International Journal of Plant Developmental Biology ©2010 Global Science Books

Sustainable *in Vitro* Propagation and Clonal Fidelity in Strawberry

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ABSTRACT

An efficient protocol was developed for sustainable mass multiplication of strawberry (*Fragaria* × *ananassa* Duch cv. 'Chandler') through multiple shoot induction. Shoot buds were most successfully induced from runner tips, when these were cultured on Murashige and Skoog (MS) medium supplemented with 0.1 mg l^{-1} α-naphthalene acetic acid and 1 mg l^{-1} 6-benzylaminopurine. Maximum shoot multiplication and proliferation occurred on MS medium with 2 mg l^{-1} kinetin. MS basal medium with 0.5 mg l^{-1} indole-3-acetic acid and 2 g l^{-1} activated charcoal proved to be the best for root induction and elongation from separated shoots. Autoclaved sand and soil with intermittent water spraying could optimize the primary acclimatization of *in vitro* generated plantlets. A pot mixture combined with sand, soil and farm yard manure (1: 1: 1 v/v) resulted in the acclimatization of 92% of plantlets. To prove the genetic uniformity of propagules, *in vitro* generated clones were DNA fingerprinted using selected ISSR primers.

Keywords: acclimatization, *Fragaria* × *ananassa* Duch, genetic uniformity, ISSR, multiple shoots
Abbreviations: AC, activated charcoal; **BA**, benzyladenine; **BAP**, 6-benzylaminopurine; **DMRT**, Duncan's multiple range test; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **ISSR**, inter simple sequence repeat; **KIN**, kinetin or 6-furfurylaminopurine; **MS**, Murashige and Skoog (1962); **NAA**, α-naphthalene acetic acid

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is an important family member of the *Rosaceae*. It is a natural hybrid of *Fragaria chiloensis* L. P. Mill. and *Fragaria virginiana* Duch. (Sakila *et al*. 2007). The cultivated strawberry is an octaploid (2n = 8x = 56) (Debnath and Teixeira da Silva 2007). This perennial stoloniferous herb produces a very popular fruit, strawberry, renowned for its aroma, taste, its fresh use and processing. It is also well adapted to all kinds of climates including temperate, Mediterranean, subtropical and taiga zones (Hancock *et al*. 1991). Conventional strawberry cultivation is labour intensive (Goutam *et al*. 2001) and propagated by runners (Kaur *et al*. 2005; Gaafar and Saker 2006; Sakila *et al*. 2007). As strawberry is a small herb, it requires 20,000 runners to plant one acre and propagation through runners results in the transmission of viral diseases such as strawberry mottle virus, strawberry mild yellow edge virus and strawberry crinkle virus (Goutam *et al*. 2001; Kaur and Chopra 2004). Therefore, *in vitro* mass multiplication through tissue culture assures to be the best alternative to traditional runner production (Mahajan *et al*. 2001) resulting in high yield and disease-free plant materials (Mohan *et al*. 2005). Additionally, strawberry plantlets can be stored *in vitro* in a small space for several years compared to the storage of runners (Kaur *et al*. 2005) to meet the demand at a commercial scale. *In vitro* micropropagation through adventitious shoot regeneration using petioles (Rugini and Orlando 1992; Passey *et al*. 2003), runner tips (Passey *et al*. 2003), stipules (Jemmali *et al*. 1994; Passey *et al*. 2003), stems (Graham *et al*. 1995), shoot tips (Preininger *et al*. 1997), leaves (Hanhineva *et al*. 2005; Hammerschlag *et al*. 2006) and through multiple shoot induction using vegetative buds (Kaur *et al*. 2005) and nodes (Sakila *et al*. 2007) were successfully investigated. The present report describes: a) the development of a novel protocol for *in vitro* mass multiplication through multiple shoot culture using runner tips as the explant; b) the establishment of an efficient acclimatization process; c) the maintenance of a sustainable pool of propagules *in vitro* and d) the assessment of genetic uniformity among *in vitro* generated plantlets.

MATERIALS AND METHODS

Explant source

Young, actively growing strawberry runner tips were collected from plants being maintained in a greenhouse of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India. The collected plant materials were washed thoroughly in running tap water and once more washed with a few drops of Teepol (Glaxo India Ltd, India) for 2 min. They were surface sterilized in 25% (v/v) Cetrimide antifungal solution (Nicholas Piramal India Ltd, India), shaken vigorously for 3 min and again washed several times with sterile water (SW) until no more bubbles were visible. The runner tip explants were then treated with 10% (v/v) NaOCl for 2 min and washed three times with SW before final treatment in 0.1% (w/v) $HgCl_2$ with occasional shaking for 3 min. Again the explants were rinsed vigorously three times in SW and both ends were trimmed to 1.5-2 cm. The whole process of surface sterilization was performed under a laminar air flow chamber.

