Chemical Science Review and Letters ISSN 2278-6783

Research Article

Assessment of Genetic Integrity in Plants Regenerated by Organogenesis from Callus Culture of Strawberry

Anuradha, S.K. Sehrawat, and D.S. Dahiya

Department of Horticulture, CCS Haryana Agricultural University, Hisar, 125004, India

Abstract

The nodal segments of strawberry cultivar Ofra were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of auxins and cytokinins for indirect regeneration via callus formation. MS medium supplemented with 4.0 mg/l NAA and 2.0 BAP yielded the highest percentage of callus. The highest response of shoot regeneration was obtained in MS medium fortified with 2.0 mg/l kinetin and 0.5 mg/l IAA for regeneration of shoots from callus. The regenerated shootlets were rooted on Half strength MS medium. The plantlets thus developed were hardened successfully. Some morphological variations were found in plants generated by callus. On the basis of variations, *in vitro* raised plantlets were subjected to evaluation of genetic uniformity using RAPD markers. Out of twenty one primer screened, six primers showed amplification and the RAPD profiles were obtained found to be reproducible and monomorphic. The results concluded that the tissue culture raised plants of strawberry were genetically identical and clonally uniform.

Keywords: *In vitro* propagation, strawberry, auxins, cytokinins, genetic fidelity

***Correspondence**
Author: Anuradha
Email: anuradha2917@gmail.com

Introduction

The cultivated strawberry is predominantly used as fresh fruit and enjoyed by millions of people in all climates The cultivated strawberry is one of the most important soft fruit of the world and an octaploid (2n = 56). It has unique position among the cultivated fruits. Among the fruits, strawberry gives the quickest returns in the shortest possible span [1]. The continuous increases in its cultivation acreage should be concomitant with the development of new production practice. The conventional way of production is not adequate to meet the commercial demand. In view of potential commercial value, it is highly desirable to develop methods for rapid, efficient and large scale propagation of crop [2]. *In vitro* production of plants involves the application of growth regulators, such as auxins, cytokinin for process of organogenesis. Cytokinins are known to play a major role in shoot multiplication [3]. Successful callus culture depends on type of plant growth regulator. Cytokinins and auxins are known to promote callus formation in tissue culture. Auxin has a wide range of effects on plant growth and morphogenesis [4].

In order to obtain true to the type plantlets with the aid of tissue culture, it is quite necessary to examine the genetic uniformity of *in vitro* raised plants from time to time. During *in vitro* cultures, however it comes to surveillance that many of regenerated plants are not genetically identical to their parents and these plants are known as somaclonal variants. Somaclonal variation although thought to be beneficial for generating better performing clones, can sometimes be considered a serious problem as it may lead to creation of non true to type plants which differ in genetic makeup from that of their parent [5].Thus clonal fidelity is one of the major concerns in commercial propagation. The generation of true to type propagules and genetic stability are the prerequisites for the application of *in vitro* strawberry propagation [6]. Strawberries are too amenable to *in vitro* somaclonal variation [7]. The most reliable methods are the molecular marker techniques that identify the variance depending on plant proteins, which are expressed from regions of DNA, or DNA polymorphisms. RAPD (random amplified polymorphic DNAs) is a powerful technique for identification of genetic variation [5]. Strawberries have been extensively analyzed for clone identification, mapping and diversity studies using random amplified polymorphic DNA (RAPD) marker [8-12].

Thus, objectives of present study was conducted to determine the appropriate growth regulator concentration and combination to establish a mass production system of callus raised plant of strawberry cultivar Ofra and assess the genetic fidelity of the *in vitro* raised plants through RAPD markers. This can be a useful for establishing a reliable

micropropagation system for the production of genetically uniform plants before they are released for large scale cultivation or commercial purposes.

Experimental

Materials and Methods

The experiment was carried out at the Centre of Plant Biotechnology and Department of Horticulture, CCS Haryana Agricultural University, Hisar during the 2014-2015.

