STUDIES ON GENETIC FIDELITY OF MICROPROPGATED PLANTS OF STRAWBERRY (*Fragaria× ananassa* DUCH.) USING MOLECULAR MARKERS.

Thesis

by

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Studies on genetic fidelity of micropropagated plants of strawberry (*Fragaria* × *ananassa* Duch.) using molecular markers"** submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Mr. CHHERING BODH (H-2014-43-M)** son of Sh. Sohan Singh under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

Place : Nauni-Solan
Dated: JULY, 2017

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Studies on genetic fidelity of micropropagated plants of strawberry (*Fragaria* × *ananassa Duch.)"** submitted by **Mr. CHHERING BODH (H-2014-43-M)** son of Sh. Sohan singh to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Studies on genetic fidelity of micropropagated plants of strawberry (*Fragaria × ananassa Duch.)*"** submitted to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni-Solan (HP) by **Mr. CHHERING BODH (H-2014-43-M)** in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY.**

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I own entire responsibility for all the errors and omissions.

Place: **Chhering**
Date:

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LIST OF ABBREVIATIONS

ºC	-	Degree celcius
%	-	Per cent
µg	-	Micrograms
µl	-	Microlitre
ADW	-	Autoclaved distilled water
AFLP	-	Amplified fragment length polymorphism
bps	-	Base pair (s)
cM	-	centiMorgan
cDNA	-	Complementary DNA
CTAB	-	Cetyl trimethyl ammonium bromide
cv.(s)	-	Cultivar (s)
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotide 5-triphosphate
EDTA	-	Ethylene diamine tetraacetic acid
EST-SSR	-	Expressed Sequence Tag-Simple Sequence Repeat
Fig.	-	Figure
Gm	-	Gram
HCl	-	Hydrogen chloride
Kb	-	Kilobase pairs
l	-	Litre
M	-	Molar
mA	-	Milliampere
mg	-	Milligrams
Ml	-	Millilitres
mM	-	Millimolar
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NCBI	-	National Centre for Biotechnology Information
ng	-	Nanograms
nm	-	Nanometres
NTSYSpc	-	Numerical Taxonomy System personal computer

PCR	-	Polymerase chain reaction
pMoles	-	Picomoles
RAPD	-	Random amplified polymorphic DNA
RFLP	-	Ristriction fragment length polymorphism
RNase	-	Ribonuclease
SAHN	-	Sequential Agglomerative Hierarchical and Nested Clustering
rpm	-	Revolutions per minute
Spp.	-	Species
SSRs	-	Simple sequence repeats
TAE buffer	-	Tris acetate EDTA buffer
Taq	-	*Thermus aquaticus*
TE	-	Tris EDTA
U	-	Unit
UPGMA	-	Unweighed pair group method with arithmetic mean
UV	-	Ultra-violet
V	-	Volts
v/v	-	Volume/volume
v/w	-	Volume/weight
Ver.	-	Version

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Chapter-1

INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is one of the most important members of family Rosacae. The cultivated strawberry is an octaploid (2n =8x = 56) stoloniferous perennial herb. It is cultivated worldwide for its fruit. The fruit, which is not a botanical berry but an aggregate accessory fruit is widely appreciated for its characteristic aroma, bright red color, juicy texture and sweetness. (Lal and Sharma 2003). Strawberry is valued for its low-calorie carbohydrate and high fiber contents. It is good source of natural antioxidants, including carotenoids, vitamins, phenols, flavonoids, dietary glutathionine metabolites and exhibit a high level of antioxidant capacity against free radicals, that are believed to reduce carcinogens in humans and protect against tumor development (Kresty *et al.* 2001)

It has a wide range of climatic adaptation which includes Mediterranean, temperate, subtropical and taiga zones (Hancock *et al.* 1994). The Huelva region in Spain is main strawberry growing area in Europe and fourth worldwide after the United States, Mexico and Turkey (FAOSTAT, 2014). In India, it is mainly grown in Himachal Pradesh, Jammu and Kashmir,Uttar Pradesh, Maharashtra, West Bengal, Punjab, Haryana, Rajasthan, Delhi and the Nilgiri. Maharashtra's Panchgani-Mahabaleshwar belt leads the country's strawberry production (Anonymous, 2011).

It is consumed in large quantities, either fresh or in prepared foods such as preserves, fruit juice, pies, ice cream, milkshakes, and chocolates. Artificial strawberry flavorings and aromas are also widely used in many products like candy, hand sanitizer and perfumes.

Strawberry fruits contain vitamin B,C and it is also a good source of meganese. Strawberry cultivation is quite labour intensive and is propagated by runners. Runner is a slender, prostrate branch with long internodes creeping at the ground, rooting at node and growing in to a new plant. Therefore, adaptation of micropropagation technique has been shown to be a viable alternative to traditional runner production. The use of micropropagation technique can play a vital role to produce large number

of true-to-type plants from a meristematic tissue in a relatively short period of time and space. Rapid proliferation obtained by *in- vitro* propagation allows farmers to meet an unexpected demand for particular cultivar. Strawberry is perhaps first fruit crop in which micropropagation technique has been standardized (Sharma *et al.* 1999). Cultivar 'Ofra' is commercially important one and has been micropropagated and multiplied.

Clonal fidelity is one of the main concerns in commercial micropropagation. True-to-Type propagules and genetic stability are prerequisites for the application of strawberry propagation *in vitro*. The occurrence of variations in plants regenerated from *in- vitro* cultures was named as 'somaclonal variation' by Larkin and Scowcroft (1981) and have been reported for morphological and yield variation in micropropagated strawberries. There is a pressing need to recommend the use of reliable methods for studying genetic fidelity of micropropagated plants

Several types of molecular markers such as RAPD, SSRs, ISSRs are most commonly used molecular markers for the assessing genetic fidelity of micropropagated plants. Random amplified polymorphic DNAs (RAPD) and inter simple sequence repeat(ISSR) evaluate the trueness to type of tissue cultured material (Anand *et al*. 1997) ; Rout *et al*. 2002 and Kaur *et al.* 2004). The use of molecular marker based on DNA result in a consistent and robust method to identify plant material based on their stability in different conditions. Though, variations can be studied through morphological and biochemical markers also, but DNA markers are stable and whereas morphological traits can be influenced by environmental and developmental stages. Molecular markers have many numerous applications in plant breeding including the anlysis of genetic similarity or diversity, cultivar identification, gene tagging and QTL analysis.

RAPD analysis a PCR–based molecular marker is a powerful technique for identification of genetic variations. It was developed initially by Williams *et al.* 1990. These DNA markers are the results of PCR amplification of random genomic DNA segments with a single primer usually 10 nucleotide long of arbitrary sequence.

Inter simple sequence Repeat (ISSRs) is another molecular marker technique given by Zietkiewicz in 1994. ISSR is a general term for a genomic region between microsatellite loci. ISSRs are ideal as markers for genetic fidelity, genetic mapping

and population studies because of their abundance and high degree of polymorphism of closely related genotypes. (Singh *et al.* 2011).

ISSR and RAPD markers have been successfully applied to detect the genetic similarity or dissimilarity in various plant species (Luisa *et al.* 2004 and Ramage *et al.* 2004; Modgil *et al.* 2005 and Kaur *et al.* 2009) . This type of analysis has additional advantages, such as a short turnaround time for results and being highly innovative (Arnau *et al.* 2003) . Such markers have been widely used because they are efficient, reproducible, and fast and generate high polymorphisms (Kuras *et al.* 2004) and Reddy *et al.* 2002). In addition, these two marker systems are, relatively, simple and cheap.

Presently availability of good plant material of a required cultivar in a large quantity is a major limitation in expansion of strawberry cultivation. Hence, non-availability of disease free planting material is barrier in its cultivation. So keeping in view this problem, regeneration through *in vitro* culture has now become a viable and alternate method to conventional one. The formation of healthy shoots and higher rates of multiplication is one of the pre-requisite of an economically viable propagation. However, somaclonal variations have been observed in the plants raised through tissue culture. Particularly which raised through callus induction, which defeats the purpose of producing true- to- type plants. Hence the need arises to study the genetic fidelity of tissue culture raised strawberry plants.

There are several commercially viable cultivars of strawberry. Cultivar ‘Ofra’ is one of them. Micropropagation makes avialable the plant material throught the year.

Keeping in mind the importance of strawberry the present studies were proposed to achieve the following objectives:

1. Maintenance of *in-vitro* cultures of “ofra”.
2. To asssess genetic ‘trueness-to-type’ of long term microprogated plants using molecular markers.

Chapter-2

REVIEW OF LITERATURE

Review is discussed in the light of available litreture relevant to the research problem on strawberry.

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2.2 Maintenance of *in-vitro* cultures of other crops

2.3 Studies on genetic stability of tissue culture raised plants

2.3.1 RAPD analysis

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2.3.3 ISSR analysis

2.3.4 ISSR analysis in other crops

2.1 Maintenance of *in-vitro* cultures of "Ofra"

Plant tissue culture technique which paved the way for development of modern plant biotechnology has become a powerful tool for studying and solving various problem of modern plant biotechnology. As our traditional wealth on plant genetic resources has been decreasing tremendously, these techniques have gained greater momentum on commercial application in the field of plant propagation. Tissue culture techniques are becoming increasingly popular as alternative means of vegetative propagation in many plant species.

The term tissue culture technique colloquially covers a wide range of techniques including *in vitro* culture of organs (shoot tips, root tips, runner tips, stem segments, flowers, anthers, ovaries, ovules, embryos, etc.), tissues, cells and protoplasts. These techniques are become useful tools for rapid and clonal multiplication of plants. Since 1902, when Haberlandt gave the idea of totipotency considerable success has been achieved in plant tissue culture.

The area of plant tissue culture has grown phenomenally since 1962 when Murashige and Skoog developed a revised medium for rapid growth and bioassay of tobacco tissue culture. Recent advances in plant tissue culture leading to the

development of high yielding, uniform virus free cultivars, are some of the major achievements. Today, its tremendous potentiality in plant productions not only provides an edge over the conventional methods of vegetative propagation in higher multiplication rates, but also makes it possible to produce plants that are resistant to abiotic and biotic agents (drought, salinity). Sufficiently wide range of variations is the base of any effective crop improvement programme.

In vitro studies on strawberry (*Fragaria* × *ananassa* Duch.) have been conducted during the last two decades. Boxus (1974) determined the proper concentration of BAP necessary to obtain a large number of shoots from meristem tip explants in strawberry. Since then it became possible to multiply strawberry plants on an industrial scale.

Battistini *et al*. (1991) conducted studies *in vitro* to evaluate the performance of somaclonal strawberry (*Fragaria* x *ananassa* cv. Brighton) variants for susceptibility to *Phytophthora cactorum*.

Bhatt *et al*. (2000) studied that strawberry nodal segments cultured on MS medium supplemented with BA 4.0 µM and NAA 0.1 µM gave the best explant establishment and shoot number per explants..

Owen *et al*. (1996) conducted study to maximize plant regeneration frequencies from cultured anthers of 'Chandler', 'Honeoye', and 'Redchief' strawberries (Fragaria x ananassa). A comparison of auxins (IAA, NAA), cytokinins (BA, BAP, KIN) and carbohydrates (sucrose, glucose, maltose) in MS medium showed that the highest shoot regeneration across cultivars (8%) occurred when using a medium containing 2 mg/l IAA, 1 mg/l BA, and 0.2 M glucose.

Mansouri *et al*. (1996). developed an efficient and reliable method for shoot regeneration from leaf disks of *Fragaria vesca*. This protocol has been successfully employed to obtain transformed plants using *Agrobacterium tumefaciens* as gene vector. Murashige and Skoog basal medium supplemented with benzyladenine (4 mg/l) and indole-3-butyric acid (0.25 mg/l) induced maximum percentage of shoot regeneration (98%) and the highest number of shoot colonies per explant (4.6) after 8 weeks of culture. They observed that isolated shoots would elongate and proliferate when the benzyladenine concentration was lowered to 0.5 mg/l.

Khan *et al*. (2004) conducted a study on the *in vitro* callus culture from the leaf disc explants in strawberry (*Fragaria* x *ananassa* cv. Tango). They reported a high percentage of regeneration and established a new protocol for the speedy micro propagation of strawberry.

Litwinczuk *et al.* (2009) reported influence of gibberellic acid on *in vitro* shoot culture growth and development of strawberry. In general, gibberellic acid improved axillary shoot elongation and reduced the growth of callus as well as the formation of roots and development of adventitious shoots. GA_3 applied at a concentration of 1.0-2.0 mg/l significantly increased the number of axillary shoots, whereas under higher (5.0-10.0 mg/l) doses it stimulated runners.

Biswas *et al.* (2009) studied the meristem culture, direct leaf organogenesis, callus culture and somatic embryogenesis of strawberry. Meristem were transferred on a filter paper bridge in a test tube containing liquid MS medium supplemented with GA_3 (0.5 mg/l) for primary establishment. For direct leaf organogenesis, leaves were cultured on MS medium supplemented with BAP (6 mg/l). For micropropagation shoots, *in vitro* plants were cultured on MS medium supplemented with BAP (2.0 mg/l) and kinetin (0.5 mg/l). For callus culture, leaves and nodal segments were placed on MS medium supplemented with 2,4-D (3 mg/l) for callus induction, whereas somatic embryogenesis induced from leaves derived callus. Three week old calli were transferred on somatic embryogenesis medium and placed in dark. They observed that medium containing 2,4-D (1.0 mg/l) and BAP (0.5 mg/l), supplemented with 25 percent proline were suitable for somatic embryogenesis.

Haddadii *et al*. (2010) developed an efficient micropropagation system for strawberry cv. Camarosa. Shoot tips derived from the *in vitro* stock plants were cultured on MS medium containing 0, 2, 4, and 8 µM thidiazuron (TDZ) and 0, 4, 9, 18, and 27 µM BAP for shoot induction. Optimum number of shoots were produced on medium containing 2 µM TDZ and 4 µM BAP. They observed that increasing the TDZ concentration in absence of BAP, increased the mean number of shoots produced per explants. However, by increasing the BAP concentration in absence of TDZ reverse was observed. Poor shoot production also occurred at high concentrations of BAP.

Moradi *et al*. (2011) carried out an experiment to examine the effects of different combinations of plant growth regulators on *in vitro* propagation of strawberry. Theyfound that the best concentration of BAP for bud induction was 0.5 mg/l plus kinetin 0.2 mg/l.

Zobayer *et al.* (2011) reported that maximum percentage (80%) of shoot was obtained from lower concentration of BAP (0.5 and 1.0 mg/l) and higher concentrations of kinetin (1-2 mg/l). More promising result was obtained by combining two concentrations kinetin (2.0 mg/l) + BA (0.5mg/l) and kinetin (1.5 mg/l) + BA (1.0 mg/l).

