vitro grown plantlets were acclimatized in a two-step process, where primarily plantlets were hardened in 5 different potting mixtures divided into two experimental sets, one with plastic material support and another without plastic material support to the stem system. Here newly regenerated plants were kept under low light intensity and covered with ventilated plastic bags for 3 days. Then they were transferred to the natural environment with 50% light intensity

using UV stabilized shade net.

DNA extraction and RAPD analysis

The genomic DNA was extracted by the cetyltrimethyl ammonium bromide (CTAB) method (Coen *et al.* 1990) from fresh leaves of conventionally acclimatized *Fragaria vesca* L., new method-oriented acclimatized *Fragaria vesca* and of the mother plant. The quality and concentration of DNA were measured by Nano Drop spectrophotometer (Nano Drop 1000, Thermo Scientific, USA). For RAPD analysis, a total of 18 arbitrary primers were used.

DNA amplification for RAPD and ISSR marker analysis was performed according to a pre-established method (Cui *et al.* 2019). The size of the amplification products was estimated by a 1.5 kbp DNA ladder. The gels were photographed using the gel documentation system (Bio-Rad, Hercules, CA, USA), and only clear and scorable DNA bands were considered.

Statistical analysis

Statistical data were analyzed based on 10 replicates with a triplicate of repetitions. All the data were collected in regular intervals and analyzed through SPSS software (version 24) along with Duncan's test with $p < 0.05$.

RESULT AND DISCUSSION

As *Fragaria vesca* grows in the alpine region, it is a challenge to grow alpine strawberries in Burdwan (West Bengal, India) having a latitude and longitude of 23.2324°N , 87.8615°E because of high temperature and humidity. Farmers can target only the winter season to grow strawberries. Lesser availability of seeds, very low seed germination rate, and less survival capacity make the cultivation of strawberries difficult in West Bengal (India). The taste of strawberry fruit and its medicinal importance draw the attention of the scientific community. So, here is the need for micropropagation technology to gain an adequate number of adapted strawberry saplings which must be healthy, disease-free and much higher survival capacity.



Fig. 1. *In vitro* propagation of *Fragaria vesca* L. A= germination of *Fragaria vesca* L. seeds, B= Plantlet obtained by seed germination, C= *in vitro* shoot Multiplication on MS+ BAP (2.0mg/l), D= *in vitro* shoot Multiplication on MS+TDZ 2.0 mg/l, E= *In vitro* shoot multiplication on MS+KIN 2.0 mg/l, F = *in vitro* root induction on MS+ IBA/BAP (1.0/0.1 mg/l).

In vitro shoot multiplication

In vitro propagation of plantlets lies on optimum concentration and a combination of plant growth regulators. The different PGRs [Cytokinins (BAP, KIN),

Cytokinin precursor (TDZ), Auxin (NAA, IBA)] were used in different concentrations and combinations. Multiple shoots were formed in BAP only (Fig. 1C), but stem length and leaf area were very small, and not suitable for photosynthesis. The vigor of a mul-

Table 1. Effect of Plant growth regulators (PGRs) on *in vitro* shoot multiplication.

PGRs (mg/l)					Shoot Multiplication (Mean±SE)	Shoot length (cm) (Mean±SE)	Root Induction	Callus Induction
BAP	KIN	TDZ	NAA	IBA				
1.0	-	-	-	-	11.67±0.23 ^b	0.79±0.23 ^a	-	-
2.0	-	-	-	-	10.39±0.20 ^{de}	0.54±0.25 ^e	-	-
3.0	-	-	-	-	6.12±0.23 ^p	0.54±0.25 ^e	-	-
4.0	-	-	-	-	3.33±0.23 ^v	0.36±0.20 ^s	-	-
1.0	-	-	0.1	-	12.33±0.40^a	2.98±0.35 ^f	-	-
2.0	-	-	0.1	-	10.66±0.25 ^d	2.98±0.60 ^f	-	-
3.0	-	-	0.1	-	8.69±0.25 ^h	2.39±0.56 ^{ghijkl}	-	-
4.0	-	-	0.1	-	5.42±0.20 ^q	2.44±0.56 ^{ghijkl}	-	-
1.0	-	-	-	0.1	11.0±0.23 ^{bc}	2.89±0.25 ^g	-	-
2.0	-	-	-	0.1	8.0±0.23 ^{hijk}	2.89±0.20 ^g	-	-
3.0	-	-	-	0.1	7.33±0.40 ^m	1.99±0.23 ⁿ	-	-
4.0	-	-	-	0.1	8.66±0.40 ^{hi}	1.66±0.32 ^o	-	-
1.0	-	1.0	-	-	10±0.45 ^{de}	2.89±0.46 ^g	-	+
2.0	-	1.0	-	-	9.66±0.40 ^f	2.89±0.56 ^g	-	+
3.0	-	1.0	-	-	8.0±0.20 ^{hijk}	1.99±0.54 ⁿ	-	-