Plant material

Explants were collected from the healthy plants (in July) maintained at experimental polyhouse of the Department of Horticulture. Young tender vegetative nodal segment of 5-10 cm length were excised. The MS medium supplemented with BAP, Kinetin IAA and NAA alone or in combination each other for direct regeneration and for callus induction by hit and trial method. Calli were cultured on MS medium supplemented with different concentration of BAP and kinetin at lower concentration in combination with auxins (NAA, IAA). The cultures were maintained thermal insulated tissue culture room with temperature of around 25±2 ^{0}C and light 4000 lux was similar in all experiment. Half strength MS medium induced rooting in all culture. The rooted plantlets were transferred to 3:1:1 mixture of coco peat, perlite and sand in plastic pots and acclimatized to greenhouse condition.

Culture media and conditions

Murashige and Skoog's (MS) medium containing sucrose 3% (w/v) sucrose (Himedia, India) was used in all the experiments. The growth regulators were added in measured quantity, whenever required. The pH of medium was adjusted to 5.8 with 1N NaOH and 1N HCl using pH meter. Agar was added to this medium followed by melting of medium in oven. The medium was stirred regularly to avoid agar clumps till boiled thoroughly. It was allowed to cool for few minutes at room temperature and poured in jam bottles. The media were sterilized in autoclave at 121^{o}C temperature and 15psi pressure for 20 minutes and were stored at room temperature for further use. The bottles containing culture media were used within a week.

Statistical analysis

The data of all the experiment recorded during the present investigation were subjected to statistical analysis using "Completely Randomized Design" All the values have be presented as the mean of repeats with the evaluation of C.D. (critical difference) at 5% level by using software OP STAT.

DNA isolation and PCR amplification

Modified CTAB (Cetyl Trimethyl Ammonium Bromide) method of Murray and Thompson [13] modified by Saghai-Maroof and Xu et al. [14-15] was used for extraction of total DNA from both tissue cultured plants and mother plants. Twenty one RAPD primer were used for assaying genetic uniformity. PCR was performed in a volume of 10µl. The variations were made in genomic DNA concentration (25ng, 50ng and 100ng), Taq. DNA polymerase concentration (1.0 unit and 2.0 units), primer concentration (0. 5µl and 1µl) and annealing temperature (36^{0}C, 37^{0}C and 40^{0}C). All possible combinations were tried in 10µl of reaction mixture for PCR amplification. Amplification was carried out in PTC-100 programmable thermal cycler. PCR conditions for RAPD analysis included an initial pre-denaturation step of 3 minutes at 94^{0}C and following 45 cycles of amplification of denaturation 94ºC at 1 min, annealing for 36^{0}C and an extension at 72^{0}C for 1 min and Final extension was carried out at 72^{0}C for 7 minutes. The amplified products were resolved on a 2% agarose gel in TBE (Tris-borate EDTA) buffer stained with Ethidium Bromide using 100bp ladder as reference. PCR amplified products were visualized under UV light and photographed using VSD image Master of Pharmacia, Biotech.

Result and Discussion

Among the used hormonal supplements, explants of strawberry showed high response to callus formation MS

medium supplemented with BAP (Table 1). The specific concentration of growth regulators, nutrients and incubation condition modify the normal physiology of explants and induce dedifferentiation and redifferentiation of tissue. Depending on the auxins and cytokinin concentration of culture media calli were initiated from cut surfaces. The application of hormones disturb the internal system and leads to dedifferentiation and differentiation of tissue.

Table 1 Effect of different concentrations and combination of growth regulators on callus induction and number of days taken for callus initiation in strawberry cultivar Ofra

Hormonal composition of media (mg/l)	Number of days taken for callus initiation	Callus induction (%)
MS media + 2.0 NAA	15.00 ± 0.58	81.67 ± 0.67
MS media + 4.0 NAA	15.00 ± 0.00	73.33 ± 1.67
MS media + 5.0 BAP	16.33 ± 0.33	70.67 ± 2.08
MS media + 5.0 BAP + 0.1 NAA	13.00 ± 0.5 8	83.33 ± 0.58
MS media + 5.0 BAP + 0.1 IAA	16.00 ± 0.58	80.33 ± 0.33
MS media + 4.0 NAA + 2.0 BAP	11.00 ± 0.58	87.33 ± 1.67
MS media + 4.0 NAA + 2.0 KIN	17.67 ± 0.33	71.67 ± 1.67
CD (P=0.05)	1.44	4.63