Culture conditions

MS (Murashige and Skoog 1962) basal medium [consisting of salts, vitamins and 3% (w/v) sucrose] was used after solidifying with 0.7% (w/v) agar. Different plant growth regulators (PGRs) like α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), kinetin or 6-furfurylaminopurine (KIN), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were added at various concentrations to MS media before the pH of the media was

Received: 19 December, 2009. Accepted: 7 July, 2010.

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adjusted to 5.7 (with 0.5 N NaOH). Media were autoclaved at 1.06 kg cm^{-2} and 121°C for 15 min. All the MS basal salts, agar, vitamins and PGRs were obtained from SRL, Mumbai, India. Cultures at all growth stages were incubated under artificial conditions: 25 ± 2°C, 60% RH and a 16-hr photoperiod (using white fluorescent tubes) under a photosynthetic photon flux density of 30 µmol m^{-2} s^{-1}.

Bud induction and multiple shoot proliferation

For shoot bud induction, MS basal media with nine different combinations of NAA and BAP were tested, considering MS medium, devoid of PGR, as a control (**Table 1**). Excised runner tips were inoculated in the media, dipping approximately up to 0.5 cm for good contact. The cultures were incubated following the above mentioned artificial growth conditions, until fresh multiple greenish buds appeared. The best composition was identified in terms of performance over response, days to bud induction and number of buds. Next, the induced buds were separated and cultured for multiple shoot proliferation. Overall, nine varying levels of BAP or KIN were used in MS media for this purpose where MS medium free of PGR acted as a control (**Table 2**), like the earlier experiment. During shoot multiplication and proliferation the cultures were incubated for 50 days. Meanwhile one subculture was performed at the 25th day of multiple shoot proliferation. The best resulting media formulation was identified in the respect of response, number and length of shoots.

Root induction and elongation

In vitro growing shoots were used for further root induction and elongation. MS basal media was supplemented with 11 different concentrations of IAA or IBA with the presence of activated charcoal (AC); simple MS medium served as the control (**Table 3**). The proliferated multiple shoots were separated and then transferred individually to rooting media. The efficiency of IAA and IBA, including AC, were used to test the response to root induction, days to root induction, number and length of roots, and the most successful media composition for this purpose.

Acclimatization

A two-step acclimatization processes were developed to maximize the survival rate. Prior to entering the major part of acclimatization, the well rooted healthy plantlets were taken out of the media, separated individually by hand and washed thoroughly under running tap water to remove all the traces of agar from the roots. Elongated roots were then pruned for easy and accelerated *ex vitro* root regeneration. In the first phase of acclimatization, plantlets were transferred into small earthen cups filled with a mixture of autoclaved sand and soil (1: 1, v/v). The process of intermittent water spraying and subsequently covering the plantlets with polythene sheet ensured high humidity. The plantlets were then allowed to grow for 15 days in this condition. The partially-acclimatized plantlets with 3-4 primary leaves were then transferred to the larger pots containing- sand, soil and farm yard manure mixture (1: 1: 1, v/v) ensuring better plantlet production. After 20 days of growth the well acclimatized plantlets were recovered and they were advanced to the clonal fidelity study.

Sustained culture of multiple shoots

In this scheme, each *in vitro* generated shoot was subcultured for a further multiple shoot proliferation in a sustainable manner. The identified, best performing, multiple shoot proliferation medium was used for this sustained culture. Five subcultures were carried out in the same medium at an interval of two months, on the whole, over a period of 10 months. The performance of these subcultures was assessed on the basis of response, number and length of multiple shoots (**Table 4**). The selected shoots from sustained culture could be used for further root induction and acclimatization process. This continuous procedure for multiple shoot production would facilitate the steady supply of propagules as and when necessary. The clonal fidelity of shoots cultured for such a long period was assessed through ISSR markers.

Clonal fidelity

Genetic uniformity of *in vitro* generated and acclimatized plantlets was estimated through DNA fingerprinting using 10 selected ISSR primers (Gantait *et al.* 2008) (**Table 5**). At first, genomic DNA was extracted from 80 mg tender leaves according to the procedure described by Chattopadhyay *et al.* (2008). The extracted DNA samples were subjected to Polymerase Chain Reaction (PCR) amplification using 10 ISSR primers as mentioned above. The 25 µl optimized PCR mixture contained 40 ng DNA, 2.5 µl 10X *Taq* polymerase assay buffer, 3.5 µl 2.5 mM dNTPs, 0.5 U *Taq* DNA polymerase (all from Chromous Biotech Pvt. Ltd., India) and 200 ng of primer (Bangalore Genei Pvt. Ltd., India). PCR performance consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at annealing temperature 90 s at 72°C and final extension at 72°C for 7 min, 4 °C for 5 min was done using Gene Amp PCR system 2400 (Applied Biosystems, USA). The annealing temperature was adjusted according to the Tm of the primer which is being used in the reaction. The amplified PCR products, along with 50 bp DNA ladder were resolved by electrophoresis on 1.5% (w/v) agarose (SRL) gel in 1X TBE buffer stained with ethidium bromide (10 µg l^{-1} TBE buffer). Bands were scored and photographed on Gel Logic 200 trans-illuminator system (Kodak). Similarly, shoots generated from sustained cultures were also examined for their clonal fidelity.

