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IN VITRO PRESERVATION AND MOLECULAR CHARACTERIZATION OF THREE STRAWBERRY VARIETIES

Hassan, Nevin A.[1] and M.A. Ragab[2]

1. National Gene Bank and Genetic Resources, A.R.C. Giza , Egypt

2. Strawberry and Nontraditional Crops Center, Agriculture faculty, Ain-Shams University.

ABSTRACT

Low temperature preservation experiment was carried out with 3 strawberry varieties. The Merstim tips were collected in June and cultured on medium contain Knob salts , BAP10 mg/L, 30-g/L sucrose and 6-g/L agars with pH5.7 at 25^{0}C then at 4 and 12 weeks plant materials were transferred to media of knob salts without hormones and different concentrations of sucrose 0,20,30,40 and 60 g/L. Two different temperatures were evaluated for preservation 25 ^{0}C and 5 ^{0}C either kept in dark or 16/8 h light/ dark. .Preservation was checked after 2,6,9 and 12 months. . Results showed that the 3 varieties could be preserved for 12 months with survival percentage of over 50%. The molecular markers of the three varieties were detected using ISSR analysis. The three primers detected polymorphisms among the three varieties and revealed 11, 12 and 8 unique markers characterizing the different varieties.. The dendogramk constructed with ISSR data to study the distance between them.

INTRODUCTION

The use of *in vitro* repositories for the maintenance of valuable plant genotypes or virus –free plantlets offers a number of advantages over conventional methods. Space requirements and costly greenhouse maintenance are reduced, and the stored material is protected from insect pests and pathogens and can be micropropagared rapidly when desired .The use of low temperatures (2-10 ^{0}C) for long –term minimal –growth storage of cultured plant cells and organs has been applied successfully to grape (Morel,1975), apple (Lundergan and Janick 1979), strawberry (Mullen and Schlegel 1979) and for age crops (Dale, 1980 and Bhojwani1981).

In vitro preservation of plant cells, embryos and Merstim have become an important tool for the long-term storage of germplasm as it offers several advantages over maintaining field collections. Moreover, with increasing interest in genetic engineering of plants, the preservation of cultured cells with unique attributes in particular, genetically modified cells and plants is becoming of greater importance. The major advantage of storage of biological material at such temperatures is that both metabolic processes and biological deterioration are considerably slowed or even halted (kartha 1981). *In vitro* storing by slow growth is achieved by modifications of culture medium (Withers 1987) and /or low temperature (Withers 1991). Cold storage conservation methods for *in vitro* cultures of prunus and pyrus were successfully used (Duruart 1985; Wanas et al.1986; Wilkins et al.1988) .Two storage procedures for strawberry was developed in the 1970s. Damiano (1979) stored plantlets on solid medium at 2 ^{0}C. Most *in vitro* germplasm collections were stored on agar solidified media (Wilkins *et al.* 1988) .The use

of osmotic regulators (manitol, sorbitol and sucrose are recommended as they are relatively metabolically inert to minimize growth by imposing a level of osmotic stress on the cultures (Westcott 1981, Espinoza *et al.*1984, Ng and Hahan 1985 and Monette 1986). The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification (Nybom 1990).The development of molecular biology techniques provided DNA based markers for detecting genetic polymophism. More recently, molecular marker techniques based on the Polymerase Chain Reaction (PCR) technique (Saiki *et al.*, 1985 and Mulis and Faloona 1987) have become increasingly popular for fingerprintings and cultivar identification. Inter-simple sequence repeats markers (ISSR, anchored microsatellites) use simple sequence repeats anchored at the 5` or 3` end by a short arbitrary sequence as PCR primers (Zietkiewiez *et al* 1994) and take advantage of simple sequence repeats (SSR) or microsatellites that are abundant in all eukaryotic genomes. SSR are short, tandem repeats that are useful as genetic markers. However, use of SSR requires knowledge of the sequence of the region flanking the tandem repeats. ISSR, in contrast, does not require any prior knowledge of genome sequence. This analysis allows amplification of the genomic segments between inversely oriented repeats (ISSRs). ISSR markers have recently become widely used in population studies because they have been found to be highly variable, require less investment in time, money and labor than other methods (Wolfe and Liston, 1998). Also, ISSRs are ideal as markers for genetic mapping and population studies because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes (Cregan *et al.*, 1994, Jarret and Bowen 1994, Lanham and Brennan 1995 and Hokanson *et al.*, 1998) and have the ability to be inherited (Gupta *et al.*, 1994; Tsumura *et al.*, 1996)

The aim of the present study was to preserve strawberry plants under different conditions of preservation such as, reducing temperature, light and addition osmotic regulators. Also,use ISSR markers to assess the polymorphism among the three varieties and to identify additional unique markers characterizing each of them.