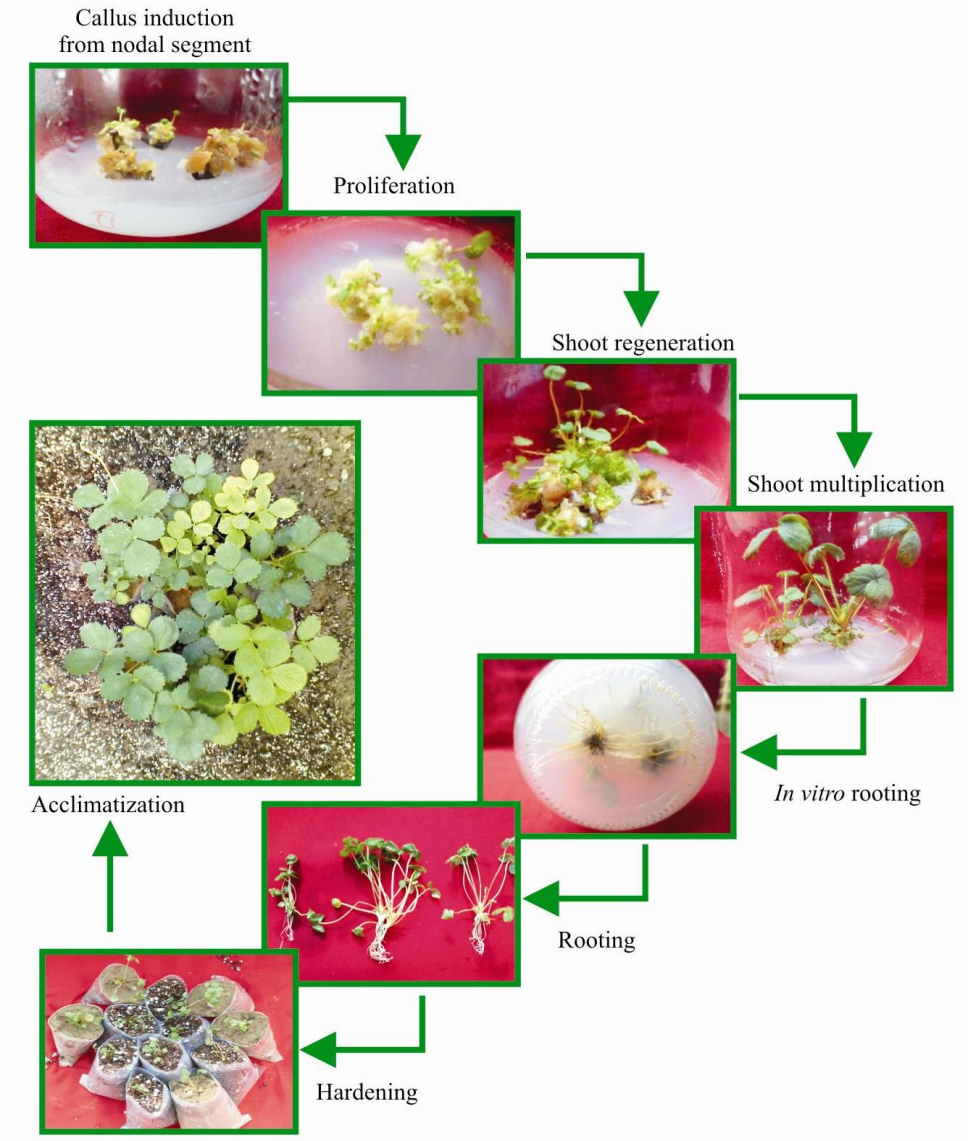

Figure 1 *In vitro* propagation cycle through indirect regeneration via callus

Among the used hormonal supplements, auxins in combination with cytokinins (NAA, BAP) Table 1 was found to be the most effective for callus induction, which is concomitant with previous reports of [4,16-17]. Callus induction is prerequisite on the way to generate somaclonal variation. Because during callus division in artificial conditions different types of abnormalities occur in the genetic constituents that ultimately contributed to the regenerated plants.