Experimental design and statistical analysis

Culture vessels were arranged according to a Complete Randomized Design (CRD) on the shelves of a growth room. Each single explant was considered as an experimental unit. All the above mentioned experiments were repeated thrice, except the sustained subculture part with six repetition using 20 explants in each replication and the standard deviation was calculated from the recorded numerical information. Data on bud establishment and multiple shoot proliferation were recorded at 18 days and 45 days, after each inoculation, respectively. In case of root induction and elongation experiment, observed data were recorded after 27 days of culture in the media. For data scoring on sustainable culture the 60th day of every subculture was chosen. The number of plantlets recovered out of those plantlets subjected to acclimatization was recorded for the analysis of the percentage of survival. Observations on cultures were carried out on every alternate day. The collected data were subjected to the analysis of variance (ANOVA) and significant variation among the treatments were tested by Duncan's multiple range test (Duncan 1955) at 5% level using WINDOWSTAT software package (UBKV, India). For ISSR profiles, the well-resolved and consistently reproducible amplified DNA fragments were scored in terms of their presence or absence. To detect the genetic uniformity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

RESULTS

Explant establishment and shoot multiplication

Fresh runner tip explants inoculated in MS basal media, supplemented with PGRs responded well in general. On the other hand, the control set (*i.e.* only MS medium devoid of any PGR) failed to compete with those with NAA-BAP combinations whereas, 0.1-0.5 mg l^{-1} NAA and 0.5-2 mg l^{-1} BAP in eight combinations served as the optimum conditions for the establishment of runner tips through shoot bud induction. A very low level of NAA (0.1 mg l^{-1}) and BAP (1 mg l^{-1}) promoted earliest bud induction, (**Fig. 1A**) within 6 days after inoculation and over all the other tried compositions. A maximum number of explants (17.67 out of 20) responded in this composition and after 18 days, around 4 buds per inoculated runner tip were observed (**Table 1**). For shoot multiplication and proliferation, freshly induced shoot buds, separated and excised, were inoculated in a strict aseptic condition. MS medium with 2 mg l^{-1} KIN proved best for this purpose in terms of the response of the buds to multiple shoot formation, number and length of multiple shoots. More than 18 buds (90.6%) out of 20 res-

Table 1 Standardization of PGRs on bud induction in strawberry.

Growth regulators (mg l^{-1})		Performance of runner tip explant		
NAA	**BAP**	**Response to bud induction**	**Days to bud induction**	**№ of buds**
0	0	0.67 ± 0.58 e	33.33 ± 2.52 a	0.67 ± 0.58 c
0.1	0.5	8.67 ± 1.53 c	11.33 ± 1.53 d	1.67 ± 0.58 bc
0.1	1.0	17.67 ± 0.58 a	5.67 ± 0.58 e	3.67 ± 1.15 a
0.1	1.5	15.33 ± 1.15 b	7.33 ± 0.58 e	2.33 ± 0.58 b
0.1	2.0	15.33 ± 0.58 b	12.33 ± 0.58 cd	1.67 ± 0.58 bc
0.5	0.5	4.33 ± 1.53 d	16.67 ± 1.15 b	1.33 ± 0.58 bc
0.5	1.0	8.67 ± 0.58 c	12.67 ± 1.53 cd	1.67 ± 0.58 bc
0.5	1.5	14.33 ± 1.15 b	14.33 ± 1.53 bc	2.00 ± 0.00 b
0.5	2.0	15.67 ± 0.58 b	11.33 ± 1.53 d	2.33 ± 0.58 b
Overall mean		11.19	13.89	1.93
SE (±)		0.5774	0.8165	0.3685
CD at 5%		1.715	2.426	1.095

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ following DMRT.

Table 2 Multiple shoot proliferation in strawberry.

Growth regulators (mg l^{-1})		Response to shoot multiplication out of 20	№ of shoots	Shoot length (cm)
BAP	**KIN**			
0	0	1.33 ± 0.58 f	1.33 ± 0.58 d	1.50 ± 0.44 f
1.5	0	12.33 ± 1.53 e	3.00 ± 1.00 cd	2.90 ± 0.20 e
2.0	0	14.33 ± 0.58 cde	3.67 ± 0.58 bc	3.60 ± 0.36 cd
2.5	0	16.33 ± 0.58 abc	5.67 ± 1.53 b	3.70 ± 0.30 cd
3.0	0	15.00 ± 2.65 bcd	4.33 ± 0.58 bc	3.40 ± 0.44 de
0	1.5	13.67 ± 1.53 de	3.33 ± 0.58 cd	3.37 ± 0.31 de
0	2.0	18.33 ± 0.58 a	10.33 ± 1.53 a	5.23 ± 0.31 a
0	2.5	17.33 ± 1.15 ab	5.67 ± 1.15 b	4.63 ± 0.31 b
0	3.0	15.67 ± 1.53 bcd	5.00 ± 2.00 bc	3.93 ± 0.25 c
Overall mean		13.82	4.70	3.59
SE (±)		0.7857	0.6759	0.1908
CD at 5%		2.334	2.008	0.567

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ following DMRT

Table 3 Root induction and elongation in strawberry.