MATERIAL AND METHODES

In this investigation three strawberry varieties (Fragaria ananassa); Tamar, camarosa and Diamond were used. Runners ranging in length from 2 to 3 cm were taken and surface sterilized by 70%ethanol for 1min followed by 30% sodium hypochlorite solution for 30 min and then rinsed three times with sterile distiller water. Merstims were excised and cultured in glass tubes (100 x25 mm) contained Knop's medium supplemented with BAP 10 mg/L, and 30-g/L sucrose and 6-g/L agars and pH were adjusted to 5.7 before autoclaving. The normal incubation conditions were at 25±2°C, photoperiod 16 hr. 1500 lux with Philips white fluorescent tubes for 4 weeks and 12 weeks before placing cultures in the storage conditions.

For cold storage and to asses tho importance of increased sugar content and osmotic stress in med –term storage the proliferated seedlings (12 weeks) were individually transferred to Knop's medium without hormones

and supplemented with different sucrose concentration (0, 20, 30, 40, and 60 g/L) and then incubated at 25 °C in 16 hr. of 1500 lux light at 25 °C in the dark and 5 °C in the dark(seedlings of 4 weeks and 12 weeks). Fifteen cultures were taken after 2, 6, 9, and 12 and survival percentage was recorded. The cultures were transferred to fresh medium containing hormones and placed under standard culture room condition for four weeks and then survival percentage were assessed. Each treatment consisted of 15 replicate .All experiments were designed in complete randomized design and obtained data were statistically analyzed using the method described by Waller and Duncan (1969).

Molecular marker

Isolation of plant genomic DNA

DNA extraction was carried out using leaf materials collected from each variety. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA).

Scoring of the data:

Scoring of ISSR data was performed from 1% agarose gel electrophoresis, as clear and distinct amplicans were scored as (1) for presence and (0) for absence.

ISSR

Inter Simple Sequence Repeats (ISSR) was carried out according to the procedure given by (Sharma *et al.*, 1995).ISSR analysis was carried out in a total volume of 50 ul containing 5 ul 19x buffer, 10 ul Q solution,5 ul of 2 mM dntps, 80 pmol primer, 0.5ul hot start taq polymerase and 25 ng DNA. The temperature profile composed of initial denaturating cycle at 95 °C for 25 min followed by 10 touch down cycles of 95 °C / 30 sec, 65-55 °C /1 min, 72 °C 90 sec. This was followed by 30 cycles of 95 °C / 30 sec, 55 °C C/ 1min, 72 °C / 90 sec and then a final extension cycle at 72 C for 7 min. The primers sequences of the three ISSR primers are presented in table (1).

Table (1): Name and sequence of the primers used in ISSR analysis.

Primer code	Nucleotide sequences 5`———3`
P1	(CA)6GT
P10	(CAC) 3GC
P11	(GAC) 3GC

RESULTS AND DISCUSSION

Storage at 25 °C in darkness:

Explants stored up to 3 months remained fully viable (100 % survival) at all sucrose concentrations after the 3 months the seedling became white and succulent (Table 3). Darkness caused the death of all the shootlets after 3 months at all sucrose concentrations (Table 2).

Storage at 25 °C with light: -

Explants with 2 months old, up to 9 months revealed 90 % healthy and green shoots on medium with 30 g / L sucrose and viability decreased to 75% after 12 months.

Table (2): Survival and recovery of *Fragaria ananassa* shootlet during storage at different temperature and light with difforont Concentrations of osmotic stress induced sucroso.