Shoot regeneration from callus

The results showed that (MS media + 2.5 mg/l KIN+ 0.5 mg/l IAA) was found best **(Fig. 1)** for shoot regeneration(Table 2) followed by (MS media + 1.0 mg/l BAP + 0.1 mg/l NAA) from nodal segment derived callus. It was observed that higher concentration of cytokinins with lower concentration of auxins promoted shoot regeneration from callus, hence it is the optimum ratio of cytokinins and auxins which is necessary for better shoot regeneration. The calli which were placed in MS medium without growth regulators failed in regenerating shoots strengthen this view point. The findings of present investigation are reflected by Karim et al. [18] who reported that addition of kinetin in combination of BA and NAA resulted in efficient shoot induction and multiplication from calli, while Ara et al. [16] reported that combination of BA and NAA produced highest number of shoots. Synergistic effect of BA and KIN has been reported to be the best for shoot regeneration in strawberry by some earlier workers [19].

The number of leaves per explants was recorded highest with (MS medium + 2.5 mg/l KIN + 0.5 mg/l IAA). This increase in number of leaves may be attributed to the working of kinetin and IAA in tandom creating a synergistic effect. Bhat et al. [20] reported that addition of kinetin with BAP resulted maximum number of leaves. Shashi [21] also reported that BAP added with kinetin increased the number of leaves in chrysanthemum.

Length of shoots was recorded maximum (5.67cm.) with (MS medium + 2.0 mg/l KIN + 0.1 mg/l IAA). It was observed that as concentrations of kinetin increased length of shoots decreased BAP at higher concentrations exhibited synergistic effect with lower concentrations of NAA (0.1 mg/l). Cytokinins are responsible for division of cells which are elongated by auxins due to their ability to increase cell wall plasticity and lowering in cell wall resistance to the penetration of solutes. Similar results were reported by Bhat and Dhar [22].

Table 2 Effect of different concentrations of growth regulators on shoot multiplication from nodal segment derived callus of strawberry cultivar Ofra

Hormonal composition of media (mg/l)	**Observation on 21th day**		
	Shoot number	**Number of leaves**	**Shoot length**
MS media (control, no growth regulators)	0	0	0
MS media + 0.5 BAP	3.07 ± 0.07	2.90 ± 0.20	3.67 ± 0.00
MS media + 0.5 BAP + 0.1 NAA	5.33 ± 0.19	4.84 ± 0.16	3.14 ± 0.23
MS media + 1.0 BAP + 0.1 NAA	6.11 ± 0.11	5.50 ± 0.28	5.11 ± 0.22
MS media + 1.0 BAP + 0.5 NAA	3.44 ± 0.29	3.64 ± 0.21	3.07 ± 0.13
MS media + 2.0 KIN + 0.1 IAA	4.33 ± 0.28	3.83 ± 0.16	5.67 ± 0.33
MS media + 2.5 KIN + 0.1 IAA	4.66 ± 0.19	4.68 ± 0.22	4.52 ± 0.26
MS media + 3.0 KIN + 0.1 IAA	6.00 ± 0.00	5.19 ± 0.09	4.07 ± 0.07
MS media + 2.0 KIN + 0.5 IAA	4.62 ± 0.03	4.54 ± 0.02	5.41 ± 0.07
MS media + 2.5 KIN + 0.5 IAA	6.96 ± 0.04	5.89 ± 0.11	5.33 ± 0.19
MS media + 3.0 KIN + 0.5 IAA	4.26 ± 0.13	3.63 ± 0.32	4.78 ± 0.22
CD (P=0.05)	0.49	0.59	0.61

RAPD analysis

In the present work, the genetic fidelity of the nuclear genome of the mother plant of strawberry and their tissue cultured clones was also determined so that true to type character of calli derived plants could be established. Twenty one primer were used, out of which six primer (Table 3) showed amplification. The DNA amplification products, could result from changes in either the sequence of the primer binding sites or changes which alter the size and present the successful amplification of target DNA.