Growth regulators (mg l^{-1})		Additive (g l^{-1})	Response to root induction out of 20	Days to root induction	№ of roots	Root length (cm)	Shoot length (cm)
IAA	**IBA**	**AC**					
0	0	0	5.67 ± 1.15 e	42.00 ± 2.65 a	1.00 ± 0.00 e	0.77 ± 0.31 ef	1.73 ± 0.41 g
0	0	1	8.67 ± 0.58 d	30.33 ± 0.58 b	1.00 ± 0.00 e	1.00 ± 0.30 ef	2.60 ± 0.30 ef
0	0	2	10.33 ± 0.58 cd	24.67 ± 2.52 c	1.33 ± 0.58 e	1.33 ± 0.35 def	2.70 ± 0.53 ef
0.1	0	2	11.33 ± 1.53 c	13.67 ± 0.58 fg	2.33 ± 0.58 cd	2.36 ± 0.46 bcd	3.37 ± 0.21 cd
0.25	0	2	18.33 ± 0.58 a	8.33 ± 0.58 h	5.33 ± 0.58 a	3.96 ± 0.21 a	5.43 ± 0.31 a
0.50	0	2	17.00 ± 0.00 a	12.67 ± 2.31 g	3.33 ± 0.58 b	2.93 ± 0.25 ab	3.73 ± 0.47 bcd
1.0	0	2	14.67 ± 1.15 b	13.33 ± 1.15 g	3.33 ± 0.58 b	2.83 ± 0.23 bc	3.93 ± 0.25 bc
0	0.1	2	9.67 ± 1.15 cd	20.00 ± 1.73 d	1.33 ± 0.58 e	2.30 ± 0.10 bcd	2.50 ± 0.53 f
0	0.25	2	11.33 ± 1.53 c	17.33 ± 1.53 de	1.67 ± 0.58 de	1.60 ± 0.17 def	3.20 ± 0.26 de
0	0.50	2	16.67 ± 0.58 a	13.00 ± 1.73 g	3.00 ± 0.00 bc	1.96 ± 0.32 bcde	2.07 ± 0.21 b
0	1.0	2	14.00 ± 1.73 b	16.33 ± 0.58 ef	2.67 ± 0.58 bc	1.83 ± 0.31 cde	3.37 ± 0.31 cd
Overall mean			12.52	19.24	2.39	2.08	3.33
SE (±)			0.6276	0.9482	0.2843	0.3445	0.2091
CD at 5%			1.841	2.781	0.834	1.010	0.613

Mean ± SD of 20 clones per treatment in three repeated experiments
Different letters indicate significant difference at $P < 0.05$ following DMRT

ponded to shoot multiplication and proliferation (**Table 2**). Overall 10 multiple shoots per inoculant were scored at the 45th day after shoot bud inoculation. At that time shoot length was also appreciable *i.e.* 5.23 cm and the multiple shoots were healthy and light green in colour (**Fig. 1B**). Whereas, only MS medium acting as control could not produce any significant result either in shoot bud induction or multiplication.

Rooting *in vitro*

Root induction was observed more or less in all tried media (**Table 3**). After almost 70 days of culture from bud induction to shoot multiplication, the regenerated clumps of multiple shoots were separated and 40 selected shoots were transferred to rooting media. Root initiation was observed within 8 days approximately and the highest response of rooting obtained was 91.65% (18.33 out of 20) in the MS medium fortified with 0.25 mg l^{-1} IAA and 2 g l^{-1} AC (**Fig. 1C**). More than 5 roots per inoculated shoot were scored after 27 days of culture in the identified medium. During this point of growth, average root length was around 4 (3.96) cm and the average length of shoot was 5.43 cm. Out of 40 shoots 37 were well rooted and advanced for acclimatization.