Time in storage (Month)	Survival rate at 25 C With light %						Survival rate at 25 C in Darkness %						Survival rate at 25 C darkness %					
	Sucrose concentration						Sucrose concentration						Sucrose concentration					
	0/g	20/g	30/g	40/g	60/g	Recv.	0/g	20/g	30/g	40/g	60/g	Recv.	0/g	20/g	30/g	40/g	60/g	Recv.
3 months	100% a	100% a	100% a	100% a	100% a	100%	100% a	100% a	100% a	100% a	100% a	100% a	83 % b	100% a	100% a	100% a	100% a	100%
6 months	10% e	74% b	90% a	40% d	30% c	100%	0	0	0	0	0	0	50 % d	100% a	100% a	85 % c	90 % b	100%
9 months	0 d	70% b	90% a	20% c	20% c	100%	0	0	0	0	0	0	0	80 % b	90 % a	70 % c	75 % c	100%
12 months	0 e	62% a	75% b	10% d	20% c	100%	0	0	0	0	0	0	0	75 % b	85 % a	50 % d	60 % c	100%

*Letters show the significances between treatments /month

Lowest percentage was obtained on medium absent of 0 g / L sucrose and remained fully viable up to 3 months (100 % survival) and decrease to10 % after 6 months (Table 2).

Table (3): Survival and recovery of *Fragaria ananassa* Merstim tips during storage at low temperature of 5 C in darkness and with different concentrations of sucrose.

Time in storage (mo.)	Survival rate at 5C in darkness %					
	Sucrose concentration					
	0/g	20/g	30/g	40/g	60/g	Recovery
2 months	100% a	100% a	100% a	100% a	100% a	100%
6 months	100% a	100% a	90% b	80% c	90% b	100%
9 months	100% a	100% a	70% c	50% d	80% b	100%
12 months	87 % b	80% b	50% d	0% f	60% c	100%

*Letters show the significances between treatments /month

Storage at 5 °C in darkness: -

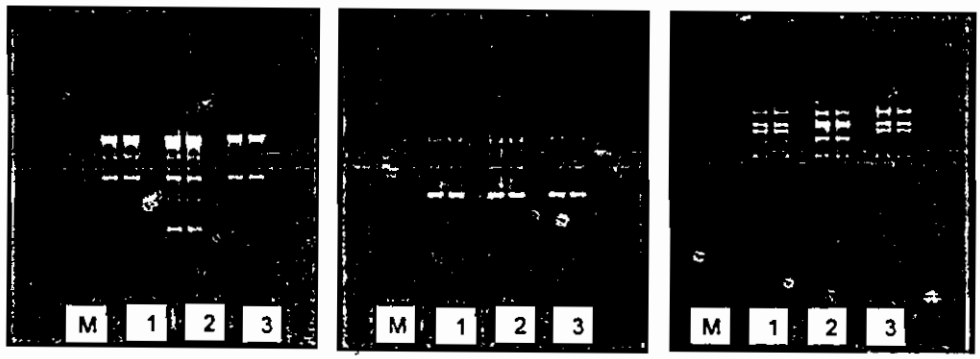
Up to 12 months 85 % of shoot culture remain healthy and green on medium with 30 g / L sucrose, while the lowest survival rate 50 % was observed on medium with 40 g/ L sucrose (Table 2). The Merstim tips showed that up to 12 months 87% of Merstim tips remain healthy and green on medium with 0 g /L sucrose while the survival rate was 50% on medium with 30 g/L sucrose (Table ٢). These results are in line with those obtained by, Damiano (1979) who mentioned that survival rate of strawberry plantlets which stored at 2 °C was 90% for plantlets of some strawberry varieties, and were successfully stored at 4 °C in the dark for 24 months and for 12 months with 74 % survival rate (Reed 1991, 1992) .In this respect, Flecher (1994) mentioned that the cultures of asparagus remained viable after 15-16 months of storage at 6 °C. The shoots of *Saussurea lappa* stored *in vitro* at 5 C in dark up to 12 months remained viable (Arora and Bhojwani 1989). Also, HaeBoong *et al.*, (1996) found that the best storage condition for *in vitro* storage of strawberry germplasm was at 2 °C in light; he also mentioned that storage was best when the previous culture period was short and when the plantlets were very young. Explants of 22 strawberry varieties were preserved at 4 °C for 4 months with a survival percentage of over 50 % (Yu GuiHong *et al.*, 2003). The use of combination of addition of osmotic regulators or growth inhibitors and low temperature storage may be of benefit. Westcott (1981) used growth inhibitors and low temperature with successfully for *solanum tuberosum* storage.