Table 3 Random primers showing amplification

Sr. No.	Primer code	Sequence
1	OPB 1	GTTTCGCTCC
2	OPB 2	TGATCCCTGG
3	OPB 3	CATCCCCCTG
4	OPB 4	GGACTGGAGT
5	A-05	GGGATATCGG
6	OPA 10	GACCGCTTGT

Figure 2 Morphological variation in plant raised through indirect regeneration

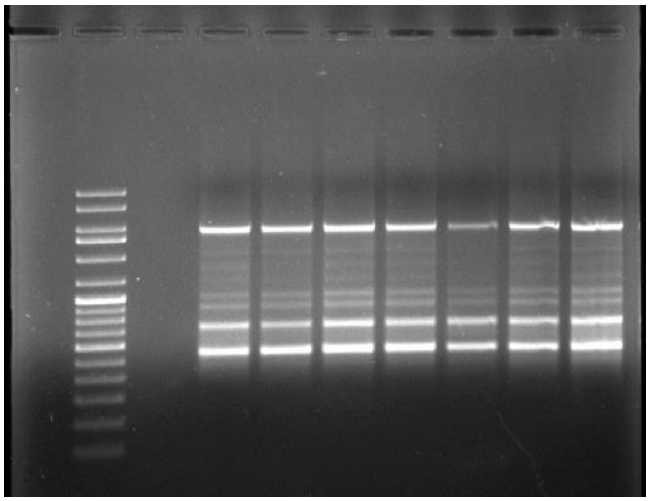

Figure 3 RAPD Profile of indirect regenerated plants showing monomorphic bands.

The results concluded that the tissue culture raised plants were genetically identical and uniform. Although, minor morphological variation **(Fig. 2)** were recorded in some plants regenerated via callus, the developed RAPD profiles **(Fig. 3)** of tissue culture raised plants were typical identical, because there were no changes in the banding pattern observed in direct regenerated, calli derived plants and mother plant irrespective of phenotypic variation. The phenotypic variations in calli derived plants might be due to higher concentrations of growth regulators (BAP and auxins). Similar results were reported by Gaffar and Saker [5] who described that epigenetic changes in tissue culture raised plants due to applications of plant growth regulators, but such trait is not passed to their offspring through sexual cycle or might entirely diaappear during plant maturation. Biswas et al. [23] reported that high concentration of BAP induced variation in plantlets. Similar results were reported by Sutan et al. [24] that epigentic variation might be found due to higher concentration of growth regulators but at molecular basis tissue cultured plants were true to the type.

Conclusion

Among the growth regulators tested, maximum callus induction percentage with minimum number of days for callus induction was observed on treatment MS basal + 4.mg/l 0 NAA + 2.0 mg/l BAP. MS medium most effective with regards to multiple shoot formation and number of leaves for direct regenerated shoots and for callus derived shoots, it was observed on MS media supplemented with 2.5 mg/l KIN and 0.5 mg/l IAA whereas, shoot length was recorded maximum on MS basal + 2.0 mg/l KIN + 0.1 mg/l IAA. All RAPD profile for *in vitro* raised plants were monomorphic and similar to their field grown mother plants. No polymorphism was detected with *in vitro* raised plants.

References

[1] Lal M, Sharma S, Micropropagation of strawberry (Fragaria x ananassa Duch.), Indian J. Agric., 2003, 37, 231-234.

[2] Vandana, Sharma, NK, Kumar M, EST SSR markers analysis of in vitro regenerated plantlets of festivel and Sweet Charlie cultivars of strawberry, Intl. J. Agric. Env. Biotech., 2012, 5(4),315-322.

[3] Haddadii F, Aziz MA, Salch G, Rashid AA, Kamaladini H, Micropropagation of strawberry cultivar Camarosa: Prolific shoot regeneration from in vitro shoot tip using Thidiazuron with N6-benzylamino-purine, Hortsci., 2010, 45(3), 453-456.

[4] Biswas MK, Hossain M, Islam R, Virus free plantlets production of strawberry through meristem culture, World J. Agric. Sci., 2007, 3(6), 757-763.

[5] Gaffar RM, Saker MM. Monitoring of cultivars identity and genetic stability in strawberry in strawberry varieties grown in Eygpt, World J. Agri. Sci., 2006, 2(1), 29-36.

[6] Debnath SC, Teixeira da silva JA, Strawberry culture in vitro: applications in genetic transformation and biotechnology, Fruit Veg. Cereal Sci. Biotech., 2007, 1(1), 1-12.