Ex vitro acclimatization

The *ex vitro* establishment of the selected plantlets was significantly affected by thorough washing and pruning of *in vitro* regenerated roots. In the first phase of acclimatization the mixture of autoclaved sand and soil (1: 1 v/v)

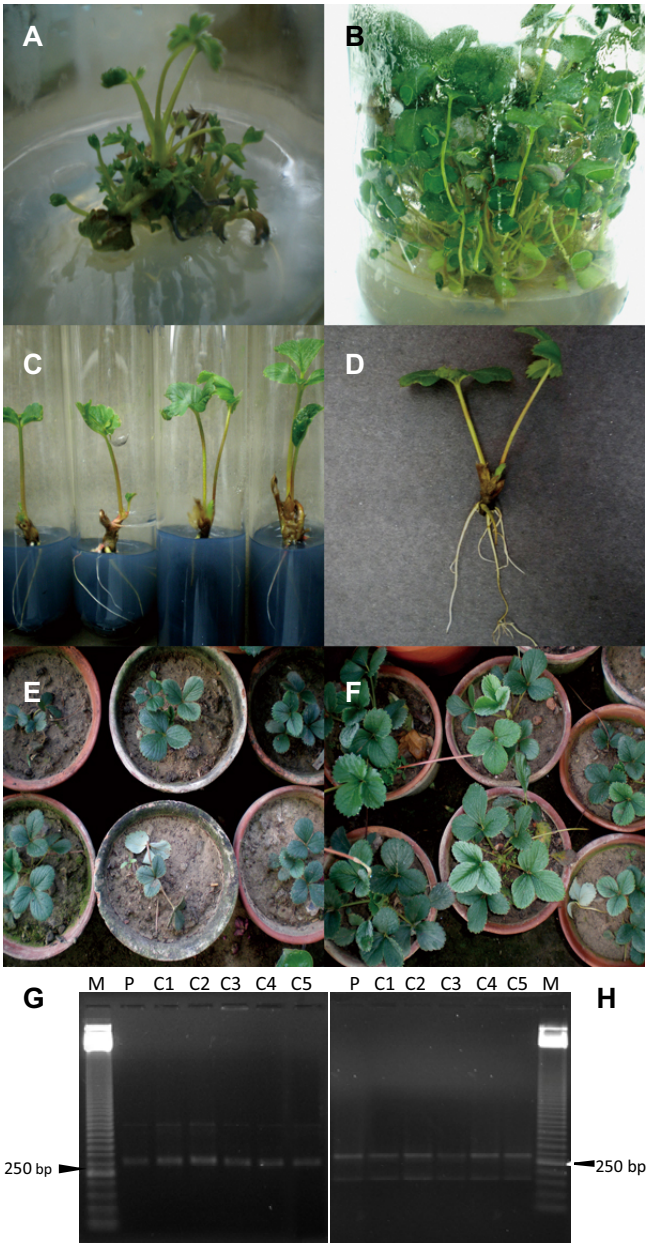

Fig. 1 Tissue culture and ISSR analysis of Chandler regenerants. (**A**) Bud break from runner tip explant, (**B**) Multiple shoot proliferation, (**C**) Rooting of shoots, (**D**) Plantlet with stout and healthy roots, (**E**) Primary acclimatization on autoclave sand and soil, (**F**) Final acclimatization on sand, soil and farm yard manure mixture (**G**, **H**) Agarose gel electrophoresis of ISSR fragments of *in vitro* regenerated clones (C1-C5) with their mother (P) showing monomorphic bands generated by primer IS-7 (5′ GTG TGT GTG TGT GTG TA 3′) and IS-65 (5′ AG AG AG AG AG AG AG AG T 3′). Lane M – 50 bp ladder (white arrow indicates 250 bp).

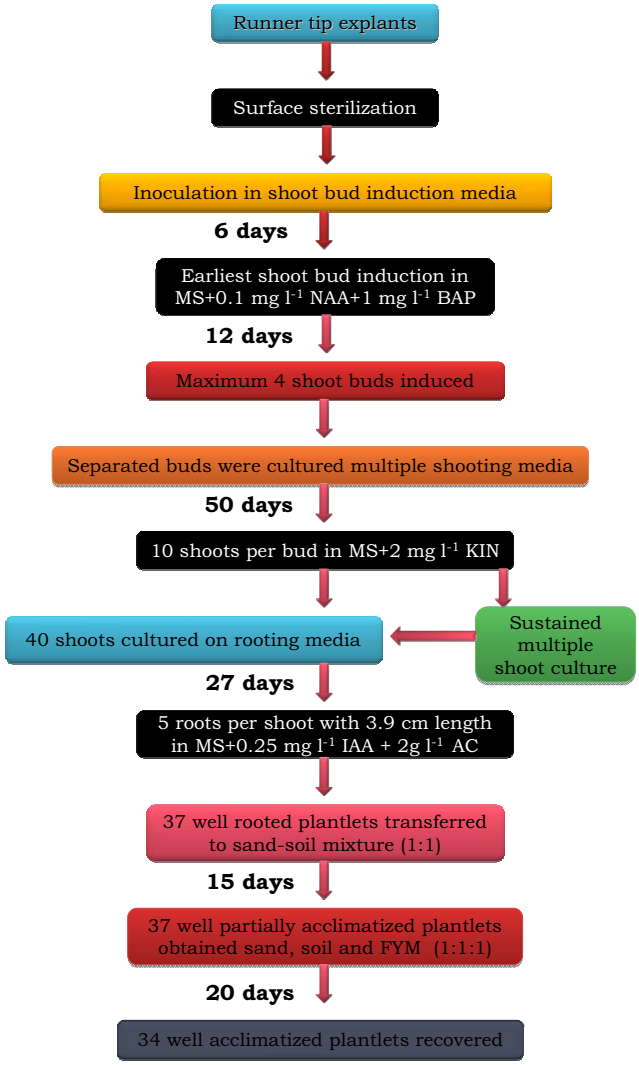

Fig. 2 Flow diagram illustrating entire activity of *in vitro* cloning and *ex vitro* acclimatization.