Ara *et al*. (2012). developed a simple and rapid protocol for micropropagation of strawberry (*Fragaria* x *ananassa* Duch.) by using runner tips and nodal segments. The excised runner tips and nodal segments were cultured on MS medium containing 6-benzylaminopurine, 6-furfuryl amino purine, indole-3-butyric acid and gibberellic acid at various levels of concentration and combination for multiple shoot induction and proliferation. The highest percentage of shoot proliferation was found when both explants (92% from nodal segments and 83% from runner tips) were cultured on MS medium supplemented with 1.5 mg/l 6-benzylaminopurine + 0.5 mg/l 6-furfuryl amino purine. Shoots were rooted most effectively in MS_0 medium. Nodal segments were found more responsive explants than runner tips for rapid clonal propagation in strawberry.

Sharma *et al.* (2012) reported that nodal segments of strawberry cultivars Festival and Sweet Charlie were cultured on MS medium supplemented with different concentration of BA and kinetin as sole and in combination. At higher of BA concentrations provided (1.5 mg/l), lower the number of shoots as well as the percentage response, whereas kinetin at 2.0 mg/l gave maximum number of shoots. They also observed combined effect of BA (1.5 mg/l) and kinetin (0.5 mg/l) which produced maximum number of shoots.

Ashrafuzzaman *et al*. (2013) conducted *in vitro* propagation of strawberry at the Biotechnology Lab. Of BARI, Joydebpur, Gazipur. For shoot induction, five BAP concentrations viz., 0.0 (Control), 0.5, 1.0, 1.5, and 2.0 mg/l and for root induction four IBA concentrations viz., 0.0 (Control), 0.5, 1.0, and 1.5 mg/l were used. The highest average number of shoots (7) and the highest average length (3.34 cm) of

shoot was observed at the concentration of 0.5 mg/l BAP. The highest average number of leaves (5) was also observed at the same concentration. Among the five rooting concentrations, IBA @ 0.5 mg/l showed the best performance in all the parameters studied. The highest number (6) of roots/culture and the longest (3.05 cm) roots were also obtained from this concentration. Half strength MS media without IBA concentration did not show any response regarding root induction.

Madhavrai *et al.* (2014) reported that shoot tip of strawberry gave highest response of shoot multiplication in MS medium containing kinetin (2.0 mg/l) + IAA (0.5 mg/l) and kinetin (1.5 mg/l) + TDZ (0.5 mg/l) for 'Ofra' and Chandler respectively.

Diengngan *et al*. (2014) developed an efficient micropropagation protocol for nodal segments of strawberry cultivar Festival. They revealed that shoot proliferation percentage was maximum in MS medium supplemented with TDZ (0.5 mg/l). The maximum number of shoots and shoot length was obtained in MS medium supplemented with TDZ (1 mg/l) was 6.40±0.37 and 3.50±0.07 respectively.

Haragude *et al*. (2014) revealed that nodal segments of strawberry cultivar Sweet Charlie give rise to multiple shoots cultured on MS medium supplemented with different concentration of BA with kinetin or GA_3. The highest response of shoot multiplication was obtained on MS medium containing BA (1.5 mg/l) + kinetin (0.5 mg/l).

Bhandari *et al*. (2015) studied *In vitro* multiplication of Strawberry plant and its genetic stability on three basal media (MS, B5 & NN) and MS showed the best response. Very little and no response was observed in B5 and NN respectively. MS medium supplemented with equal concentration (5mg/l) of NAA and BAP each and low concentration (0.5mg/l) of GA_3 showed maximum response (86%) with maximum number of shoot bud proliferation (60 per explant). Increase in growth regulator concentration decreased the shoot multiplication response. No rooting was observed in auxin free media. MS media supplemented with IBA (0.5mg/l) and IAA (0.5mg/l) initiated better rooting. They also performed Genetic stability test with isozymic analyses (esterase, peroxidase, α-amylase and acid phosphatase) of the in vivo and in vitro grown plant tissue using crude enzyme/ total protein as a starting sample. Among the four isozymes tested, esterase and acid phosphatase showed high

polymorphism and two others were low in polymorphic band. The isozyme profiling of both *in vivo* and *in vitro* plants exhibited common banding pattern indicating their same genetic fidelity.

2.2 Maintenance of *in-vitro* cultures of other crops

Mikula *et al*. (2002) experimented with three years old embryogenenic suspension cultures of *Gentiana pannonica*. The initial explant for the suspension determined both, the embryogeneic charcters and embryo production. The hypocotyls, cotyledon and root explants were used for embryo production on MS medium supplemented with 1.0 mg/ml Kn, 0.5 mg /ml 2,4-D, which were later transferred and maintained in liqud MS medium with 1.0 mg dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80.0 mg/l Adenine sulphate (AS). Regeneration medium included 0.0-1.0 mg/l GA_{3} + 0.0-2.0 mg/l Kn. To complete the embryugenesis, the medium was changed from liquid to solid in presence of the same plant growth regulator combinatons. The most of the embroyonic cultures, but no embryos were found by using hypocotyls as an explant, while highest embryonic cultures, bit no embryos were obtained from roots, Almost 50% of embryos from all studied suspensions posed conservation into germ ling stage and plants were regenerated.

Mala *et al*. (2003) micropropagated the critically endangered plant species. *Gentiana verna* L. for its preservation organogenesis on the vegetative shoot was sucessful on the MS medium with increased concentration of Auxin IBA (0.5mg/l) with 70-80 % sucrose. The mortality during acclimatization was not over 5 %. At present five multi-topped cultures from each clone are stored in the explant bank.

Cao *et al*. (2005) induced callus and regenerated plants from leaves and hupocotyls of *G.macropylla*. The optimum medium found for callus induction was MS medium supplemented with 2.0 mg/l 2,4- D and 0.5 mg/l BA. 100% callusing was observed under such conditions and induced calluses were transferred to MS medium supplemented with 2.0 mg/l 2,4-D, 0.5 mg/l Kn and 500 mg/l BA to differentiate. The differentiated buds from the calluses were cultured on MS medium without harmones and developed into differentiated seedling.

Castillo *et al*. (2000) reported plants regenerated from callus and suspension cultures of *Velerianna edulis*. For the induction of callus, leaves were cultured on

semi- solid medium supplemented with 2, 4-D and Kn. Embrogenic and organogenic callus development was observed after 16 weeks. Calli were transferred to semi-solid and liquid MS medium supplemented with 2, 4-D and NAA. Appearance of shoot somatic embryo ocurred four weeks on medium supplemented with Kn and NAA. A better organogenic and embryogenic response was observed from suspension cultures. Histological observations of morphological callus revealed that both somatic embryos and shoot arose from the same type of callus.

Kaur *et al*. (2000) studied chemical characters, height and root weight in callus regenerated plants of valeriana officinalis L. Plants were successfully regenerated from callus and these regenerated plants had large variations in height and root weight.

Pretto and Santerm (2000) successfully formed callus and then plant regeneration from Hypericum perforatum leaves. Highest cell proliferation was obtained when halved leaf explants were cultured in MS medium containing 4,4 µM 2,4-D in the dark and shoot induction was obtained from callus induced on 4.6 µM Kn and 0.45 µM 2, 4-D 6 weeks after transfer to MS medium containing 4.4 µM BA. Highest rooting frequencies were obtained ½ MS, regardless of the presence of IBA, Regenerated plants were acclimated in greenhouse conditions.

Reddy *et al*. (2001) developed callus cultures from mature leaves of Coleus forskohlii on MS medium supplemented with 2.4 µM Kn and callus generated in to shoots on MS medium containing 4.6 µM Kn and 0.54 µM NAA.More than 150 shoots produced per callus after the sixth subculture. Rooted plantlets were transferred to soil and grown successfully.

Pandf *et al*. (2002) reported *in vitro* micropropagation of *lepidium sativum* Linn. Commonly known as “garden cress”. Various juvenile as well as mature explants callussed on MS medium supplemented with NAA + BA + Casein hydrolyzate (CH). Regeneration from hypocotyls callus and nodal segments ocurred after NAA/BA was replaced with IAA/Kn.

Britto *et al*. (2002) studied callus-mediated regeneration of shoot leaf explants of *Solanum incanum* L. on MS medium supplemented with 0.15 mg/l IAA + mg/l BA

combination. The regenerated shoots rooted on MS medium fortified with different IBA concentrations.

Chandra and Bhanja (2002) studied organogenesis *in vitro* from callus tissue of *Flacourtia jangomas* (Lour). Induction of callus was obtained in MS basal medium supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l BAP. Highest number of shoot bud(7.4 +/- 0.20) was noted in 2.0 mg/l BAP. Rhizogenesis was achieved in1.0 mg/l NAA from both internodes and leaf explants.Shoot bud organogenesis was obseved through histological and SEM (Scanning Electron Microscopic) study.

Chen *et al*. (2003) reported rapid clonal propagation of *Dioscorea Zingiberensis*- an important medicinal plant using stem as explants. MS medium with macroelements at half strength and supplemented with 20.0 gm/l sucrose and 8.0 gm/l agar was as basal medium. Lateral buds on nodal cuttings grew into shoots within 20 days after culture on basal medium supplemented with 4.4 mµM BAP and 1.1 mµM NAA. These shoots formed callus on medium supplemented with 8.9mµM BA and 5.4 mµM NAA after 30 days, which later on regerated multiple shoots (87.5%) within 50 days on medium containing 22.2 mµM BAP and 1.1mµM NAA.

Raha and Roy (2003) studied efficient pant regeneration in *Holarrhena antidysenterica* Wall. From shoot segment-derived callus. Callus was regenerated on MS medium supplemented with 15 mµM BA. A white friable type of calus was obtined in 4.52 mµM 2, 4-D and 2.32 mµM Kn which did not have the potentiality to regenerate.High frequency shoot differentiation was achieved on MS medium with 17.8 mµM BA and 8.0 mµM NAA

Pandey *et al*. (2004) studued th induction of callus in Aconitum Stapf., an important medicinal plant of Indian Himalayan alpines callus was induced on MS medium having 4.5 mµM BA and 26.9 mµM NAA, the highest value for shoot induction were obtained on the same medium supplemented with same concentration (4.5 mµM) of BA, however a lower concentration of NAA(1.4 mµM). Shoot were rooted efficiently on MS medium having 12.3 mµM IBA and acclimatized I greenhouse conditions.

Cheong *et al*. (2004) conducted experiments *In vitro* to determine comparative response of different explants on shoot regeneration of callus formed from shoot tip

and nodes of Gypsophilla paniculata L.(Bristol Fairy). Callus from shoot tip cultures supplemented with NAA and TDZ was compact and showed severe browning. The best callus quality was obtained on MS media supplmented with 1.0 mg/l NAA+ 05-1.0 mg/l BA.

Azad *et al*. (2005) reported that leaf explants of *Phellodendron amurense* prooduced callus on 2.0 µM TDZ and 4.0 µM NAA. The adventitious shoots were regenerated from the leaf-derived callus within 4 weeks of culture on MS medium.

Unda *et al*. (2007) used leaf explants of Exacum styler group for organogenesis plant regeneration. MS media supplemented with combinations of BA(0.44, 2.22, 4.44 or 8.88µM) and NAA(0.05, 0.54 or 2.69µM) was used for direct regeneration without an intervening callus phase in four Exacum genotypes. Regression analysis were used to analyze and interpret the data. Genotypes 01-09-01 and 01-31-61 had the highest no. shoots per explant across media (10.2 and 6.6, respectively) while the 4.4.4 µM BA plus 0.54 µM NAA treatment induced the greatest no. of shoots among the genotypes evaluated.

Adebola and Afolayan (2007) regenerated plants from seeds derived callus of Arctotis arctotides, a medicinal herb of family asteraceae. MS basal supplemented with 3% sucrose and 11% Agar at different concentration of 2,4-D were used for callus induction. Morphogenic responses were evident in cultures after two weeks and deeply stained, light yellow callus formation was observed on MS medium suppemented with 2.0 mg/l, 2,4-D in the dark. When callus material was repeatedly sub cultured in 1/2 MS medium with 16/8 h photoperiod, adventitious shoots wereproduced eight weeks of culturing, which then rooted two weeks later.

2.3 Studies on genetic stability of tissue culture raised plants

2.3.1 RAPD analysis

Hancock *et al.* (1994). screened eight strawberry cultivars for polymorphism using the PCR and 43 random 10-base DNA primer. Ten primers exhibited high levels of amplification profile and polymorphism generated was used to identify each of the eight genotype uniquely.

Parent and Page (1995) performed RAPD anlysis of thirteen raspberry cultivars of quebac Certification Programme. A combination of two primers was found to be sufficient to report relatedness in different cultivars.

Landry *et al*. (1997) charterized 75 genotypes of strawberry using RAPD markers. On the basis of the marker they could contruct a consensus cladogam which repersented the geneticn relatonship among 75 genotypes.

Dengani *et al.* (1998) studied DNA fingerprinting of strawberry (*Fragaria* × *ananassa*) cultivars using RAPD markers. The results obtained in this study demonstrate that RAPD markers can easily distinguish different strawberry cultivars, even those closely related ones. A minimum of ten markers derived from seven primers distinguish 41 cultivars, some of which were full sib or parents of each other.

Kumar *et al*. (1999) carried out the molecular analysis of genetic stability in micropropagated strawberry (Fragaria x ananassa) using RAPD markers. RAPD markers were used to determine if cold storage or supra-optimal level of benzyl adenine (BA) in the culture medium caused genetic changes leading to somaclonbal variations. RAPD markers detected no mutation in the micropraopagated plants.

Zhou and Li (2000) and Oraguzie *et al*. (2001) suggested that RAPD markers are very useful tool assessing genetic diversity in apple. They found PCR_RAPD relative simpler to generate large number of polymorphisms to determine genetic relatedness.

Burgher *et al*. (2002) selected 26 genotypes of lowbush blueberry representing four geographical zones to obtain DNA fingerprints and to estimate genetic similarity by using 30 decamer RAPD primers.

Rout and Das (2002) evaluated the application of RAPD in assessing the genetic integrity of the micropropagated *Plumbago spp*. plants by PCR. Twenty arbitrary decamers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material to assess the genetic fidelity. All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown mother plants. No polymorphism was detected within micropropagated plants of *Plumbago zeylanica*.

Gaafer and Saker (2006) reported the use of RAPD-PCR for identification of different cultivars of strawberry and detection of genetic variations in micro propagated strawberry plants. Seven varieties of the cultivated strawberry, grown in Egypt, were screened using RAPD-DNA markers. Only four RAPD primers (among 20 tested) were chosen as producing polymorphic DNA bands differentiating the investigated cultivars. Based on those identity markers, the genetic distances between varieties were determined and their genetic relationships were estimated. The phylogenetic tree revealed that the seven studied cultivars showed close similarity within the group. Although minor morphological variations were recorded in the leaves of some clones, the developed RAPD profiles of different micro propagated clones were typical to that of the donor mother plant.