[7] Kaushal K, Nath AK, Kaundal P, Sharma DR, Studies on somaclonal variation in strawberry (Fragaria × annanasa Duch.) cultivars, Acta hort., 2004, 662, 269-275.

[8] Hancock JF, Callow PA, Shaw DV, Randomly amplified polymorphic DNA in cultivated strawberry (Fragaria x ananassa.), J. Amer. Soc. Hort. Sci., 1994, 119, 862-864.

[9] Dengani C, Rowland LJ, Levi A, Hortynski JA, Galleta GJ, DNA fingerprinting of strawberry (Fragaria x ananassa) cultivars using randomly amplified polymorphic DNA (RAPD) markers, Euphytica, 1998, 102, 247-253.

[10] Zebrowska JI, Tyrka M, The use of RAPD marker for strawberry identification and genetic diversity studies. Food Agri. Env., 2003, 1(1), 115-117.

[11] Mohamed AES, Somaclonal variation in micropropagated strawberry detected at molecular level. Int. J. Agric. Biol., 2007, 9(5), 721-725.

[12] Gantait S, Mandal N, Bhattacharya S, Das PK, Field performance and molecular evaluation of micropropagated strawberry, Rec. Res. Sci. Tech., 2010, 2(5), 12-16.

[13] Murray MG, Thompson WF, Rapid isolation of high molecular weight plant DNA, Nucleic Acids Res, 1980, 8, 4321-4325.

[14] Sagai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW, Ribosomal DNA spacer-length polymorphisms in barley : Mendelian inheritance, chromosomal location and population dynamics. Prac Natl. Acad. Sci. U. S. A., 1984, 81, 8014-8018.

[15] Xu GW, Magill CW, Schertz KF, Hart GE, An RFLP linkage map of Sorghum bicolor (L.). Moench. Theor. Appl. Genet., 1994, 89, 139-145.

[16] Ara T, Karim R, Karim MR, Islam R, Hossain M, Callus induction and shoot regeneration in strawberry (Fragaria x ananassa Duch.), Int.J. Biosciences (IJB), 2012, 10(1), 93-100.

[17] Karim R, Ahmed F, Roy UK, Ara T, Islam R Hossain M, Varietal improvement of strawberry (Fragaria x ananassa Duch.) through somaclonal variation using in vitro techniques, J. Agric. Sci. tech., 2015, 17,1-10

[18] Karim MR, Aziz MA, Roy UK, Hoque MA, Hossain MM, In vitro response of strawberry (Fragaria × ananassa Duch.) for callus induction and shoot regeneration, Int. J. Agr. Agric. R., 2011, 1, 29-36.

[19] Sood N, Srivastava R, Singh OS, Gosal SS, Enhancing micropropagation efficiency of strawberry using bandage in liquid medium, J. Appl. Hort., 2000, 2(2), 92-93.

[20] Bhatt RP, Kheroda DM, Jayaluxmi H, Sophia I, Prajani S, Effect of plant growth regulators on establishment and growth of strawberry (Fragaria x ananassa Duch.) var. Chandler in vitro, Agri. Sci. Res. J, 2012, 2(12), 623-632.
[21] Shashi, In vitro propagation and genetic fidelity studies in chrysanthemum, M.Sc. thesis. CCS Haryana Agricultural University, Hisar, 2011.
[22] Bhatt ID, Dhar U, Micropropagation of Indian Wild strawberry, Plant Cell, Tiss. Org. Cul., 2000, 60(2), 83-88.
[23] Biswas MK, Dutt M, Roy UK, Islam R Hossain M, Development and evaluation of in vitro somaclonal variation in strawberry for improved horticultural traits, Sci Hort., 2009, 122, 409-416
[24] Sutan AN, Popesscu A, Gheorghe R, Popescu CF, Isac V, Molecular markers for genetic stability of intergeneric hybrids (Fragaria x potentilla) derived from tissue culture, The Annals of Oradea University, Biology Fascicle, XVI (2), 2009, 146-149.

Publication History
Received 18th Jan 2017
Revised 13th Feb 2017
Accepted 13th Feb 2017
Online 25th Feb 2017