Table 4 Performance of sustainable subcultures on shoot multiplication of strawberry.

Treatments	Responding shoots out of 20	№ of shoots	Shoot length (cm)
Subculture I	18.33 ± 0.52 b	10.33 ± 1.03 c	5.23 ± 0.27 b
Subculture II	19.33 ± 0.52 a	10.50 ± 1.05 bc	5.33 ± 0.24 ab
Subculture III	19.67 ± 0.52 a	11.33 ± 0.82 ab	5.42 ± 0.10 ab
Subculture IV	19.67 ± 0.52 a	11.67 ± 0.52 a	5.53 ± 0.24 a
Subculture V	19.67 ± 0.52 a	11.50 ± 0.55 a	5.45 ± 0.18 ab
Overall mean	19.33	11.67	5.39
SE (±)	0.2108	0.3367	0.0881
CD at 5%	0.614	0.981	0.257

Mean ± SD of 20 clones per treatment in six repeated experiments
Different letters indicate significant difference at $P < 0.05$ following DMRT

provided optimum anchorage to the plantlets and helped in primary regeneration of roots (**Fig. 1D**) for 15 days. Intermittent spraying of water and coverage of transparent polyethylene ensured high humidity which clearly encouraged the quick acclimatization process. For the next 20 days, a balanced mixture of sand, soil and FYM (1: 1: 1 v/v) resulted to the 34 well acclimatized plantlets (**Fig. 1E**) out of 37. Overall 92% success was achieved following the above mentioned protocol.

A flow-diagram is presented to illustrate the entire protocol in **Fig. 2**.

Propagule maintenance

To examine the sustainable performance, regenerated shoots were subcultured at an interval of two months, over a period of 10 months on MS medium with 2 mg l^{-1} KIN (**Table 4**). The performance was meticulously monitored in respect of multiple shoot proliferation which revealed that there was no significant variation in performance except improvement in response. Morphogenetic efficiency continued to remain unaltered with sustained subculturing. To ascertain the genetic uniformity at molecular level, DNA fingerprinting was performed using ISSR primers.

Clonal fidelity

In the present study, 10 selected ISSR primers were used for checking the fidelity of *in vitro* generated clones among which IS-6 (5′ GAG AGA GAG AGA GAG AC 3′), IS-9 (5′

Table 5 ISSR primers used for fidelity test of *in vitro* generated clones.

Oligo name	Tm (°C)	5′-3′ motifs	Anchoring	Reaction to strawberry DNA	Number of scorable bands per primer	Total number of scorable bands	Size range (bp)
IS-6	52	$(GA)_8C$	3′ anchor	Negative	-	-	-
IS-7	50	$(GT)_8A$	3′ anchor	Positive, reproducible, monomorphic	2	12	300-600
IS-8	52	$(AG)_8C$	3′ anchor	Positive but not reproducible	-	-	-
IS-9	46	$(TG)_7TA$	3′ anchor	Negative	-	-	-
IS-10	52	$C(GA)_8$	5′ anchor	Negative	-	-	-
IS-11	52	$(CA)_8G$	3′ anchor	Positive but not reproducible	-	-	-
IS-12	52	$(GT)_8C$	3′ anchor	Negative	-	-	-
IS-61	50	$(GA)_8T$	3′ anchor	Positive but not reproducible	-	-	-
IS-63	52	$(AG)_8C$	3′ anchor	Positive but not reproducible	-	-	-
IS-65	50	$(AG)_8T$	3′ anchor	Positive, reproducible, monomorphic	2	12	150-300
Total					4	24	

TGT GTG TGT GTG TGT 3′), IS-10 (5′CGA GAG AGA GAG AGA GA 3′) and IS-12 (5′GTG TGT GTG TGT GTG TC 3′) did not react with strawberry DNA. Rest of the primers (*i.e.* IS-7, IS-8, IS-11, IS-61, IS-63 and IS-65) displayed a positive interaction but only IS-7 (5′ GTG TGT GTG TGT GTG TA 3′) and IS-65 (5′ AG AG AG AG AG AG AG AG T 3′) among these confirmed to be reproducible (**Table 5**). A total number of 24 reproducible monomorphic bands were scored from the clones including their mother. In our study, the primers amplified distinct bands between 150 bp to 600 bp molecular size range. None of the primers showed any difference in the banding pattern (**Fig. 1F, 1G**), proving that the uniformity of the *in vitro* regenerated clones was maintained. On the other hand, similar result was displayed in the case of sustained multiple shoot culture, and in addition, there was no variation in major reproducible bands among the prolonged cultured clones.