56 genotypes (Japanese and European plum cultivars) of plum were used for molecular variation study using 24 RAPD, 10 ISSR primers, and 21 SSR primer pairs by Qiao *et al*. (2007). They detected 201 bands, 86 bands, 102 bands with RAPDs, ISSRs and SSRs markers. Based on all the RAPD, ISSR and SSR markers a Dendrogram was constructed. The dendrogram based on Jaccard's similarity coefficient clearly showed a separation between European and Japanese plum cultivars. They concluded that cultivars from the USA and Japan were grouped into one cluster, and the majority of the native Chinese cultivars were clustered in two separate groups corresponding their geographic location.

Mohamed (2007) evaluated *in vitro* derived plantlets and standard to detect drift in genetic stability of micropropagated plants. No variation was observed in the pattern obtained with RAPDs from the two sources of strawberry plants.

Martelli *et al*. (1999) assessed nineteen cultivar and breeding parental lines of strawberry using 15 decamer primers. RAPD pattern revealed a high level of polymorphism. The results obtained with one of the primers allowed a characterization of all genotypes and three other could characterize all but two genotypes. Comparison of results with the pedigree information confirmed the genotypes percentage contribution.

Sutan *et al.* (2009) examined the effect of growth regulators, explant source and culture age on genetic stability of plants obtained from tissue culture propagation of ornamental strawberry "Serenata". Genomic DNA of *in vitro*-derived shoots and

control plant were extracted and compared by RAPD-PCR analyses. Ten primers (from 48 previously tested) were selected and used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived ornamental strawberry plants The lack of polymorphisms in micropropagated plants screened through molecular markers was used to suggest genetic fidelity.

Celik *et al*. (2017) studied the genetical relationships between the Ottoman strawberry cultivar and other early-period strawberry cultivars planted in Istanbul and Karadeniz Ereğli regions of Turkey, is the first on this field. Seven strawberry cultivars were analyzed by using RAPD. 10 random primers carried out DNA fingerprinting analyses of these cultivars. The average polymorphism rate was determined as 87.1%. The most polymorphic primer was OPH01 which produced 13 bands. The cluster dendrogram presented that the similarity coefficients were between range of 0.03 and 0.73. Ottoman cultivar showed higher similarity with Tüylü more than Kara cultivar which is reported as the mother cultivar. RAPD method was sufficient to assess the phylogenetic relationship between Ottoman cultivar and other early-period strawberry cultivars. The data of this study brought forward the necessity of further genetic analysis to prove the phylogenetic relationship among Tulyu and Ottoman cultivars.

2.3.2 RAPD analysis in other crops

Kelley *et al*. (2004) did DNA fingerprinting of *Hydrastis canadensis* by using RAPD markers. This plant has been used as anti inflammatory. Antibotic and antipyretic. DNA profile was generated from individual plants and used to asess genetic relatedness within cultivated and wild population of *Hydrastis canadensis.*to ensure consistency and reproducibility, ready to go RAPD analysis, bead Kits were used for ampifications. 72-82 % similarity was shown by cultivated material whereas, 20-67 % similarity was found among wild populations.

Kawaiak and Ojkoska, (2004) studied genetic fidelity of two micropropagated *Drosera* species, *D.angelica* and *D. binata* by using RAPD markers. These species were regenerated by adventitous buddding from leaf explants and shoot tips, respectively. Twenty arbitrary decamer primer used to screen 15 randomly selected plantlets of each species. No genetic variation was detected among *D. binata*

regenerants, whereas 0.08% polymorphism frequency was estimated for *D. anglica* plantlets.

Nas *et al*. (2004) carried out RAPD and Phenotypic analysis to assess clonal stability of Hazelnut regenerated from axillary buds cultured *in vitro* for long term consrvation. RAPD analysis did not reveal any somaclonal variation between donor plants from which *in vitro* cultures wereinitiated and micropropagated (6-years cultures), and no somaclonal variation was detcted among *in vitro* propagated plants. However, polymorphism(15.6%) was detected between parent plant and in its propagated progenies (from seedlings). These showed a good dicriminating power of RAPD to detect polymorphism between sampls wher it is expected, andit can be effectively used for genetic assessment of micropropagated hazelnuts.

Roy and Chakraborty (2007) evaluated genetic diversity in Tea of Darjeeling foot-hills using RAPD and ISSR markers. A high level of polymorphism was found with both RAPD and ISSR markers. A total of 26 polymorphic bands in RAPD and 14 Polymorphic bands in ISSR were scored. The mean polymorphism was 60.66% in RAPD and 64.28% in ISSR markers. The scored binary data were used in cluster analysis to construct dendogram using UPGMA method in NTSYS pc software. Cluster analysis suggesting that 10 tea cultivars could be discriminated from on another using these markers, which also revealed their genetic relationships.

Kumar *et al*. (2007) carriid out RAPD analysis of two closely related species of i.e. *Senna surattensis* Burm, f. And *S.sulfurea* D C. Ex Collad. To solve the taxonomic problem between the two. Amplification with 10 decamer primers was performed under pre-standardized condition of 38 accessions along with Senna *occidentalis*, *Calia fistula*, *Senna Tora* and *Senna siamea* collected from different mixed populations. Out of sixty primers utilized, fifty-four were sucessful in amplification and among them one was species specific. The results demonstrated the ability of RAPD markers to reliably differntiatebetween *S. Surattensis* an *s.sulfurea*.

2.3.3 ISSR analysis

Corvo *et al*. (2001) used Inter-simple sequence repeat (ISSR) markers for cultivar identification and for determination of the phenetic relationships among 24 pear cultivars (*Pyrus communis* L.). The ability of several molecular marker systems

including randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), and selective amplification of microsatellite polymorphic loci (SAMPL) to detect variation among clones of the most significant Portuguese cultivar, Rocha, was also investigated. Each of the eight ISSR primers tested was able to distinguish the 24 pear cultivars. The ISSR primers generated 337 markers, 79.5% of which were polymorphic. The cultivar dendrogram obtained with the ISSR marker data was very similar to that obtained with previous RAPD+AFLP analysis, confirming the genetic divergence of 'Pérola', 'Carvalhal' and 'Lawson' from the other cultivars. Eight out of 15 apple [*Malus sylvestris* (L.) Mill. var *domestica* (Borkh.) Mansf.] SSR primers tested also amplified microsatellites in pear. None of the five molecular marker systems analyzed (with a total of 1082 markers) detected reproducible polymorphisms among the nine 'Rocha' clones, in spite of the presence of clear phenotypic differences.

Hussien *et al*. (2008) investigated to molecularly identify and fingerprint six strawberry varieties. The genetic relationships among the six strawberry varieties were achieved using the Inter Simple Sequence Repeats (ISSRs) technique with nine primers. Primers generated 102 total amplified fragments, of which 86 (84.3%) polymorphic fragments discriminated the varieties under the present investigation. A dendrogram-tree generated across the analysis demonstrated that six strawberry varieties are grouped into two clusters. The first cluster contained the Capitola variety only, while Tamar, Chandler, Sweet Charl, Rosa and Diamond comprised the second one. The Sweet Charl variety showed the same high genetic similarity index (83%) to Diamond and Chandler. On the contrary, the most genetically distant varieties were Tamar and Capitola with 45% similarity index. It is concluded that the ISSR technique along with the nine primers in this study were useful tools for the identification of the six strawberry varieties.

Debnath *et al*. (2009) determined the level of genetic diversity and relatedness among 16 strawberry (*Fragaria* x *ananassa* Duch.) cultivars and 11 breeding lines developed in Canada, using Inter Simple Sequence Repeat (ISSR) markers. Sevnteen primers generated 225 polymorphic bands. Cluster analysis by the unweighted pair-group method with arithmetic averages (UPGMA) revealed a substantial degree of genetic similarity among the genotypes ranging from 63 to 77% that were in

agreement with the principal coordinate (PCO) analysis. Geographical distribution for the place of breeding program explained only 1.4% of total variation as revealed by analysis of molecular variance (AMOVA). The ISSR markers detected a sufficent degree of polymorphism to differentiate among strawberry genotypes, making this technology valuable for cultivar identification and for the more efficient choice of parents in current strawberry breeding programs.

Debnath and Ricard (2009) used inter simple sequence repeat (ISSR) anaysis, anthrocyanin contents and antioxidant activities to characterize 10 strawberry (*Fragaria x ananassa* Duch.) cultivars and nine breeding lines. 15 primers generated 240 polymorphic bands. Cluster analysis by the UPGMA revealed a substantial degree of genetic similarity among the genotypes ranging from 45% to 73% that were in agreement with principal coordinate (PCO) analysis. Wide genetic diversity was observed among the strawberry genotypes for anthocyanin contents and antioxidant activities .The ISSR analysis together with data for antioxidant activities and anthocyanin contents in strawberries could be used for germplasm management and more efficient choices of parents in current strawberry breeding programs.

Gantait *et al*. (2010) transferred micropropagated and conventionally propagated plantlets of strawberry (*Fragaria x ananassa* Duch. cv. Chandler) to the similar field condition and growth stage. A comparative study was conducted based on morphological parameters as well as genetic assessments using ISSR markers. The *in vitro* generated strawberry plants exhibited significantly vigorous morphological growth and earlier flower induction when compared to le plants propagated through planting of runners. Genetic assessment through ISSR showed no polymorphism in banding pattern and thus it was revealed that, there was no significant variation between micropropagated and conventional propagated plants at molecular level.

Sen and Dhawan (2008) ascertained the genetic uniformity of the nuclear genome of mother plant and its tissue cultured progenies of strawberry in order to establish true-to-type character through enhanced axillary branching micropropagation by employing ISSR-PCR molecular markers. The study yielded monomorphic bands in all the seven UBC dinucleotide motif primers. The results concluded that the tissue culture raised plantlets of strawberry are genetically identical and clonally uniform at least up to the 15th culture passage.

Morales *et al*. (2011) Started a breeding program necessary for the genetic characterization of the avilable germplasm. Molecular markers are important tools that can be used for this purpose. The objectives of the present study were to assess the genetic similarity among 11 strawberry cultivars using RAPD and ISSR molecular markers and to indicate the possible promising crosses. The DNA of the eleven strawberry cultivars was extracted and amplified by PCR with RAPD and ISSR primers. The DNA fragments were separated in agarose gel for the RAPD markers and in polyacrylamide gel for the ISSR markers. The genetic similarity matrix was estimated by the Jaccard coefficient. Based on this matrix, the cultivars were grouped using the UPGMA method. The dendogram generated by the RAPD markers distributed the cultivars in three groups while the ISSR markers generated two groups. There was no direct relationship between the marker groups when the two types of markers were compared. The grouping proposed by the ISSR markers was more coherent with the origin and the genealogy of the cultivars than that proposed by the RAPD markers, and it can be considered the most efficient method for the study of genetic divergence in strawberry. The most promising crosses, based on the genetic divergence estimated from the RAPD and ISSR molecular data were between the Tudla and Ventana and the Oso Grande and Ventana cultivars, respectively.

Debnath *et al*. (2013) developed the reliable methods for identifying strawberry germplasm and for assessing genetic diversity/relatedness in berry genotypes for practical breeding purposes and proprietary-rights protection. The introduction of molecular biology techniques, such as DNA-based markers, allows direct comparison of different genetic material independent of environmental influences. This review presents the progress in-depth of various aspects of molecular diversity analyses in strawberries. Significant progress has been made in diversity analysis in strawberry cultivars and advanced lines developed in Canada. Inter simple sequence repeat (ISSR) markers detected a sufficient degree of polymorphism to differentiate among strawberry genotypes, making this technology valuable for cultivar identification and for the more efficient choice of parents in the current breeding programs. The paper also discusses the issues that still need to be addressed to utilize the full potential of molecular techniques including expressed sequence tag-polymerase chain reaction (EST-PCR) analysis to develop improved strawberries

environmental friendly cultivars suited to the changing needs of growers and consumers.

Guasmi *et al*. (2016) evaluated two markers (RAPD and ISSR) were evaluated for potential use in fingerprinting and determination of the similarity degree between 3 accessions of apple “Douce Djerba” and 8 grafting between Douce Djerba and Anna. A total of 4 ISSR primers was used and 28 polymorphic alleles were amplified. Four RAPD primers yielded a total of 20 bands, of which 4 (20%) were polymorphic. all accessions were easily distinguishable employing both methods. The similarity coefficient between accessions ranged from 0.692 et 0.923 for ISSR analysis and from 0.875 et 0.933 using the RAPD methodology. This study indicates that the results obtained based on the RAPD, and ISSR techniques are not significantly correlated. The marker index, based on the effective multiplex ratio and expected heterozygosity, was calculated for both analyses (MI = 2.9 for RAPD and MI = 6.7 for ISSR assays). The ISSR markers were found to be useful for cultivar identification and assessment of phenetic relationships, revealing advantages, due to higher reproducibility, over other RAPD.

2.3.4 ISSR analysis in other crops

Shi *et al.* (2008) investigated genetic variations among 214 individuals of *Coptis chinensis* including both wild and cultivated populations using seven ISSR markers. Seven ISSR primers generated 91 loci, out of which 84 were polymorphic. Among the seven wild populations, the mean percentage of polymorphic bands, Nei’s genetic diversity and Shannon’s information index values obtained were 52.4%, 0.15 and 0.24, respectively, while among the three cultivated populations, the values were 65.2%, 0.16 and 0.25, respectively. Molecular analysis following Neighbour-joining approach, demonstrated that wild populations and cultivated populations were not separated into two groups. Their study revealed that cultivation did not seriously influence genetic variation of present-day cultivated populations of *Coptis chinensis*.

Genetic diversity among 10 natural *Prunus pseudocerasus* cultivars was studied by Li *et al.* (2009). 18 selected ISSR primers generated 150 loci, with an average of 8.33 bands per primer. Their results showed that the percentage of polymorphic bands was low at the population level (32%), but relatively high at the species level (84%). They also used the Mantel test which revealed a significant

correlation between genetic and geographic distances among the populations. They concluded that the relatively high inter-population genetic differentiation was attributed to its small population size.

Ganopoulos *et al*. (2011) studied genetic diversity in 19 Greek traditional sweet cherry cultivars and two international cultivars, by using 15 SSR primers and 10 ISSR primers. The 15 SSR loci produced a total of 92 bands, with an average of 9.2 bands per primer with average similarity value of 0.683 and ISSR primers generated 91 bands, with an average of 9.1 bands per primer with average similarity value of 0.37. For ISSR markers, the dendrogram generated from the UPGMA cluster analysis based on the similarity index, classified the 21 sweet cherry cultivars in three main groups while for SSR markers, was divided into two main groups. Their study revealed that SSR and ISSR were related to different morphological characters.