Molecular fingerprints of strawberry variety based on (Inter Simple Sequence Repeats (ISSR):

All primers used in the present investigation resulted in the appearance of PCR products with varied band number and sizes .A total of 52 DNA bands were detected across the 3 ISSR primers; 30 of them were polymorphic (Table 4,5).

Primer P1showed 20 bands with 86.3%polymorphism (Table5), the fragment sizes ranged from 237 to 1825 bp Fig (1). Primer P10 revealed 17 bands with 76.4% polymorphism (Table5), the fragment sizes ranged from

263 to 1700 bp Fig (2). Primer P11 produced 13 bands with 92.3% polymorphism (Table5), the fragment sizes ranged from 259 to 927 in Fig (3). The Tamar variety was characterized by 11 unique markers (P1-1008, P1 739, P1-464, P1 – 326, P1-279, P10 –1375, P10- 1000, P10 –627, P10 –457 and P11- 577) and three negative markers (P1-572, P1- 451 and P10 –483). Diamont variety had discriminated 12 positive markers (P1- 1225, P1- 718 P1- 500, P1- 335, P1-289, P11- 680, P11- 435, P11 354, P10 –1700,P10 – 757,P10 – 418 and P10 –331. and 3 negative markers (P10 –570, P10 –365 and P11- 373). Camarosa variety revealed 8 positive markers (P1-271, P1-1202, P1- 1825P10 655, P10- 1425, P11 –608, P11- 811 and P11 –927) and 6 negative markers (P1- 379, P1- 1128, P1- 1643, P11- 876, P11- 758, P11 525 and P11-259)



A B C

Fig.(1): ISSR profiles of the three strawberry varieties as detected by different ISSR primers (1) Tamar (2) Diamont (3) Camarosa as detected by (A) p1 (B) P10 (C) P11, M = 100 bp marker.

Table (4): Unique ISSR markers showing polymorphism among the three *Fragaria ananassa* Varieties.

Primer	Band size in bp	Tamar	Diamont	Camarosa
P1	1825	0	0	1
	1643	1	1	0
	1306	1	0	1
	1225	0	1	0
	1202	0	0	1
	1128	1	1	0
	1008	1	0	0
	739	1	0	0
	718	0	1	0
	572	0	1	1
	500	0	1	0
	464	1	0	0
	451	0	1	1
	379	1	1	0

Continued Table (4): Survey of ISSR – PCR markers in three *Fragaria ananasa* Varieties using 3 primers

Primer	bp	Tamar	Diamont	Camarosa
	335	0	1	0
	326	1	0	0
	289	0	1	0
	279	1	0	0
	271	0	0	1
	237	1	1	1
P10	1700	0	1	0
	1567	1	1	1
	1425	0	0	1
	1375	1	0	0
	1238	1	1	1
	1000	1	0	0
	800	1	1	1
	757	0	1	0
	655	0	1	1
	627	1	0	0
	570	1	0	1
	483	0	1	1
	457	1	0	0
	418	0	1	0
	365	1	0	1
	331	0	1	0
	263	1	1	1
P11n2.tif	927	0	0	1
	876	1	1	0
	811	0	0	1
	758	1	1	0
	680	0	1	0
	608	0	0	1
	577	1	0	0
	525	1	1	0
	435	0	1	0
	373	1	0	1
	354	0	1	0
	311	1	1	1
	259	1	1	0

Notes: (1) means presence band, (0) means absent band

Table (5):Total number of amplicans and the level of polymorphism among the three strawberry varieties as revealed by ISSRs.

Primer ID	Total number of amplicans	Polymorphic amplicans	% of polymorphism
P1	22	19	86.3%
P10	17	13	76.4%
P11	13	12	92.3%

Cluster analysis as revealed by ISSR:

ISSR dendogram obtained from UPGMA cluster analysis of genetic distances is presented in Fig. (4). The similarities and dissimilarities between the examined three varieties of *Fragaria ananasa are* illustrated in dendogram using the linkage between varieties. It is clear that the two varieties Diamont and Tamar are closer related than the camarosa.