Table 1. Continued.

BAP	PGRs (mg/l)				IBA	Shoot Multiplication (Mean±SE)	Shoot length (cm) (Mean±SE)	Root Induction	Callus Induction
	KIN	TDZ	NAA						
4.0	-	1.0	-	-	5.33±0.23 ^{qr}	1.66±0.25 ^o	-	-	
-	-	1.0	-	-	7.66±0.23 ^l	2.45±0.48 ^{ghi}	-	-	
-	-	2.0	-	-	6.66±0.23 ⁿ	2.89±0.25 ^{fg}	-	-	
-	-	3.0	-	-	5.33±0.20 ^{qr}	1.66±0.20 ^{no}	-	-	
-	-	4.0	-	-	4.0±0.54 ^{tu}	1.66±0.23 ^{no}	-	-	
-	-	1.0	0.1	-	8.33±0.20 ^{hi}	2.66±0.56 ^{gh}	+	+	
-	-	2.0	0.1	-	7.33±0.35 ^m	2.66±0.25 ^{gh}	+	+	
-	-	3.0	0.1	-	4.66±0.20 ^s	1.33±0.25 ^{no}	-	+	
-	-	4.0	0.1	-	4.33±0.23 st	1.66±0.23 ^{no}	-	+	
-	1.0	-	-	-	8.33±0.25 ^{hi}	4.66±0.26^a	+	-	
-	2.0	-	-	-	10.0±0.25 ^{de}	4.66±0.45^a	+	-	
-	3.0	-	-	-	7.0±0.40 ^m	3.33±0.25 ^e	+	-	
-	4.0	-	-	-	5.33±0.45 ^{qr}	2.45±0.60 ^{ghij}	+	-	
-	1.0	-	0.1	-	8.33±0.20 ^{hi}	4.12±0.48 ^{abc}	+	-	
-	2.0	-	0.1	-	7.66±0.56 ^l	3.66±0.52 ^d	+	-	
-	3.0	-	0.1	-	6.33±0.60 ^s	3.33±0.46 ^e	+	-	
-	4.0	-	0.1	-	5.33±0.23 ^{qr}	2.45±0.25 ^{ghij}	+	-	
-	1.0	-	-	0.1	9.33±0.20 ^{fg}	4.66±0.25^a	+	-	
-	2.0	-	-	0.1	7.66±0.20 ^l	4.33±0.32 ^{ab}	+	-	
-	3.0	-	-	0.1	6.33±0.30 ^o	3.33±0.45 ^e	+	-	
-	4.0	-	-	0.1	5.33±0.60 ^{qr}	2.33±0.65 ^{ghijk}	+	-	
-	1.0	1.0	-	-	7.66±0.60 ^l	4.33±0.23 ^{ab}	+	+	
-	2.0	1.0	-	-	7.33±0.54 ^m	4.33±0.22 ^{ab}	+	+	
-	3.0	1.0	-	-	6.66±0.45 ⁿ	3.66±0.35 ^d	+	+	
-	4.0	1.0	-	-	5.33±0.30 ^{qr}	2.45±0.25 ^{ghij}	+	-	

(The results were expressed as the (Mean±SE) of 7 samples for each treatment with three iterations. Using Duncan's multiple range test, values represented by the same letter did not vary significantly ($p>0.05$). The standard error of the mean is shown here by SE).