DISCUSSION

Establishment of primary aseptic cultures of explants, collected and excised, from *ex vitro* grown plants was difficult due to the high incidence of microbial contamination as reported by Mills *et al*. (1997). This problem has been solved during the present investigation by the use of Cetrimide, NaOCl and $HgCl_2$, where cent percent aseptic cultures were procured.

Direct organogenesis: Media formulations

To optimize the NAA and BAP concentrations for explant establishment, runner tip explants were inoculated in MS media with low levels of NAA and BAP. Induction of multiple buds in MS medium fortified with 0.1 mg l^{-1} NAA and 1 mg l^{-1} BAP proved the earlier observations of Hashemabadi and Kaviani (2008), which states that low levels of auxin and cytokinin stimulate the number and elongation of shoot buds. Positive effect of auxin and cytokinin combination with diverse concentration in the present study supports the earlier reports of several authors (Burrit and Leung 1996; Kiyakawa *et al*. 1996). In order to establish an efficient PGR supplement for multiple shoot proliferation, two different types of cytokinin (*i.e.* BAP and KIN) were tried individually in eight different levels (1.5-3 mg l^{-1}) with MS basal media. MS medium with 2.0 mg l^{-1} KIN proved as the most effective formulation for multiple shoot formation and proliferation than any other tried combinations confirmed by DMRT. The results of multiple shoot proliferation demonstrated that cytokinin alone, without auxin, successfully induced a maximum number of multiple shoots (Jajoo 2010). Again, the higher concentration of cytokinin, used in the present study for shoot multiplication, reduced the number of shoot gradually (Teixeira and da Silva 1990; Bhatt and Dhar 2000). At the same time, in this experiment, KIN performed better in all respect than BAP as the cytokinin source which supports the earlier observations in *Allium* (Kamstaityte and Stanys 2004). Profuse *in vitro* rooting was recorded in familiar combination of MS basal medium with 0.25 mg l^{-1} IAA and 2 g l^{-1} AC. Here IAA performed better than IBA in this respect, in contrary to the report of Sakila *et al*. (2007). A very low concentration of IAA and the presence of AC proved to be very competent in the induction as well as promotion of root growth for strawberry. The addition of AC plays an effective role in the enhancement of root induction because it absorbs the polyphenols produced through chemical processes within the media which may act as growth inhibitors during morphogenesis (George and Ravishankar 1997; Wann *et al.* 1997; Madhusudhanan and Rahiman 2000). Moreover, AC helps to eliminate light and provides a reasonable physical environment for the rhizosphere and also helps rooting (Nissen and Sutter 1990).

Acclimatization

For better acclimatization process it is necessary for each shoot to have a number of well developed healthy roots. Henceforth, after optimum root formation the selected 37 plantlets were washed with double distilled water to remove all the traces of medium adhering to the roots. This was very effective to restrict any kind of microbial contamination in the present study (Sharry and Teixeira da Silva 2006). Pruning of roots at the time of planting made the plantlets easy to be established *ex vitro*, obtain their vigour and increase the root regeneration potential. In addition, root pruning helped in reducing the variability, arose due to the difference in the numbers and lengths of the *in vitro* generated roots and gave more uniform plants (Thomas and Ravindra 1997). Mainly, the credit of this successful acclimatization went to the retention of high humidity. Excepting the utilization of the intermittent water spraying, along with the covering with transparent polyethylene sheet (Thomas and Ravindra 1997), we assumed that the use of FYM also played an important role in retaining the moisture. It was observed that, out of 37 regenerated shoots, from the single runner tip, 34 acclimatized healthy growing plants were ready for field transfer.

This technique efficiently produced a high frequency of plantlets within a limited time span. It is clear from the activity chart (**Fig. 2**) that it would take around 125 days or almost 14 weeks (from induction of bud to complete acclimatization of plantlets) to raise as many as 34 well acclimatized plants from a single runner tip.

Propagule maintenance

There are two distinct advantages to maintain a steady supply of propagules from the regenerated shoots under prescribed MS medium and the acclimatization process as described. Initially, there was no need to start with a fresh runner tip and as a result, time lag would be curtailed by more than two weeks. Moreover, since the duration of multiple shoot culture was short (60 days), each subculture resulted in an exponential increase in the number of shoots (Ray *et al*. 2006). Finally, a balanced propagule supply can possibly be sustained over a period of time, without any genetic damage. In the present study, morphological para-

Table 6 Comparison of the novelty of the present study with the earlier reports.