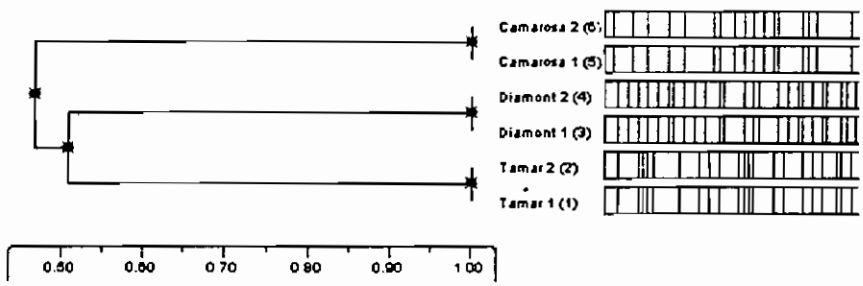


Fig. (4): Dendogram showing the genetic relatedness of strawberry (*Fragaria ananasa)* varieties based on the analysis of ISSR data.

Different authors reported on the usefulness of ISSR for cultivar identifications The ISSR technique, technically not much more difficult for marker development than RAPD, and also, requiring a small amount of DNA for amplification, enables the detection of the genome (Zietkiewicz *et al.*, 1994). ISSRs are ideal as markers for genetic mapping and population studies because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes (Cregan *et al.*, 1994, Jarret and Bowen 1994, Hokanson *et al.*, 1998 and Lanham and Brennan 1998). Arnaud *et al.*, (2000) tested ISSR analysis for the identification of strawberry varieties that had previously been tested with AFLP. They concluded that ISSR is a good alternative to AFLP, being cheaper, more rapid and more reproducible. Korbin *et al.* (2002) used molecular marker generated in RAPD and ISSR for genetic identities of 12 strawberry genotypes and their results confirmed the usefulness of RAPD and ISSR markers in determining genetic distance between genotypes in germplasm, dividing them into relatedness group, and, based on this selecting the most interesting parental forms were chosen for crossing. Also, Arnaud *et al.* (2003) confirm that ISSR technique is a potentially useful tool for the identification of strawberry varieties because it is simple, fast, cost-effective, highly discriminate and highly reliable.

In conclusion, the low temperature storage and modified sugar concentrations seem to be most applicable to the *in vitro* germplasm storage of *Fragaria ananassa* in order to expand the duration of reculturing and subsequently to save the expensive sub culturing requirements. The storage was best when the plantlets were very young and previous culture period was short. Also, ISSR technique used to identify the three strawberry varieties and ISSR dendogram divided them into 2 groups according to their relatedness.

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استخدام تقنية زراعة الأنسجة في حفظ ثلاث أصناف من الفراولة وإجراء البصمة الوراثية لها.

نيفين عبد الفتاح حسن[1] و محمد إمام رجب[2]

١-البنك القومي للجينات ــ مركز البحوث الزراعية.

٢-كلية الزراعة- جامعة عين شمس.

أجريت هذه الدراسة على ثلاث أصناف من الفراولة (تمار- د دياموند- كمـاروزا) . للحصول على مزارع النموات الخضرية تم زراعة المرستيمات على بيئة تحتوى علـى أمـلاح كنوب كما يضاف إلى البيئة سيتوكينين بينزيل امينو بيورين بتركيز ١٠ مليجرام/ لتر و٣٠ جرام/ لتر سكروز و٦ جرام / لتر أجار ثم يتم ضبط درجة الحموضة على ٥,٧. ولدراسة تأثير إضـافة منظمات الضغط الاسموزى (السكروز) بتركيزات مختلفة (صفر،٤٠،٣٠،٢٠، ٦٠ جرام /لتر) و كذلك استخدام الإضاءة أو الإظلام و أيضا استخدام درجات حـرارة مختلفـة (٥م^0 ،٢٥ م^0) علي حفظ هذه الأصناف باستخدام تقنية زراعة الأنسجة. أيضا تم تحديد البصمة الوراثية باستخدام تقنية ISSR للثلاث أصناف وقد أظهرت النتائج الآتي:

.يمكن حفظ الثلاث أصناف من الفراولة حتى ١٢ شهر بنسبة مئوية تزيـد عـن ٥٠ % تحـت درجات الحرارة المنخفضة (٥م^0) وتحت إظلام تام وبتركيز سكروز ٣٠ جرام/ لتر وذلك باستخدام فريخات بعمر ١٢ شهر . كما تم عمل البصمة الوراثية للثلاث أصناف باستخدام ثلاث بادئـات وراثية كما تم تحديد القرابة الوراثية حيث تم تقسيم الثلاث أصناف إلى مجموعتين تبعـا للقرابـة الوراثية لهم.