multiplied shoot can be better by adding a small amount of auxin (NAA or IBA) with BAP. Optimum shoot multiplication ($12.33±0.45$) was obtained by applying BAP (1.0 mg/l) + NAA (0.1mg/l) among 10 different combinations of PGRs (Table 1). Here, most of the combinations and concentrations of TDZ showed callus induction which was not desirable for direct *in vitro* regeneration (Table1). TDZ provided a good morphological and huge number of multiple shoots in combination with KIN, but it showed a tendency to produce callus along with multiplied shoots (Table 1, Fig. 1D). Similar phenomenon was reported in strawberries by Chung and Ouyang (2021) and other plants by Huang *et al.* (2020), Nikule *et al.* (2020). TDZ is also very active in root formation in combination with NAA (Table 1), but callus induction at the same stage may cause somaclonal variation which is not desirable for direct *in vitro* multiplication. Whereas only Kinetin induced multiple shoots with healthy morphological responses(Naing *et al.*

Table 2. Effect of Plant growth regulators (PGRs) on *in vitro* root induction.

PGRs (mg/l)		Roots/explants (cm) (Mean±SE)	Root length (cm) (Mean±SE)
NAA	-		
0.5		0.96±0.34 ^a	3.66±0.36 ^j
1.0		3.54±0.45 ^l	3.66±0.45 ^j
1.5		1.33±0.34 ^p	1.93±0.23 ^l
2.0		0.0	0.0
NAA(1/2 MS)	-		
0.5		0.0	0.0
1.0		2.11±0.24 ^a	2.66±0.20 ^k
1.5		1.9±0.20 ^p	1.33±0.25 ^m
2.0		0.0	0.0
IBA	-		
0.5		3.66±0.35 ^k	5.66±0.45 ^d
1.0		8.33±0.23 ^f	6.00±0.20 ^e
1.5		7.33±0.25 ^h	4.33±0.25 ^g
2.0		2.69±0.25 ^m	3.97±0.36 ⁱ
IBA(1/2 MS)	-		
0.5		9.54±0.20 ^e	6.00±0.25 ^e

Table 2. Continued.

PGRs (mg/l)		Roots/explants (cm) (Mean±SE)	Root length (cm) (Mean±SE)
1.0		13.66±0.25 ^a	6.33±0.45 ^b
1.5		9.66±0.45 ^d	5.66±0.25 ^d
2.0		4.33±0.20 ⁱ	5.00±0.25 ^f
IBA	BAP		
0.5	0.1	7.66±0.23 ^e	5.33±0.45 ^e
1.0		13.33±0.45^b	6.66±0.25^a
1.5		10.33±0.45 ^e	5.33±0.20 ^e
2.0		5.21±0.20 ⁱ	4.00±0.36 ^h

(The results were expressed as the (Mean±SE) of seven samples for each treatment with three iterations. Using Duncan's multiple range test, values represented by the same letter did not vary significantly ($p>0.05$). The standard error of the mean is shown here by SE)

2019). The number of multiplied shoots was lesser (10.0 ± 0.25) in KIN (2.0mg/l) (Table 1, Fig. 1E) than BAP (1.0mg/l) + NAA (0.1mg/l), but due to longer shoot length KIN at 2.0 mg/l concentration was selected as optimum for shoot multiplication, although KIN along with IBA also provided a good number of multiplied shoots (Table 1).

In vitro root induction

Different root induction strategies showed variable outcomes. As it was mentioned earlier that KIN also induced root formation, IBA and NAA were used as auxin for root induction. The rate of root induction and the number of roots were higher in IBA than in NAA. IBA (1.0mg/l) was optimum for *in vitro* rooting with half-strength MS-Medium (Table 2). But healthier plantlets with profuse root formation were obtained when 0.1mg/l BAP along with 1.0mg/l IBA were added (Table 2, Fig. 1F).

Acclimatization of *in vitro* grown plantlets

The whole experiment for the acclimatization of *in vitro* regenerated plantlets was conducted between November to March from the year 2019 to 2023. In the acclimatization process, primarily a suitable potting mixture was selected from 5 different combinations (Table 3). During the acclimatization process, several problems were observed- fungal infection, rotting of the stem, wrinkling of leaves. It was nec-



Fig. 2. Hardening/acclimatization of *in vitro* propagated plantlets A= Hardening on sand: soil (3:1) with plastic support system, C = Flowering on the Hardened *Fragaria vesca* L. plant, D= Fruit on the hardened *Fragaria vesca* L. plant.