Perez *et al.* (2012) analysed genetic diversity among 35 accessions of *Calibrachoa caesia* by using 13 ISSR primers. Out of 13, seven were polymorphic (69.2%) which yielded a reproducible banding pattern, with 701 amplified loci. The ANOVA test for all populations showed highest genetic variation within populations. These results showed that ISSR primers clearly showed intrapopulation genetic diversity.

Marsafari *et al.* (2013) studied 15 native cultivars of date palm collected from South and Southwest of Iran by using 10 RAPD and 14 ISSR primers. RAPD and ISSR markers exhibit 132 and 162 amplicons respectively, representing a level of polymorphism of 92.4% and 95.67%, respectively. The genetic relationships among cultivars were estimated in terms of similarity using Dice coefficients. The genetic similarity ranged from 94.5% to 99.3% and from 94.1% to 99.4% as given by RAPD and ISSR markers respectively. A dendrogram based on Neighbor joining divided the population into four suitable groups. Analysis of molecular variance (AMOVA) revealed 95% (ISSR) and 97% (RAPD) of variability among the cultivars. These results represent the efficiency of ISSR and RAPD markers to evaluate genetic relationships of the date palm cultivars.

Hassanpour *et al*. (2013) evaluated the genetic diversity among 40 accessions of Cornelian cherries with 20 ISSR primers. These primers showed high level of polymorphism ranging from 80-100%. They reported the average polymorphism

information content (PIC) of 0.46, which reflected that the majority of primers were informative. Based on these results, it was concluded that ISSR markers can be used for the characterization and grouping of Cornelian cherry.

Nunes *et al.* (2013) used five ISSR Primers to determine the genetic variability among 84 strawberry hybrids. The hybrids were produced from crosses involving the following progenitors: 'Toyonoka' x 'Sweet Charlie', 'Camino Real' x 'Sweet Charlie', 'Oso Grande' x 'Sweet Charlie', 'Oso Grande' x 'Toyonoka', 'Dover' x 'Oso Grande', and 'Camino Real' x 'Toyonoka'. 14 genotypes were randomly sampled for each hybrid combination and evaluated. The five ISSRs were capable of screening 41 loci, generating 100% polymorphic bands considering all individuals assessed. An average of 10.69 bands per ISSR primer were observed. The dendrogram divided panel into two basic groups. The small group included the progeny from the crosses 'Toyonoka' x 'Sweet Charlie' and 'Oso Grande' x 'Sweet Charlie' and the parents 'Oso Grande,' 'Sweet Charlie,' and 'Toyonoka.' Whereas bigger group included the progeny from the remaining crosses. Genetic profiles of the hybrids, as demonstrated by were recorded to be very diverse, most likely due to the high heterozygosity of the genome of each progenitor involved.

Athanasiadis *et al.* (2013) analysed 26 genotypes of *Prunus* species, by using 30 RAPD and 10 ISSR primers producing in total 150 loci, of which 116 were polymorphic. Both techniques were highly informative and had a discrimination power greater than 0.9. Dendrogram constructed from RAPD and ISSR data were fairly correlated. The accessions were clustered according to ploidy and species. All *Prunus domestica* genotypes were grouped together and showed greater similarity to *P. insititia* and *P. cerasifera* genotypes as compared to *P. salicina*, which was found genetically diverse. Analysis revealed significant admixture among genotypes. Greek varieties *P. domestica* 'Goulina' and 'Asvestochoriou' exhibited a distinctive genetic background, differentiating them from foreign varieties. This feature could make them attractive for breeding programs, since they can increase genetic diversity.

Safari *et al.* (2013) studied 401 *Brassica napus* genotypes using 12 RAPD and 15 ISSR markers and they revealed that all RAPD and ISSR markers showed polymorphism. Cluster analysis of data using Dice dissimilarity matrix and Neighbor Joining algorithm divided genotypes into seven, five and three main clusters by

RAPDs, ISSRs and both markers, respectively. Non accordance of genetic diversity obtained from this study to origin of these genotypes, showed that screening of genotypes for breeding programs cannot rely on geographical origin and it must be carried out by exact genetic studies.

Chapter-3

MATERIAL AND METHODS

The present investigations entitled "Studies on genetic fidelity of micropropagated plants of strawberry (Fragaria x ananassa Duch.) using molecular markers." were carried out in Department of biotechnology of Dr. Yashwant Singh Parmar, University of Horticulture and Forestry, Solan . The outlines of methodology followed to carry out the above mentioned investigations have been described under following captions.

3.1 Maintenance of *in-vitro* cultures of strawberry cv "Ofra"

3.1.1 Source plant material

3.1.2 Cleaning of glass ware

3.1.3 Media preparation

3.1.4 Aseptic manipulations and culture conditions

3.2 To assess genetic trueness-to-type of long term micropropagated plants of strawberry using molecular markers: RAPD and ISSRs

3.2.1 Source plant material

3.2.2 Isolation and purification of DNA

3.2.3 Quantitative and qualitative assessment of genomic DNA

3.2.4 RAPD –PCR

3.2.4.1 DNA amplification

3.2.4.2. Standardization of RAPD –PCR procedures

3.2.4.3. Electrophoresis of amplified DNA

3.2.5 ISSR -PCR

3.2.5.1 DNA amplification

3.2.5.2 Standardization of ISSR –PCR procedures

3.2.5.3 Electrophoresis of amplified DNA

3.2.6 Analysis of data

3.1 Maintenance of *in vitro* cultures of strawberry cv 'Ofra'

3.1.1 Source plant material

In vitro plant material is being multiplied in the Department of Biotechnology, University of Horticulture and Forestry, Nauni, Solan for the last three years, formed the source plant material to be used in present studies.

Different procedures followed during present investigations are s follws:

3.1.2 Cleaning of glassware

The new glassware were cleaned by washing in a solution of 10% teepol(v/v) in hot water with the help of test tube brush. The glasswares were then soaked in the dilute solution of chromic acid for overnight and rinsed thoroughly with hot water. Finally rinsed with double distilled water and dried at 150ºC in hot air oven for an hour.

The used glass-wares containing the contaminated cultures were first autoclaved to kill the contaminating microorganisms. The molten warm medium was disposed off to empty the culture vessels. The culture vessels were washed with hot water containing 10% teepol and rinsed with distilled water followed by sterilization in hot air oven at 150ºC for one hour.

3.1.3 Media preparation

Explants were cultured on Murashige and Skoog (1962) medium supplemented with different concentrations of growth hormones.

Separate stock solutions of inorganic nutrients and organic nutrients were prepared and stored at 4ºC for maximum of one month. Plant growth regulators were prepared fresh each time; auxins were dissolved in minimum volume of alcohol, where as cytokinins were dissolved in dilute NaOH before making of final volume in water. Stock solutions were stored at 4ºC till further use.

For preparing medium each stock is added one by one in specified quantity in distilled water, after bringing them to room temperature. Sucrose and meso-inositol were added in the concentration of 30 g/l and 100 mg/l respectively. Then added the standardized amount of different growth hormones depending upon type of medium

to be prepared such as for establishment, multiplication and rooting. Then pH of the medium was adjusted at 5.6 to 5.8 with 1 N HCl or 1 N NaOH. Final volume was adjusted with distilled water. Thereafter agar-agar @ 8.0 g/l was added and homogenized by heating the media. The media was finally dispensed into sterilized culture vessels and plugged the mouth of vessels with non- absorbent cotton plugs.

3.1.4 Aseptic manipulations and culture conditions

All the equipments, metal instruments which were used in culturing were wrapped with aluminium foil and sterilized by autoclaving. All the aseptic manipulations were carried out in laminar air flow chamber fitted with ultra violet light, HEPA filters (High efficiency Particulate Air Filters) and Fluorescent tubes. Before starting the aseptic manipulations, the working surface was thoroughly wiped with absolute alcohol. Thereafter, culture vessels containing medium, autoclaved equipments, cotton, 70% alcohol and spirit lamp were kept inside the laminar air flow cabinet and UV light was switched on before starting.

During culturing UV light was switched off, and fluorescent tube and air flow were switched on. Wiped the hands with 70% alcohol, thereafter, flame sterilized the working surface and metal instruments prior to culturing. After culturing, flame sterilized the rim of the culture vessel and quickly plugged the mouth of the vessel. In all the experiments cultures were incubated in culture room at temperature of 25 $\pm$ 2ºC, with 60 $\pm$ 5 per cent relative humidity under white fluorescent light emitted by 40 W Phillips tubes, programmed for 16 hour photoperiod and 8 hour dark conditions.

3.2.1 To assess genetic trueness-to-type of long term micropropagated plants of strawberry using molecular markers : RAPD and ISSRs

3.2.1 Source plant material

In vitro plant material being multiplied in the Department of Biotechnology, University of Horticulture and Forestry, Nauni, Solan for the last three years as well as the plant material being freshly multiplied formed the source of plant material to be used in present studies. Young and healthy leaves of fourteen cultures of strawberry were excised from the tissue cultured plants in the lab and stored at -80ºC till further use. Additionally, material was also collected from mother plant.

3.2.2 Isolation and purification of DNA

3.2.2.1 Isolation of genomic DNA

Genomic DNA from the collected leaves of different *in vitro* cultures and mother plant was isolated following CTAB method of Doyle and Doyle, 1987 with some modifications wherever required.

Reagents used for DNA isolation were as following:

Reagents:

a) 10% CTAB	10 gm of CTAB was dissolved in 100ml of distilled water by warming the solution at 65^0C.
b) 0.5M EDTA (pH 8.0)	18.61 gm of EDTA was dissolved in 80ml distilled water pH of the solution was adjusted to 8.0 with 1N HCL Final volume of the solution was made to 100 ml with distilled water and the solution was sterilized by autoclaving.
c) 4M NaCl	23.37 g of NaCl was dissolved in minimum amount of distilled water and the final volume was made to 100 ml using distilled water, and was then sterilized by autoclaving.
d) 1M Tris HCl (pH 8.0)	15.76 gm of Tris HCl was dissolved in 80 ml distilled water. The pH was adjusted to 8.0 with 1N NAOH The final volume was made to 100 ml with double distilled water and the solution was sterilized by autoclaving.
e) DNA extraction buffer	100 ml of the extraction buffer contained : 1. 10 ml 1M Tris HCl 2. 4 ml 0.5M EDTA 3. 20 ml 10% CTAB 4. 35 ml 4M NaCl 5. 31 ml distilled water 6. 0.2% β-mercaptoethanol
f) Chloroform : Isoamyl	96 ml of chloroform and 4ml of isoamyl alcohol were mixed (24:1,v/v) together and the mixture was kept in a closed container at room temperature.

g) 70% ethanol	70 ml of absolute alcohol was mixed with 30 ml of distilled water to make it 100 ml.
h) T E buffer	0.1576 gm of Tris HCl and 0.0372 gm of EDTA were dissolved in 100 ml of distilled water. The pH was adjusted to 8.0 with 1N NAOH.

Procedure:

Step 1	Collected approximately 500mg of young and healthy leaves and homogenize completely to fine powder with liquid nitrogen using prechilled pestle and mortar.
Step 2	Transferred leaf powder to 50 ml tube containing 10 ml pre-warmed (at 65^0C) DNA extraction buffer. Leaf powder should not get moist because under wet conditions DNase digests total DNA.
Step 3	Incubated the tubes for one - two hours at 65^OC in a water bath. During incubation it is recommended to mix the sample well by inverting the tubes every five minutes.
Step 4	To each tube 10 ml of chloroform : isoamyl alcohol (24:1, v/v) was added and the contents were mixed gently by hand inversions till the colour in the lower portion of the tube turned dark green.
Step 5	Centrifuged the above suspension at 12000 rpm for ten minutes at room temperature.
Step 6	Transferred the aqueous phase gently without disturbing the inter phase to fresh autoclaved centrifuge tubes.
Step 7	Added 2/3rd volume of prechilled isopropanol, mixed gently by hand inversions and incubated at -20^0C for 1hour or overnight so that DNA precipitated out.
Step 8	Spooled out the precipitated DNA with a sterilized glass hook or pelleted it by centrifugation at 10000 rpm for ten minutes at 4^0C.
Step 9	Washed the DNA with 500µl of 70% ethanol and centrifuged at 5000 rpm for five minutes at 4^0C.

Step 10	Decanted off the supernatant and dried the pellet overnight to completely evaporate the alcohol.
Step 11	Dissolved the DNA pellet in 200 µl TE buffer.

Purification of genomic DNA

Reagents:

a) RNase (10mg/ml)	Dissolved RNase in buffer containing 15 mM NaCl and 10 mM Tris HCl (pH 8.0). Heated the suspension to 100^0C for 15 minutes to denature contaminating DNase and allowed to cool slowly to room temperature.
b) Phenol : chloroform (1:1v/v)	Mixed 50 ml phenol and 50 ml chloroform properly. Stored at 4^0C in a covered container.
c) Chloroform : isoamyl alcohol (24:1v/v)	96ml of choloroform and 4ml of isoamyl alcohol were mixed together and the mixture was kept in a closed container at room temperature.
d) 3M sodium acetate	Dissolved 24.609 gm of sodium acetate in 80 ml distilled water and adjusted the pH to 4.8 using glacial acetic acid. Made the final volume to 100 ml with distilled water.
e) Absolute ethanol (95%)	The solution was procured from Merck chemicals.
f) 70% ethanol	70ml of absolute alcohol was mixed with 30ml of distilled water to make the final volume 100ml

Procedure:

Step 1	Added 0.5 µl of RNase to the100µl isolated DNA samples and incubated at 37^0C for one hour.
Step 2	Added equal volume phenol : chloroform and mixed gently.
Step 3	Centrifuged at 11000 rpm for two minutes at room temperature and transferred the aqueous phase to fresh eppendorf tubes.

Step 4	Extracted twice with equal volume of chloroform : isoamyl alcohol (24:1, v/v) and spun at 11000 rpm for two minutes.
Step 5	Separated the aqueous phase. Added $1/10^{th}$ volume of 3M sodium acetate and 2.5 volume of absolute ethanol. Mixed gently and incubated at 4^0C.
Step 6	Pelleted the DNA by centrifugation at 11000 rpm for five minutes.
Step 7	Decanted the supernatant. Washed the pellet with 70% ethanol, and air dried the pellet.
Step 8	Resuspended the pellet in 100 µl TE buffer.

3.2.3 QUANTITATIVE AND QUALITATIVE ASSESSMENT OF DNA

3.2.3.1 Agarose gel electrophoresis

The genomic DNA was electrophoresed on 0.8% agarose gel at 80V –100V and 70mA for two hours in 1X TAE buffer. The gel was stained with ethidium bromide (0.5µg/ml) and observed on UV-transilluminator. Quality of DNA samples was judged on the basis of whether sample DNA formed a single high molecular weight band or smear.