Relevent Reports	Basal medium	Physical Condition	Shoot multiplication/ regeneration		Rooting *in vitro*		Acclimatization			Clonal fidelity assay	Sustained culture	Overall success
			PGRs	Result	PGRs	Result	Media	Duration	Success			
López-Aranda *et al.* 1994	Modified Boxus	25 ± 1°C, 16-hr photoperiod with 40 µE m^{-2} s^{-1} irradiance	1.46 µm BA or $N_{45}K$	Av. 5.4-7.14 shoots with 1.5 cm length	500 mgl^{-1} AC without PGR	7 roots with 3.6 cm length	Peatmoss :perlite (3:1)	Not mentioned	90%	Not studied	Eight subcultures, but decreased performance	Not dercribed in detail
Barceló *et al.* 1998	$N_{30}K$ with MS	25 ± 1°C, 16-hr photoperiod with 40 µmol m^{-2} s^{-1} irradiance	2.46 µm IBA + 8.88 µm BA	2.9 shoot colonies per leaf disk	Not studied	Not studied	Not studied	Not studied	Not studied	Not studied	Not studied	47% shoot regeneration
Present study	MS	25 ± 2°C, 60% RH, 16-hr photoperiod with 30 µmol m^{-2} s^{-1} PPLD	2 mgl^{-1} KIN	Av. 10 shoots with 5 cm length	0.5 mgl^{-1} IAA + 2 gl^{-1} AC	5 roots with 3.9 cm length	Sand:soil: FYM (1:1:1)	35 days	92%	Using ISSR	Five subcultures with unaltered performance and fidelity	34 plantlets from a single runner tip within 125 days

meters like shoot numbers and length, determining the performance of *in vitro* propagules do not differ significantly with further sustained subculturing, whereas, Naik *et al.* (2003) observed a decrease in performance with the increase in the passage of subculturing in *Gmelina arborea*.

Clonal fidelity test

The test of clonal fidelity using ISSR primers was successfully attempted in different micropropagated plant species (Joshi and Dhawan 2007; Lakshmanan *et al.* 2007; Bhatia *et al.* 2009). However, this particular study was not extensively investigated in strawberry though there are some reports on the use of ISSR for genetic diversity analysis in strawberry genotypes (Arnau *et al.* 2002; Reddy *et al*. 2002; Debnath *et al.* 2008). In our study, use of 2 out of 10 selected ISSR primers revealed distinct monomorphic bands when the mother and *in vitro* generated propagules were subjected to clonality test. It was proven from this experiment that 1) the clones developed from *in vitro* direct organogenesis are true to its genetic identity and 2) *in vitro* direct organogenesis is the safest mode of micropropagation which comprises true to type progeny. The reports of Shu *et al*. (2003), Carvalho *et al*. (2004) and Martins *et al.* (2004) also cite similarity with this result. As for the concerned clones of the prolonged cultures, this conclusion was equally applicable as they also maintained their genetic uniformity. This uniformity would help to maintain sustained activity for tissue culture in strawberry.

Novelty of the present study

There are some earlier reports related to micropropagation of strawberry cv. 'Chandler'. But this advanced study claimed to be novel in several aspects. The major differences and likely issues have been discussed in **Table 6**. Though it is clear from the table that the earlier reports of López-Aranda *et al.* (1994) and Barceló *et al.* (1998) were based on *in vitro* study of this cultivar, but the present study proves its innovativeness in specific aspects such as basal media formulation, PGRs level, acclimatization procedure, clonal fidelity assessment and finally the unique output of these criteria. López-Aranda *et al.* (1994) studied chiefly the effect of mineral salts, benzyladenine (BA) levels and number of subcultures on *in vitro* and *ex vitro* behaviour of cv. 'Chandler'. Neither the acclimatization procedure in detail nor the clonal fidelity study was reported in it, moreover, the percentage of success in acclimatization was relatively less (90%) in comparison to the present study (92%). Soil: perlite (1: 1, v/v) was used as the acclimatization medium in the previous report whereas sand: soil: FYM (1: 1: 1, v/v) served the purpose in our study. Though the assessment of morphological performance on succeeding subcultures was reported as parallel to the present study, the results were opposite. Interestingly, the successful use of AC for *in vitro* rooting was similar to the present study. On the other hand, the report of Barceló *et al.* (1998) mainly focused on shoot regeneration from the leaf disks *in vitro* and *Agrobacterium*-mediated transformation. In the aforementioned study, the essentiality of the presence of auxin even in very low level with cytokinin for shoot regeneration was described which finds resemblance with the bud induction procedure in our study, but there was no further study on *in vitro* rooting, acclimatization or assessment of genetic clonality of *in vitro* regenerated shoots. In view of these key differences, the present study can be considered as a unique and complete micropropagation protocol of strawberry cv. 'Chandler'.

CONCLUSION

The present protocol of *in vitro* mass multiplication detains huge potential for large scale exploitation of strawberry. This will also help in preventing waste of labour and cost over raising the plantlets through conventional propagation method. Else, the regenerated multiple shoots raised from the mother explants retain their regeneration potential and genetic identity over the year. Hence, the overall study promptly determines its potentiality in the field of commercial activity for aromatic plants like strawberry, through *in vitro* mass propagation.

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