Table 3. *In vitro* acclimation of *Fragaria vesca* L. using different potting mixtures with (W) and without (WS) stem support system under 50% natural light irradiation.

Potting mixture		Survival (%)	No. of leaf	Response
Sand:Soil=1:1	WS	42	6.00±0.25 ^g	Expanded small size leaves, mild fungal infection in leafy stems.
	S	66	9.25±0.25 ^c	Expanded small size leaf with no fungal infection.
Sand:Soil=3:1	WS	52	9.00±0.10 ^{cd}	Expanded large size leaf, mild fungal infection.
	S	98	17.50±0.10^a	Expanded large size dark green leaves with no fungal infection.
Sand:Soil=1:3	WS	14	6.50±0.05 ^f	Expanded small size leaves having fungal infection both in roots and leaves.
	S	68	8.00±0.05 ^e	Expanded small size leaves having mild fungal infection both in roots and leaves.
Soil:Sand:Cocopeat =2:1:1	WS	15	4.00±0.10 ^j	Huge fungal infection, wrinkled yellowish leaves.
	S	38	6.00±0.10 ^{gh}	Relatively less fungal infected shoots with wrinkled yellowish leaves.
Soil:Sand:Cocopeat =1:2:1	WS	24	4.00±0.10 ^j	Huge fungal infection, wrinkled yellowish leaves.
	S	56	9.50±0.05 ^b	Relatively less fungal infected shoots with expanded small leaves.

(The results were expressed as the (Mean±SE) of five samples for each treatment with three iterations. Using Duncan's multiple range test, values represented by the same letter did not vary significantly ($p>0.05$). The standard error of the mean is shown here by SE).

essary to avoid the contact between the stem and the soil mixture. So here, a plastic support material was introduced for that purpose (Fig. 2A & 2B). Mostly potting mixture or soil mixture containing cocopeat showed the least survival support to these newly grown plantlets due to higher water retention. Extra moisture is harmful to roots but suitable for plant pathogens. On the other hand, higher volume soil compared to sand is also unable to provide a sufficient rate of hardening. Whereas, sand: soil= 3:1 with plastic material support showed the highest survival rate (98%) with the best morphological responses (Table 3, Fig. 2A-2B). So, an excess amount of sand is required to encounter these particular issues. On the other hand, several environmental factors like- light source and intensity can be key factors for successful hardening (Ali *et al.* 2005, Faisal and Anis 2010). Plantlets with an optimized potting mixture were al-

lowed to acclimatize under different light intensities. Under 50% shadenet became an ideal environment for hardening where puckering of leaves was initially a problem due to excess transpiration. But adequate irrigation could revive plant condition within 7 days of transplantation. After 30 days of the experiment, 50% shaded condition showed the least number of plant death which was nearly 2%. After standardizing a protocol of hardening leftover plastic bottles, jars and bowls were used as a pot to acclimatize *in vitro* grown *Fragaria vesca* L. and satisfactorily the results were the similar to the previous experiment. This protocol for hardening was standardized for the first time and no such report was found in this context.

Assessment of genetic stability

Long-term *in vitro* culture and environmental factors

Table 4. List of ISSR primers and banding pattern.

Primer code	Primer sequence(5'-3')	No. of scorable bands	Approximate range of amplification (bp)
UBC-813	CTCTCTCTCTCTCTT	9	250-1250
UBC-819	GTGTGTGTGTGTGTGA	12	150-1100
UBC-829	TGTGTGTGTGTGTGTC	6	190-1250
UBC-836	AGAGAGAGAGAGAGGYA	11	210-1000
UBC-847	CACACACACACACARC	9	150-780