3.2.3.2 DNA quantification

The quantity of DNA was assessed by picodrop (Picodrop Ltd. Cambridgeshire, UK). Concentration of DNA was quantified on the basis of absorbance at 260 nm using formula:

The ratio of absorbance at 260 nm and at 280nm was mesured to check the contamination of protiens. (Table.1)

Table 1: Purity check of DNA on the basis of A_{260}:A_{280} ratio.

Sr. No.	A_{260}:A_{280} ratio(absorbance ratio)	Indication
1.	Above 1.8	Protein contamination
2.	1.4-1.7	Good quality DNA
3.	Below 1.4	RNA contamination

$$\text{DNA } (\mu g/ml) = \frac{\text{OD}(260) \times \text{dilution factor} \times 50}{1000}$$

3.2.4 RAPD STUDIES

3.2.4.1 DNA amplification

DNA from *in* vitro cultures and mother plant were amplified using Polymerase Chain Reaction (PCR) using the protocol of Williams *et al.* (1990) with a few modifications. PCR protocol was standardized for carrying out the amplification using RAPD ans ISSR primers. Reaction conditions and optimum concentration of various components viz., primer, template DNA, $MgCl_2$, dNTPs and Taq DNA polymerase for 20 µl reaction mixture were standardized.

3.2.4.2 Standardization of PCR-RAPD procedure

Concentration of different components standardized for RAPD –PCR in 20µl of reaction mixture given in table2.

Table2: Different concentration of components for genetic fidelity analysis in *in vitro* cultures and mother plants using RAPD primers.

Sr. No.	Reagents	Variable Quantity
1.	10X PCR buffer	1X
2.	$MgCl_2$ (25 mM)	1.5 mM ,1.5 mM, 2 mM, 2.5 Mm, 3.0 mM, 4.0 mM
3.	dNTPs (10 mM)	1.0 mM-5.0mM
4.	Primer	30-50 pm
5.	Taq DNA Polymerase	0.5U-3.0U
6.	Template DNA	20-60 ng
7.	Sterile distilled water	to make the final volume 20 µl

Different quantities in 'µl' of each reaction component were taken to prepare the reaction volume. The volume was completed using sterile distilled water. The reagents were mixed thoroughly in a 2.0 ml eppendorf tube and vortexed for few seconds. 17µl of the prepared volume of RAPD- PCR was distributed to each 0.2 ml thin walled PCR reaction tube (Axygen Scientific Pvt. Ltd, New Delhi, India) and then 3.0 µl of DNA was added separately to each tube to make 20µl of the reaction

mixture.The tubes with reaction mixtures were placed in a thermal cycler (Applied Biosystems Thermal Cycler) for cyclic amplification.

Various thermal profiles were tried in which number of cycles was varied from 32-40 and annealing temperature varied from 32ºc to 35ºc and the finally followed profiles is as follows:

Thermal profile followed fo RAPD amlification was as follows:

Step	**Temperature (^{o}C)**	**Time (minutes)**
1. Initial Denaturation	95ºc	3 min
2. 35 cycles each of		
i) Denaturation	94 ºc	30 second
ii) Annealing	35 ºc	30 second
iii) Extension	72 ºc	1 min
3. Final Extension	72 ºc	10 min

3.2.4.3 Electrophoresis of amplified DNA

The amplified DNA was mixed thoroughly with 6X loading dye and then electrophoresed in 1.2% agarose gel in 1X TAE buffer. The gel was run at constant voltage at the rate of 5V/cm under submerged conditions for about 2 hours. Ethidium Bromide @ of 0.5µg/ml was incorporated in the gel.

The size of the amplified product was determined by co-electrophoresis of standard molecular weight marker (Hind III/EcoRI, double digest, GeNei, Bangalore, India). DNA profile was visualized on UV transilluminator and photographed on Gel Documentation System (Syngene, USA).

3.2.5 ISSR STUDIES

3.2.5.1 DNA amplification

ISSR technique is PCR based molecular marker tehnique that involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction (zietkiewicz et al 1994). The DNA samples from *in vitro* cultres and mother plant were amplified following PCR using 20 primers procured from M/S Metbion (international AG, Deutschland, Germany) and Bioserve (Bioserve, Bettsville USA). PCR conditions and

optimum concentration of various components viz., primers, template DNA, $MgCl_2$, dNTPs and Taq DNA polymerase for 20 µl reaction mixture were standardized.

3.2.5.2 Standardization of PCR-ISSR procedure

Concentration of different components stndardized for ISSRS-PCR in 20µl of reaction mixture is given in table3.

Table:3 Different concentration of components for genetic fidelity analysis in *in vitro* culture and mother plant using ISSRs primers:

Sr. No.	Reagents	Variable Quantity
1.	10X PCR buffer	1X
2.	$MgCl_2$ (25 mM)	1.0mM,1.5mM,2.0mM,2.5mM,3.0mM
3.	dNTPs (10 mM)	1.0 mM,2mM,3mM,4mM,5mM
4.	Primer	30-50 pmoles/µl
6.	Taq DNA Polymerase	0.5U-3U
7.	Template DNA	20-60 ng
8.	Sterile distilled water	to make up the final volume

Different quantities in 'µl' of each reaction component were taken to prepare the reaction volume in a 2.0 ml eppendorf tubes. The final volume was made with autoclaved distilled water and all the reagents were mixed thoroughly and vortexed for few seconds. 17µl of the prepared mixture of ISSR-PCR was distributed to each 0.2ml thin walled reaction tube an then 3µl DNA was added separately to each tube to make 20 µl of reaction volume for ISSR studies, various thermal profiles were tried in which number of cycles was raised 35-45 cycles and annealing temperature varied with the primer sequence, and finally the profile adopted is as below.

Thermal profile followed for ISSR amplification was as follows:

Step	Temperature (^oC)	Time (minutes)
1. Initial Denaturation	95ºc	2 min
2. 35 cycles each of		
i) Denaturation	94 ºc	10 seconds
ii) Annealing temperature varied with primer sequence		30 seconds
iii) Extension	72 ºc	65 seconds
3. Final Extension	72 ºc	10 min
4. Hold at 4ºc		

The tubes with reaction mixture were then placed in a thermal cycler (Applied Biosystems, Foster city, California, USA) for cyclic amplification.

3.2.53 Electrophoresis of amplified DNA

The amplified DNA was mixed thoroughly with 6X loading dye and then electrophoresed in 1.2% agarose gel in 1X TAE buffer. The gel was run at constant voltage at the rate of 5V/cm under submerged conditions for about 3 hours. Ethidium Bromide at rate of 0.5µg/ml was incorporated in the gel.

The size of the amplified product was determined by co-electrophoresis of standard molecular weight marker (Hind III/EcoRI, double digest, GeNei, Bangalore, India). DNA profile was visualized on a UV transilluminator and photographed by using Gel Documentation System (Syngene, USA).

3.2.6 Analysis of data:

Banding patttern generated by various RAPD and ISSR primers was recorded for monomorphism/polymorphism.

Chapter-4

RESULTS AND DISCUSSION

The results obtained in the present investigations on "Studies on genetic fidelity of tissue culture raised plants of strawberry cv. Ofra" are presented under following headings:

4.1 Source plant material

4.2 Direct regeneration and shoot multiplication

4.2 Genomic DNA isolation and purification

4.3 Qualitative and quantitative assessment of genomic DNA

4.4 Survey of polymorphism in Strawberry cv "Ofra"

4.4.1 Using ISSRs

4.4.2 Using RAPD

4.4.3 PIC value (Polymorphism Information Content) of various primers

4.5 Data analysis

4.5.1 Similarity coefficient (Jaccard's coefficient) analysis

4.5.1.1 Based on ISSR analysis

4.5.1.2 Based on RAPD analysis

4.6.2 Cluster analysis

4.6.2.1 Based upon ISSR profile

4.6.2.2 Based upon RAPD profile

4.1 SOURCE PLANT MATERIAL

In vitro Strawberry collections were used to carry out the study, which were maintained from last three years in tissue culture laboratory of the Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.).

4.2 Direct regenerations and shoot multiplications:

The inherent capacity of plant cell to give rise to whole plant, a capacity which is often retained even after a cell has undergone a final differentiation in plant body is described as 'totipotency' from time to time (Steward *et al*.1958; Haberlandt, 1902; Narayan *et al*. 1989 and Cuenca *et al.* 2000). Keeping this in view: *In vitro* multiplying shoots for the last three years in the Department of biotechnology, UHF Nauni, Solan became the source plant material and explants namely runner tips and internodal segments for direct regeneration were excised. Small pieces of about 0.5-1.0 cm of runner tips and internodal segments of forementioned *in vitro* multiplying shoots of strawberry cv " ofra" were cultured on MS medium supplemented with different concentrations and combination of plant growth regulator i.e 2.0 mg/l BA, 2.0 mg/l GA_3, 30g/L of sucrose, 100mg/L Mesoinositol and 8g/L f the agar. Nodal segments and runner tips of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of BA with KIN or GA_3 (Sakila *et al.* 2007; Haddadi *et al.* 2010 ; Ashrafuzzaman *et al.* 2013; Litwinczuk *et al.* 2009; Ara *et al* 2012).

Intially 45 flasks were cultured and monitored continuously for contamination, showed survival rate of 100% during first day. After one week, three flasks were found to be contaminated with bacterial infection showed survival rate of 93.33%. During 2^{nd}, 3^{rd} and 4^{rth} week, 12, 7, 8 flasks were found to be contaminated, showing 66.67%, 51.1% and 33.33% of survival rate, respectively. So at the end 15 flasks which also includes two mother culture flasks were survived (fig1,2).

4.2 GENOMIC DNA ISOLATION AND PURIFICATION

Genomic DNA was isolated from all the 15 Strawberry collections following CTAB method (Doyle and Doyle, 1987). About 500mg of young leaves were used for each sample. Some modifications in the protocol were made. The isolated DNA contained number of phenolics, so PVP and mercaptoethanol were added in DNA extraction buffer during isolation (Majeed *et al*. 2009; Kaur *et al*. 2009; Hassanen and Khalil in 2013; Soni and Kaur, 2014 and Thakur, 2013). The isolated DNA was treated with RNase, Chloroform and isoamylalcohol for purification of DNA.

a)

b)

c)

Fig 1: a),b),c) Shoot initiation in sub cultured strawberry 'Ofra' explants

d) Sub-culturing

e) Strawberry cv 'Ofra'

Fig 2: *In vitro* cultures of Strawberry cv 'Ofra'

4.3 QUALITATIVE AND QUANTITATIVE ASSESSMENT OF GENOMIC DNA

The quality of DNA was assessed by running it on 0.8% agarose gel (stained with 0.5µg/ml of ethidium bromide) along with lambda DNA marker of known concentration at 80 to 100 V and 70mA for two hours in I X TAE buffer. The gel was then observed on UV-transilluminator. Quality of DNA samples was judged on the basis of whether sample DNA formed a single high molecular weight band or smear.

The quantity of DNA was estimated spectrophotometrically. All the samples were found to contain DNA in the weight range of 14 µg/ml to 18 µg/ml.

4.4 SURVEY OF MONOMORPHISM IN MICROPROPAGATED MATERIAL OF STRAWBERRY CV "OFRA"

Genetic relationship analysis based on molecular markers has been carried out in many wild species, cultivars of various horticultural crops (Liu *et al.* 2007 in Plum; Qiao *et al.* 2007 in Japanese plum; Vaidya *et al.* 2012 in cauliflower; Safari *et al.* 2013 in *Brassica napus*; Saxena *et al*. 2011 in cabbage; Kaur *et al*. 2014 in peach)

Table 4: Details of ISSR primers used for present study

Sr. No.	Primer Name	Primer code	Primer sequence	T_m (^oC)	GC%	Length in bp
1	UBC-840	T1	GAGAGAGAGAGAGAGACTC	57	52.6	14
2	UBC 841	T2	GAGAGAGAGAGAGAGACTC	57	52.9	9
3	ISSR 5	T3	AGAGAGAGAGAGAGAGYC	55	52.8	32
4	ISSR3E	T4	TCTCTCTCTCTCTCTCA	50.4	47.1	9
5	UBC 829	T5	TGTGTGTGTGTGTGTGC	52	52.9	12
6	ISSR 808	T6	AGAGAGAGAGAGAGAGC	52	52.9	15
7	IISRS 3M	T7	ACACACACACACACAC	49.2	50	9
8	ISSR8	T8	CACACACACACACACA	48	50.0	13
9	UBC894	T9	TGGTAGCTCTTGTCAGGCAC	60	50	12
10	UBC 854	T10	TCTCTCTCTCTCTCTCCAGC	60	55	18
11	UBC-855	T11	ACACACACACACACACCTT	55	47.4	21
12	ISSR-4	T12	AGAGAGAGAGAGAGAGYT	53	47.2	18
13	ISSR-2	T13	CAGAGAGAGAGAGAGAYT	53	47.2	18
14	ISSR-844B	T14	CTCTCTCTCTCTTGC	47.8	53.3	15
15	ISSR17898B	T15	GATCGAGAGATGCT	44	44	14
16	UBC886	T16	ACGAGTACGCTCTCTCTCTCTCT	65	52.2	23
17	UBC890	T17	ACGACTACCGTGTGTGTTTGTGT	63	47.8	23
18	UBC-850	T18	GTGTGTGTGTGTGTGTCTC	57	52.6	11
19	ISSR 7	T19	ACACACACACACACACYC	55	52.8	33
20	UBC 848	T20	CACACACACACACACAAGG	57	52.6	20

In present study, 20 ISSR and 15 RAPD primers were used to carry out DNA amplification in 14 *in vitro* raised plants and mother plant of Strawberry cv "Ofra".(table 4) Data was recorded based on agarose gel images for both i.e ISSR and RAPD profile. DNA amplified products were analyzed using 1.2% and 2% agarose gel (Morales *et al.* 2011). Out of 15 RAPD and 20 ISSR primers, 7 RAPD and 14 ISSR primers were found to be informative.

4.4.1 Using ISSR

The 20 primer pairs amplified a total 28 bands in all the collections, majority of the bands were monomorphic. Maximum number of bands was 4 produced by single primer **'T5'**. Eight primers '**T2', 'T4', 'T5', 'T6', 'T7', T10',** produced only single Band (table-5).