Total number of scorable bands= 47

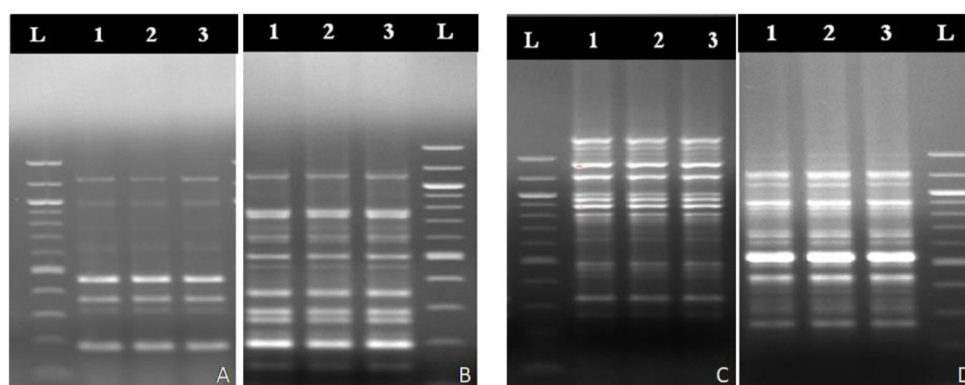


Fig. 3. Genetic homogeneity assessment through molecular markers A= UBC 829 (ISSR Marker), B=UBC819 (ISSR Marker), C= OPD 16 (RAPD Marker), D= OPP13 (RAPD Marker), L= ladder, 1= mother plant, 2-3= *in vitro* propagated plantlets.

Table 5. List of RAPD primers and banding pattern.

Primer code	Primer sequence (5'-3')	No. of scorable bands	Approximate range of amplification (bp)
OPA-15	TGCCGAGCTA	17	280-1900
OPAB-04	GGCACGCGTT	11	400-1600
OPC-5	GATGACCGCC	10	320-1600
OPD-1	ACCGCGAAGG	12	320-1800
OPD-3	GTCGCCGTCA	14	400-1600
OPD-16	AGGGCGTAAG	14	280-2000
OPL-12	GGGCGGTACT	11	320-1600
OPP-13	GGAGTGCCTC	11	250-1500
OPV-5	TCCGAGAGGG	12	350-2500
OPV-14	AGATCCCGCC	8	450-1800

Total number of scorable bands= 120

during acclimatization may cause genetic variability. As *Fragaria vesca* L. is diploid, it is a suitable choice for genomic study (Żebrowska *et al.* 2018). 5 Inter simple sequence repeat (ISSR) DNA markers generated 47 scorable bands ranging from 150-1250bp with an average of 9.4 bands/marker (Table 4). UBC819 produced the highest number of bands (12). All the ISSR markers showed no polymorphic bands with 100% genetic stability (Fig. 3A-3B). Another, 10 different Random Amplified Polymorphic DNA (RAPD) were used to test genetic uniformity. Here a total of 120 bands was generated, ranging from 250 to 2500bp with an average of 12 bands per primer (Table 5). The number of bands generated by a single RAPD primer varied from 8 to 17. RAPD analysis detected no polymorphic bands between the mother plant and regenerated plants (Table 5, Fig. 3C-3D). Both RAPD and ISSR markers both are denoted as

Dominant DNA markers and they are easy to handle (Rathore *et al.* 2014, Butiuc-Keul *et al.* 2016, Jena and Chand 2021). *In vitro* grown *F. vesca* showed genetic uniformity and stability as compared to *in vivo* grown plants. There are many reports of genetic homogeneity assessment of other species of *Fragaria* sp. exposed to different environmental conditions (Congiu *et al.* 2000, García *et al.* 2002, Morales *et al.* 2011, Naing *et al.* 2019).

CONCLUSION

In the present investigation, the micropropagation protocol of *Fragaria vesca* L. has been established. A new hardening protocol has been standardized which is cost-effective and the survival rate is maximum i.e., 98% approx. genetic stability of the *in vitro* grown plants has been confirmed by using different DNA

markers. Therefore, somaclonal variation is absent in newly grown plants.

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DECLARATIONS

The authors declare that the work contained in this paper is original and has not been submitted for publication to any other journal.

AUTHOR CONTRIBUTIONS

The experiment was designed by Sushobhan Sen and Dr. Indrani Chandra. Collection of plant, sample preparation and mostly research work was executed by Sushobhan Sen. Dr. Pulak Das helped in molecular marker analysis. Moupriya Mondal Significantly contributed in harding of plantlets. Moushumi Chatterjee statistically analyzed all the datasets. Dr. Sabyasachi Chatterjee monitored the research significantly. All authors read the manuscript, revised critically and approved the final version.

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