Table-5 Details of amplification given by 14 ISSR primers in *in vitro* raised plant material of Strawberry cv "Ofra"

Sr. No.	Primer code	Primer code	Total number of amplified bands	Polymorphic bands	Monomorphic bands	Unique Bands
1	T1	A	1	1	1	0
2	T2	B	1	0	1	0
3	T3	C	1	0	3	0
4	T4	D	1	1	0	0
5	T5	E	1	0	1	0
6	T6	F	1	0	1	0
7	T7	G	1	0	1	0
8	T8	H	2	1	1	0
9	T9	I	2	0	2	0
10	T10	J	1	0	1	0
11	T11	K	3	0	3	0
12	T18	L	3	0	3	0
13	T19	M	3	0	3	0
14	T20	N	4	0	4	0

Table 6 reveals the total number of bands amplified for each primer. 20 primers amplified a total a total of 277 fragments among the plant material used in previous study. Primer 'T11' which is denoted by alphabet 'K' in Table 3, amplified a

Plate 1: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC-840

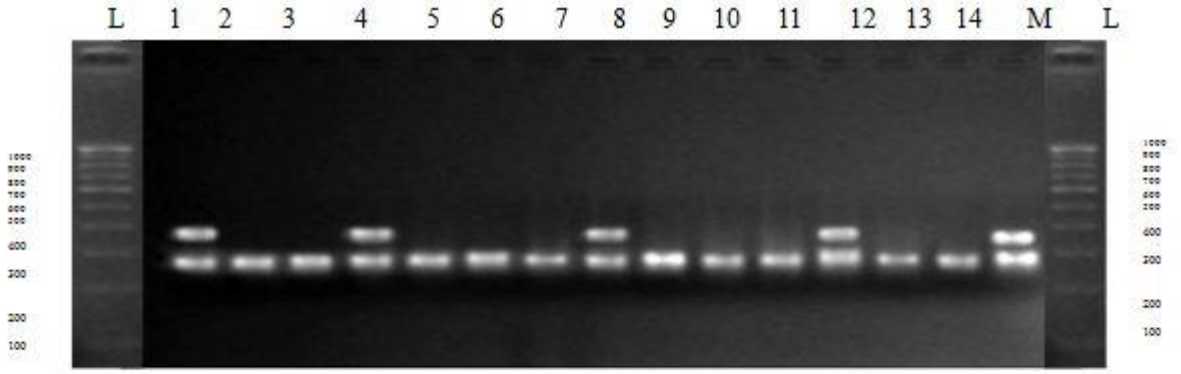
L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*In-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*In-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*In-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*In-vitro* raised plant 7		
8.	*In-vitro* raised plant 8		
9.	*In-vitro* raised plant 9		
10.	*In-vitro* raised plant 10		
11.	*In-vitro* raised plant 11		

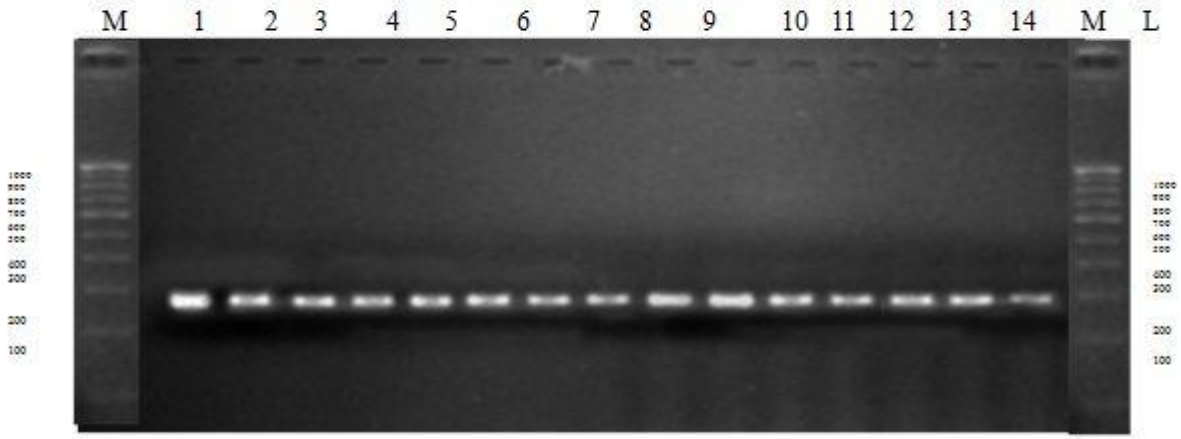
Plate 2: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC-841

L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*In-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*In-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*In-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*In-vitro* raised plant 7		
8.	*In-vitro* raised plant 8		
9.	*In-vitro* raised plant 9		
10.	*In-vitro* raised plant 10		
11.	*In-vitro* raised plant 11		



UBC-840
Plate 1



UBC-841
Plate 2

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 3: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer ISSR- 5

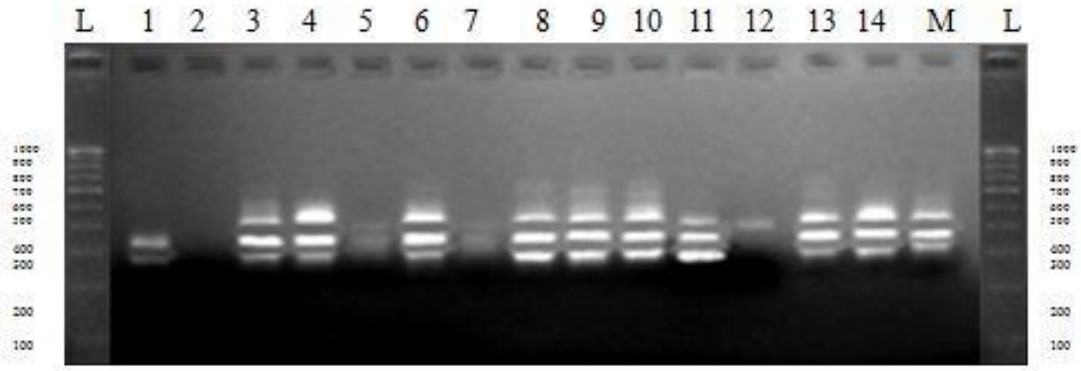
M: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*In-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*In-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*In-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*In-vitro* raised plant 7		
8.	*In-vitro* raised plant 8		
9.	*In-vitro* raised plant 9		
10.	*In-vitro* raised plant 10		
11.	*In-vitro* raised plant 11		

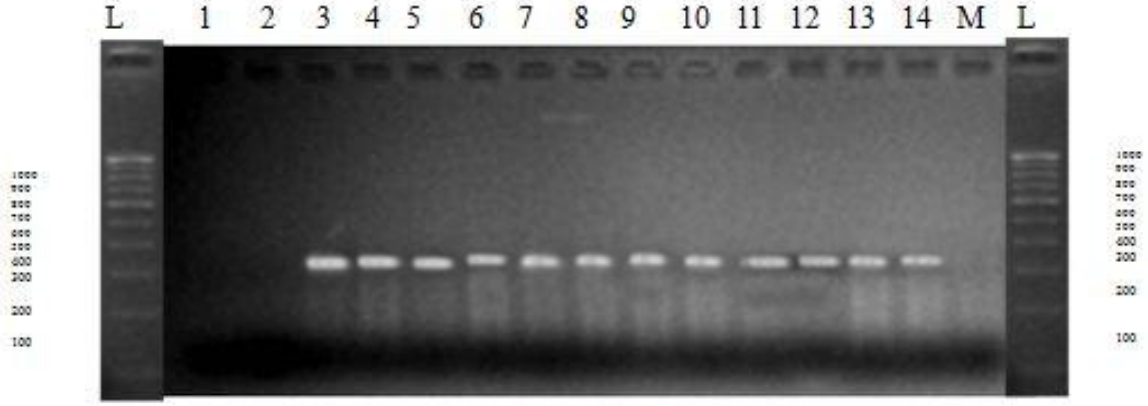
Plate 4: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer ISSRs-3-E

L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*In-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*In-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*In-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*In-vitro* raised plant 7		
8.	*In-vitro* raised plant 8		
9.	*In-vitro* raised plant 9		
10.	*In-vitro* raised plant 10		
11.	*In-vitro* raised plant 11		



ISSR- 5
Plate 3



IISRs-3-E
Plate 4

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 5: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC-829

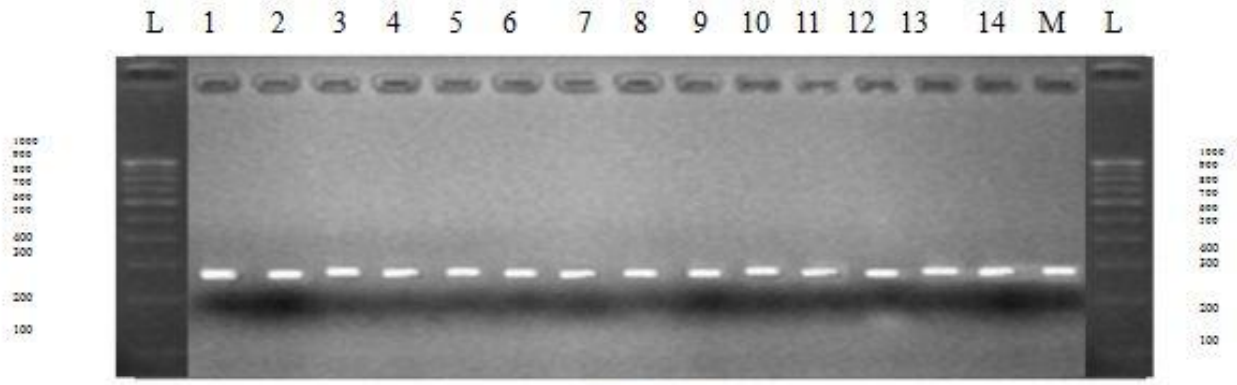
M: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant

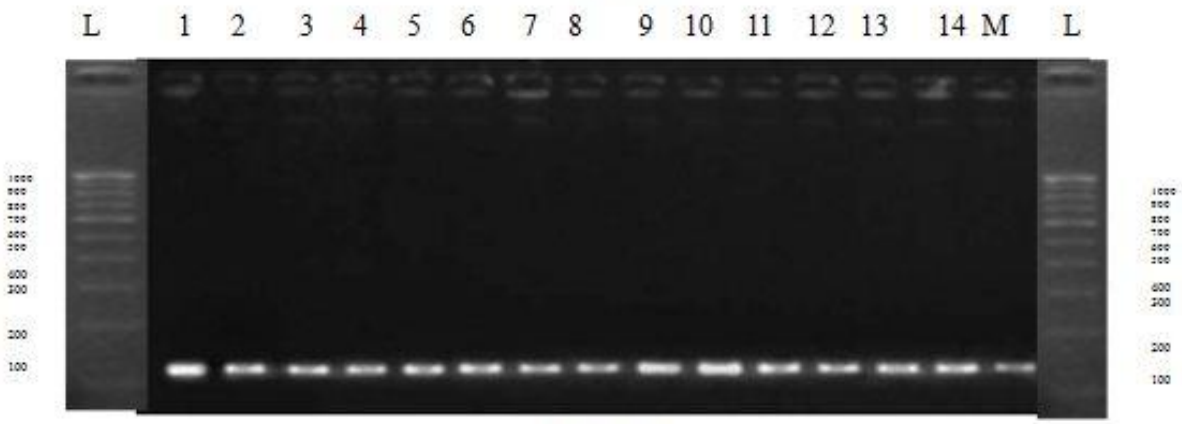
Plate 6: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC-808

M: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



UBC-829
Plate 5



UBC-808
Plate 6

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 7: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer IISSRs-3-M

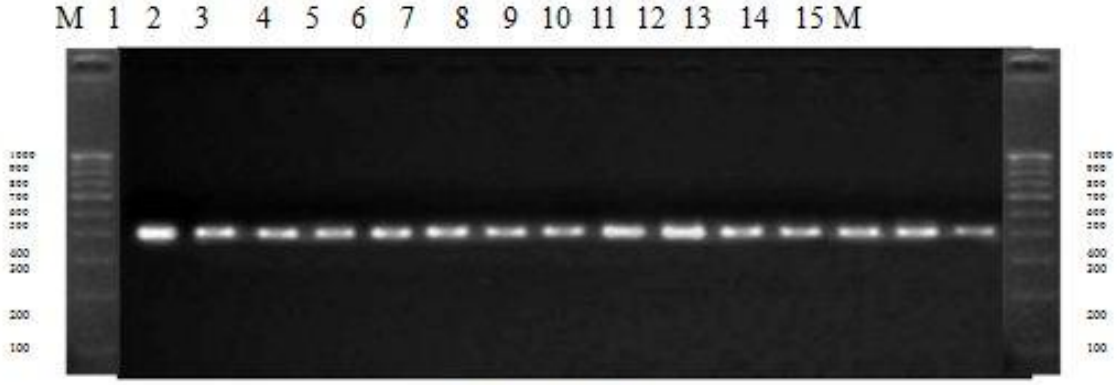
L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant

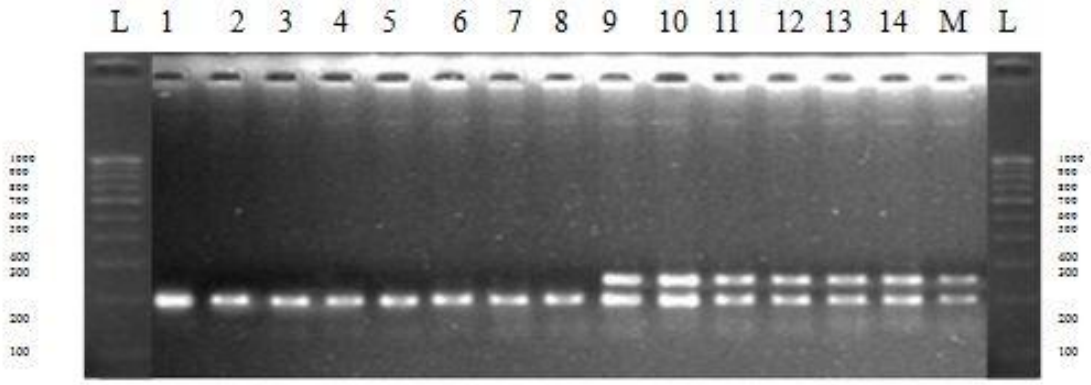
Plate 8: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer ISSR-8

L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



IISSRs- 3-M
Plate 7



ISSR-8
Plate 8

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 9: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC- 894

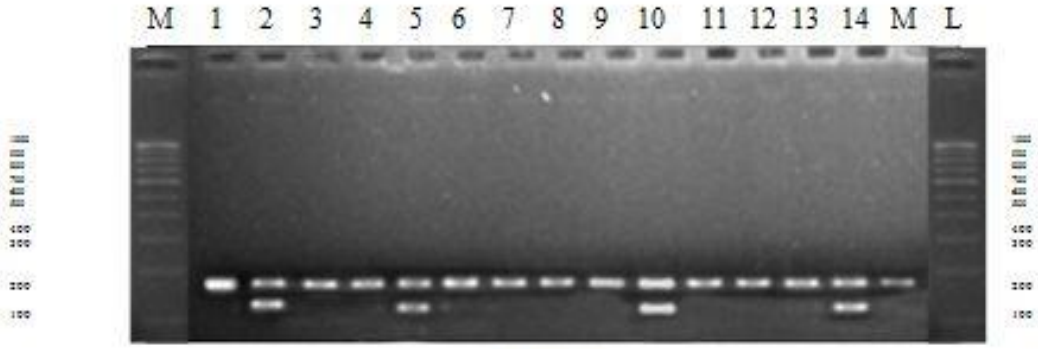
L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant

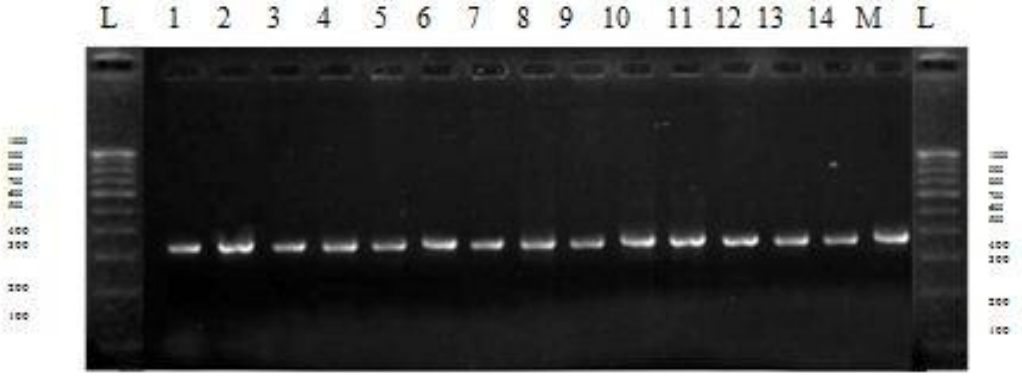
Plate 10: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC- 854

L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



UBC- 894
Plate 9



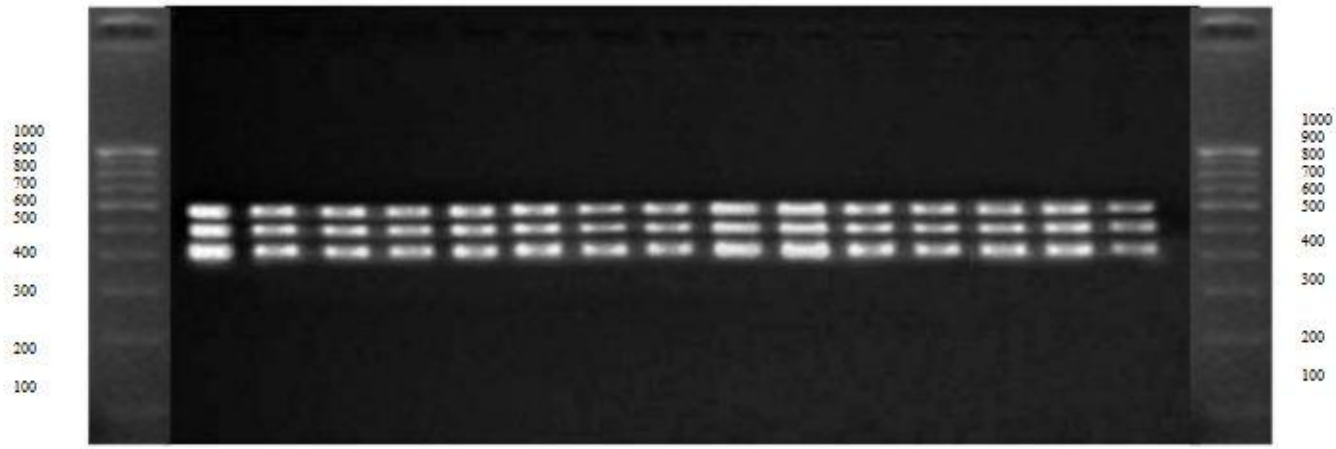
UBC- 854
Plate 10

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 11: ISSR pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer UBC-855

L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



UBC-855
ACACACACACACACACCTT
Plate 11

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

maximum 45 fragments. Minimum number of fragments i.e. nine were produced by primer '**T4**' denoted by alphabet 'D' and '**T9**' denoted by alphabet 'I' Table 3. The maximum number of amplified fragments i.e four were produced with primer T18 denoted by alphabet 'L' in,S_6,S_7,S_8,S_9,S_{11},S_{13},S_{14} and in mother plant. (table-3).Collection S_9 produced maximum amplified fragments ie. 23 with all primer pairs tested and collection S_5 produced minimum number of amplified fragments ie. 13 with all primer pairs used. Majority of the bands produced were monomorphic (table 7).

Table-6 Alphanumeric representation of amplification profile observed among *in vitro* raised plant material of Strawberry cv "Ofra". Alphabet denotes each primer and number denotes number of amplified fragments produced by the primer.

Species	Primers													Total	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L1**	**M**	**N**	
S_1	A_2	B_1	C_1	D_0	E_1	F_1	G_1	H_1	I_1	J_1	K_3	L_0	M_0	N_2	**15**
S_2	A_1	B_1	C_0	D_0	E_1	F_1	G_1	H_1	I_1	J_2	K_3	L_0	M_2	N_1	**14**
S_3	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_1	I_1	J_1	K_3	L_0	M_1	N_1	**16**
S_4	A_2	B_1	C_3	D_1	E_1	F_1	G_1	H_1	I_1	J_1	K_3	L_1	M_1	N_2	**20**
S_5	A_1	B_1	C_0	D_1	E_1	F_1	G_1	H_1	I_1	J_1	K_3	L_0	M_1	N_0	**13**
S_6	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_1	I_0	J_1	K_3	L_4	M_1	N_1	**20**
S_7	A_1	B_1	C_0	D_1	E_1	F_1	G_1	H_1	I_0	J_1	K_3	L_4	M_0	N_1	**16**
S_8	A_2	B_1	C_3	D_1	E_1	F_1	G_1	H_1	I_0	J_1	K_3	L_4	M_0	N_0	**19**
S_9	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_2	I_0	J_1	K_3	L_4	M_2	N_2	**23**
S_{10}	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_2	I_2	J_1	K_3	L_0	M_1	N_2	**20**
S_{11}	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_2	I_0	J_1	K_3	L_4	M_0	N_2	**21**
S_{12}	A_2	B_1	C_1	D_1	E_1	F_1	G_1	H_2	I_0	J_1	K_3	L_0	M_1	N_3	**18**
S_{13}	A_1	B_1	C_3	D_1	E_1	F_1	G_1	H_2	I_0	J_1	K_3	L_4	M_0	N_1	**20**
S_{14}	A_1	B_1	C_3	D_0	E_1	F_1	G_1	H_2	I_2	J_1	K_3	L_4	M_0	N_1	**21**
Mother plant	A_2	B1	C_3	D_0	E_1	F_1	G_1	H_2	I_0	J_1	K_3	L_4	M_1	N_1	**21**
Total	**20**	**15**	**32**	**9**	**15**	**15**	**15**	**23**	**9**	**15**	**45**	**33**	**11**	**20**	**277**

Table-7 Summary of amplified products obtained in *in-vitro* raised plant material of strawberry cv. 'Ofra' using ISSR primers

Total number of primers examined	20
Number of informative primers	14
Number of polymorphic primers	8
Total number of scorable bands	28
Number of polymorphic bands	17
Number of monomorphic bands	11
Average number of polymorphic bands per primer	0.85
Total number of amplified fragments	277
Average number of amplified fragments per collection	18.47
Average number of amplified fragments per informative primer	19.79

4.4.2 Using RAPD

15 RAPD primers were used for the monomorphism study among 15 different strawberry cv "Ofra" collections (Table-8). 7 were found to be informative. Maximum number of bands was three produced by two primers i.e 'CH_4' and 'CH_6'. Minimum number of bands i.e one was produced by single primer 'CH_1'. (Table 7)

Table-8 Details of RAPD primers used for present study

Sr. No.	Primer Name	Primer code	Primer sequence	Length in bp
1	5383-053	CH1	CCTCACGTCC	10
2	5383-024	CH2	ACGATGAGCC	10
3	5383-073	CH3	CCAGATGCAC	10
4	5383-063	CH4	CATGACAGGC	10
5	5383-089	CH5	CACTGTTCGG	10
6	5383-085	CH6	CTCTGTTCGG	10
7	5383-056	CH7	GTGCTCCCTC	10
8	5383-07	CH8	ACCCGGTCAC	10
9	5383-088	CH9	CGTCCTCAGG	10
10	5383-094	CH10	AGAGATGCCC	10
11	5383-064	CH11	TGGAAGAGGC	10
12	5383-050	CH12	AGT TCC ACG G	10
13	168919	CH13	GGAGCCTCAG	10
14	5383-017	CH14	GGC ATG ACC T	10
15	5383-018	CH15	TGG GCG TCA A	10

Plate 12: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-053

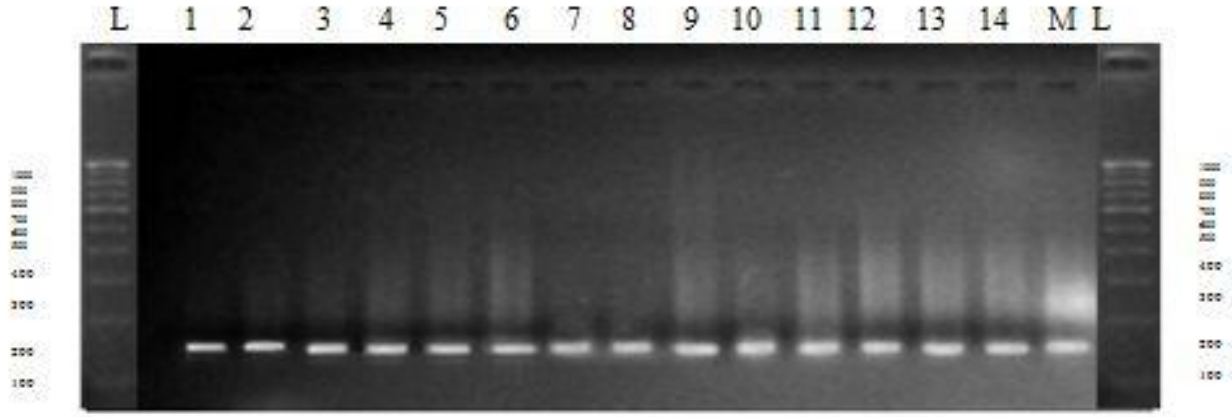
L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*n-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*n-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*n-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*n-vitro* raised plant 7		
8.	*n-vitro* raised plant 8		
9.	*n-vitro* raised plant 9		
10.	*n-vitro* raised plant 10		
11.	*n-vitro* raised plant 11		

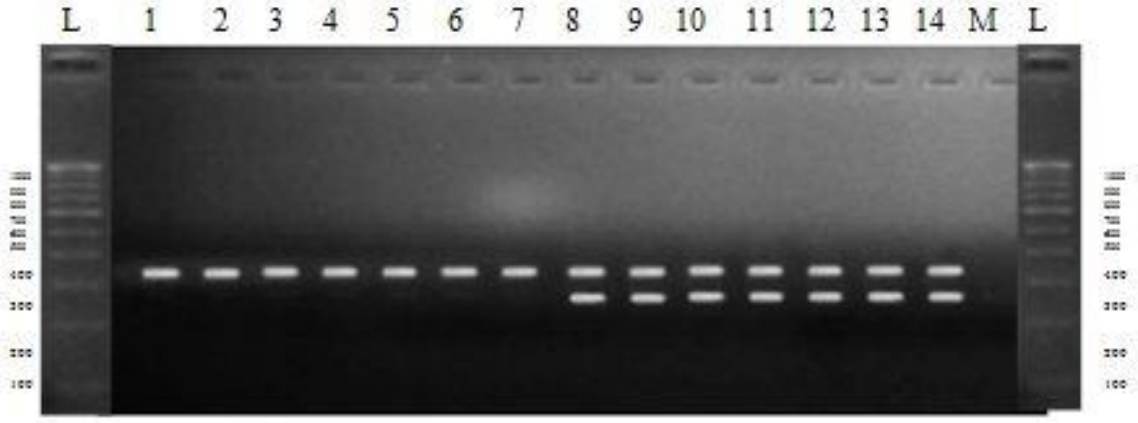
Plate 13: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-024

L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As	S. No.	Denoted As
1.	*In-vitro* raised plant 1	12.	*n-vitro* raised plant 12
2.	*In-vitro* raised plant 2	13.	*n-vitro* raised plant 13
3.	*In-vitro* raised plant 3	14.	*n-vitro* raised plant 14
4.	*In-vitro* raised plant 4	15.	Mother plant
5.	*In-vitro* raised plant 5		
6.	*In-vitro* raised plant 6		
7.	*n-vitro* raised plant 7		
8.	*n-vitro* raised plant 8		
9.	*n-vitro* raised plant 9		
10.	*n-vitro* raised plant 10		
11.	*n-vitro* raised plant 11		



5383-053
CCTCACGTCC
Plate 12



5383-024
ACGATGAGCC
Plate 13

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 14: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-073

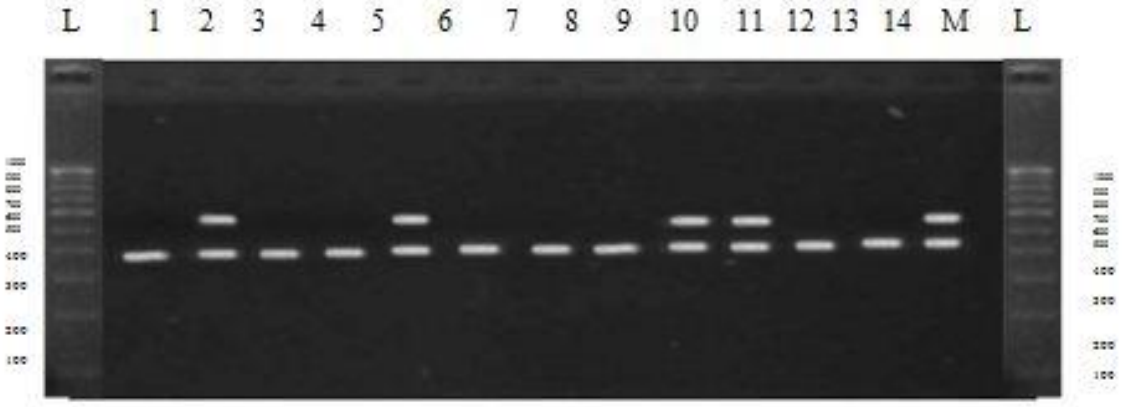
M: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant

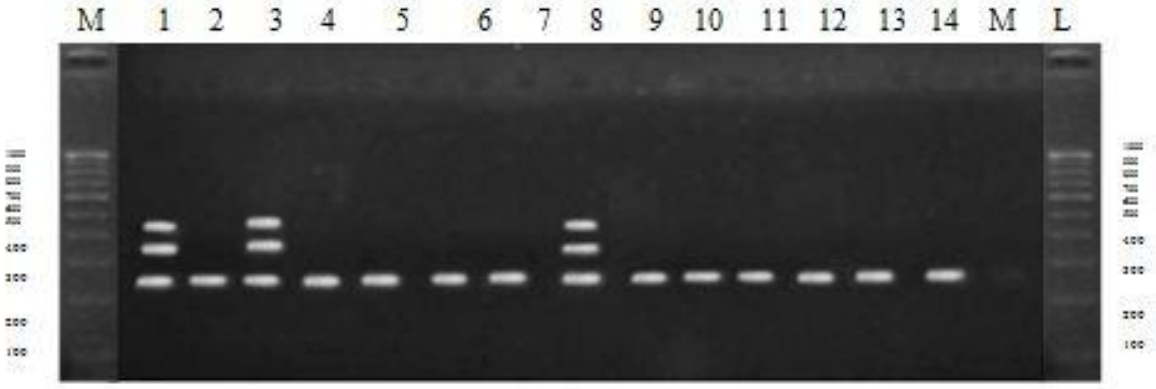
Plate 15: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-063

L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



5383-073
CCAGATGCAC
Plate 14



5383-063
CATGACAGGC
Plate 15

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 16: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-089

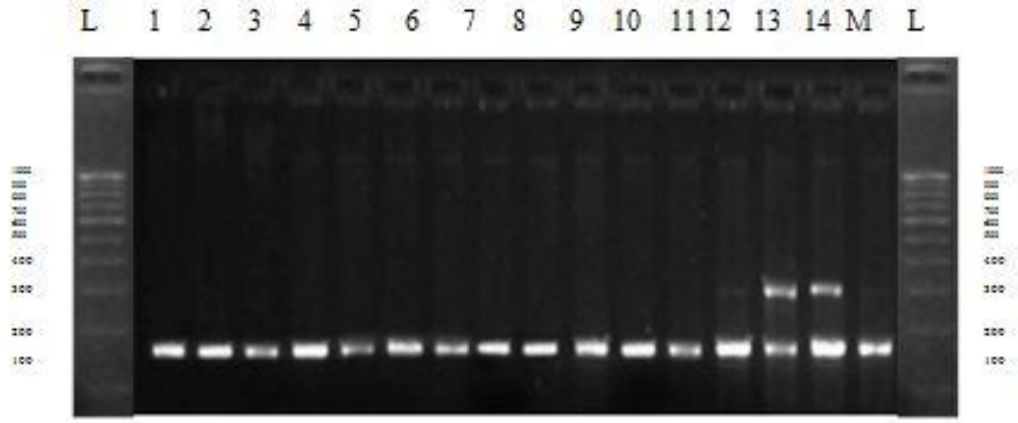
L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant

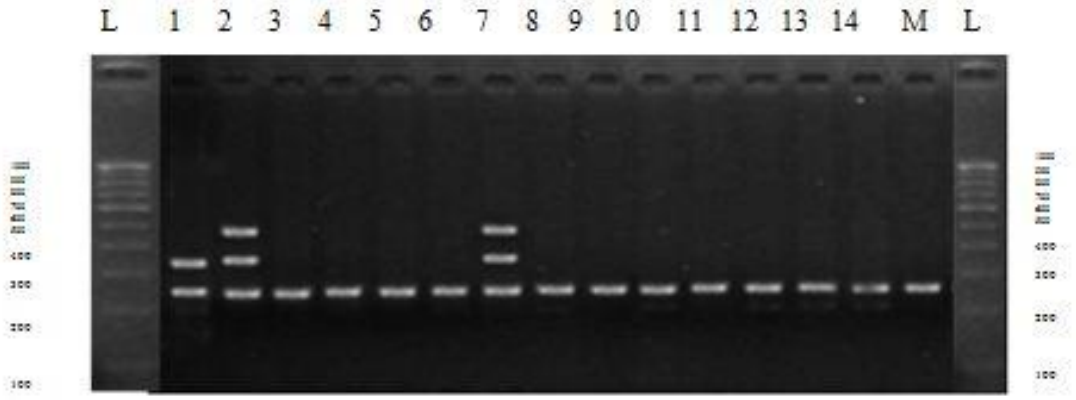
Plate 17: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-085

L : Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



Primer – 5383-089
CACTGTTCGG
Plate 16



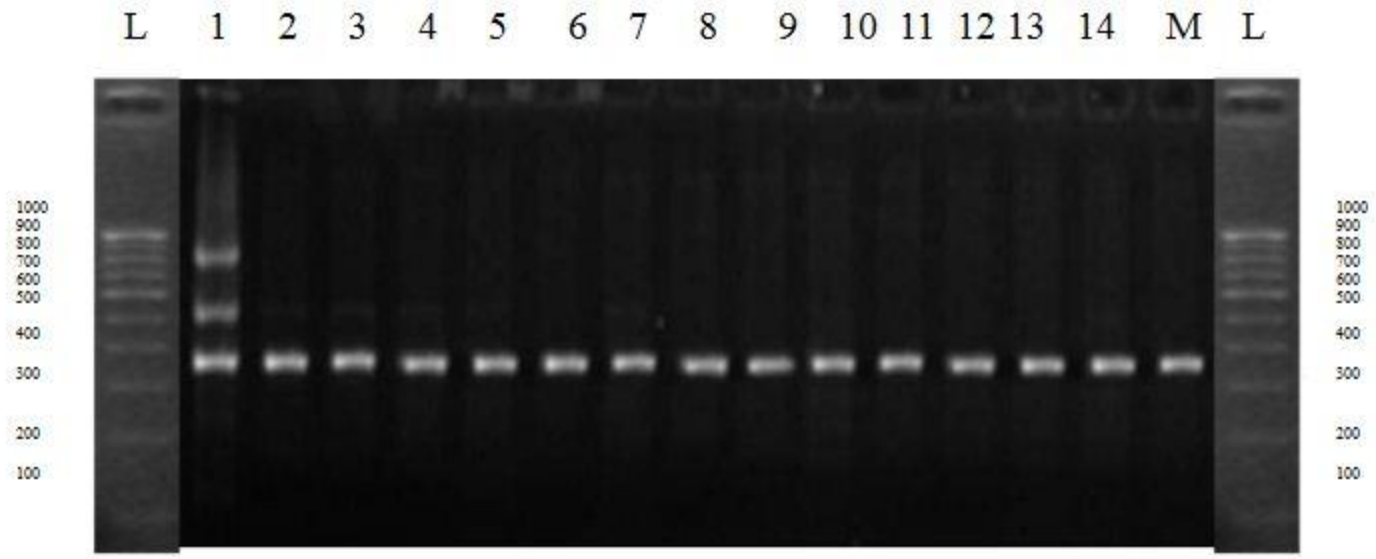
Primer - 5383-085
CTCTGTTCGG
Plate 17

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Plate 18: RAPD pattern of 14 in vitro plants and one mother plant of strawberry cv 'Ofra' generated by primer 5383-056

L: Known Molecular Weight Marker (100 bps)

S. No.	Denoted As
1.	*In-vitro* raised plant 1
2.	*In-vitro* raised plant 2
3.	*In-vitro* raised plant 3
4.	*In-vitro* raised plant 4
5.	*In-vitro* raised plant 5
6.	*In-vitro* raised plant 6
7.	*In-vitro* raised plant 7
8.	*In-vitro* raised plant 8
9.	*In-vitro* raised plant 9
10.	*In-vitro* raised plant 10
11.	*In-vitro* raised plant 11
12.	*In-vitro* raised plant 12
13.	*In-vitro* raised plant 13
14.	*In-vitro* raised plant 14
15.	Mother plant



Primer - 5383-056
GTGCTCCCTC
Plate 18

L denotes 100 bps ladder
G1: G2: G3:G4: G5: G6: G7: G8: G9: G10:G11: G12: G13: G14: In-vitro raised plants,.
M: Mother plant

Table-9 Alphanumeric representation of amplification profile observed among *in vitro* raised plant material of Strawberry cv "Ofra" collections. Alphabet denotes each primer and number denotes number of amplified fragments produced by the primer

Species	Primers							Total
	a	b	C	D	e	f	g	
P1	a1	b1	c1	d3	e1	f2	g3	**12**
P2	a1	b1	c2	d1	e1	f3	g2	**11**
P3	a1	b1	c1	d3	e1	f1	g2	**10**
P4	a1	b1	c1	d1	e1	f1	g2	**8**
P5	a1	b1	c2	d1	e1	f1	g2	**9**
P6	a1	b1	c1	d1	e1	f1	g1	**7**
P7	a1	b1	c1	d1	e1	f3	g2	**10**
P8	a1	b2	c1	d3	e1	f1	g1	**10**
P9	a1	b2	c1	d1	e1	f1	g1	**8**
P10	a1	b2	c2	d1	e1	f2	g1	**10**
P11	a1	b2	c2	d1	e1	f1	g1	**9**
P12	a1	b2	c1	d1	e2	f2	g1	**10**
P13	a1	b2	c1	d1	e2	f2	g1	**10**
P14	a1	b2	c2	d1	e2	f2	g1	**11**
P15	a1	b1	c1	d1	e1	f1	g1	**7**
Total	**15**	**22**	**20**	**21**	**18**	**24**	**22**	**142**

The total number of bands amplified for each primer-collection combination are represented in table-9. A total of 142 fragments were amplified in the whole plant material of strawberry used in the study with 15 RAPD primers. Primer CH_6 is denoted by alphabet 'f' in(table-6) amplified 24 fragments. Minimum number of fragments ie. 15 were produced by primer 'CH_1'denoted by alphabet 'a' (table-9). Maximum number of amplified fragments i.e three were produced with primer 'CH4','CH6' and 'CH7' denoted by alphabet 'd','f' and 'g; in 'P1', 'P2', 'P3' and 'P4'. P2 in- vitro raised plant produces maximum number of amplified fragments i.e 11 with all primer pair tested and P6, P15 produced minimum number of amplified fragments i.e seven with all primer pairs used

Out of 15, only 7 primers used were able to produce amplification on PCR analysis and hence were called informative. Among the 142 fragments produced; maximum bands were found to be monomorphic, representing 46.67% polymorphism. Average number of polymorphic bands per primer was recorded to be 0.6 and average

number of amplified fragments per collection was 9.5. Average number of amplified fragments per informative primer was found to be 20.29. (Table 10,11)Similarly, Hashmi *et al.* 1997 demonstrated the feasibility of using RAPD markers to identify somaclonal variants of Peach and provides evidence for the existence of genetic differences among the variants, however, Khanuja *et al.* 2000 asssessed genetic relationships in *Mentha* species, where out of 630 bands, 598(93.5 %) bands were found polymorphic. Vasil(1985) has clearly explained that cellular and tissue organization has a significant bearing on the process of mitosis. Terefore, variation can be expected in regenerants. However, it is very rare that cytological and molecular changes take place in organ or meristem cultures(Valles *et al.* 1993 and Lattoo et al. 2006)

All ISSR and RAPD primers produced 70% and 46.67% polymorphism respectively. In earlier studies, percentage of polymorphism with RAPD has also been found i.e 53% in strawberry (Zebrowska *et al*. 2003), 44-74% in strawberry (Radmann *et al* 2017), 29% in apple (Guimaraes *et al* 2013), 55% in blackberry (Stafne *et al* 2005), 56.3% in pear (Lisek *et al* 2010), 54.33 % in apple (Singh et al 2011). ISSR primers showed, 56.02% in apple (Singh et al 2011), 77.13% in *Rubus* (Sedighi et al 2015), 71.5% in pear (Lisek *et al* 2010), 87.4% in plum cultivar (Gaulao 2001), 89.6% in Chilean strawberry (Carrasco *et al*. 2007), supported the polymorphism results of the present study.

Table-10 Details of amplification given by 10 RAPD primers in 15 Strawberry cv "Ofra"

Sr. No.	Primer code	Total number of amplified bands	Polymorphic bands	Monomorphic bands	Unique Bands
1	a	1	0	1	0
2	b	2	1	1	0
3	c	2	1	1	0
4	d	3	2	1	0
5	e	2	1	1	0
6	f	3	2	1	0
7	g	2	1	1	0

4.4.3 Polymorphism Information Content (PIC value) of various primers

PIC value of ISSR primers was calculated which ranged from zero to 0.749 in primers **'T2', 'T4', 'T5', 'T6', 'T7', 'T10'** minimum PIC value i.e zero was obtained and maximum PIC value i.e 0.749 was obtained with primer **'T19'**. The average value was found to be 0.38 (table -10).

Table:11 Summary of amplified products obtained in 15 Strawberry cv "Ofra" collections using RAPD primers

Total number of primers examined	15
Number of informative primers	7
Number of polymorphic primers	6
Total number of scorable bands	16
Number of polymorphic bands	9
Number of monomorphic bands	7
Average number of polymorphic bands per primer	0.6
Total number of amplified fragments	142
Average number of amplified fragments per species	9.5
Average number of amplified fragments per informative primer	20.29

While the PIC value for RAPD primers ranged from 0 to 0.588 (Table 9). Minimum PIC value i.e 0 was obtained with primers **'CH1'** and maximum PIC value i.e 0.588 was found in primer '**CH6'** with the average value being 0.30 (Table 8). Thus, from the PIC values obtained, the conclusion was drawn that ISSR primers **'T_{19}'** and RAPD primer **'CH_6'** were highly informative in the present research. PIC values of primers are mentioned below in the Tables 12, 13.

Table:12 PIC values for RAPD

Primer name	PIC value	No. of fragments
CH1	0	15
CH2	0.433	22
CH3	0.375	20
CH4	0.449	21
CH5	0.277	18
CH6	0.588	24
CH7	0.459	22

Table13: PIC values for ISSRs

Sr. No	Primer name	PIC value	No. of fragments
1	**T1**	0.375	20
2	**T2**	0	15
3	**T3**	0.666	32
4	**T4**	0	9
5	**T5**	0	15
6	**T6**	0	15
7	**T7**	0	15
8	**T8**	0.434	23
9	**T9**	0.42	9
10	**T10**	0	15
11	**T11**	0.667	45
12	**T18**	0.565	33
13	**T19**	0.644	11
14	**T20**	0.749	20

Pattern of molecular markers (Both ISSR, RAPD) was compared with that of mother plant. The pattern of 14 randomly selected *in-vitro* raised plants was found to be largely monomorphic. Hence denoting the genetic stable nature of micropropagated plants of about 3 years old cultures of strawberry cv‘Ofra’.

The present method of tissue culture of strawberry cv. ‘Ofra’ was found to be an efficient method which was indicated by healthy multiplying cultures. Further genetic fidelity was established by RAPD and ISSR analysis showing the importance of tissue culture in cv ‘ofra’ without any risk of variations.

Chapter-5

SUMMARY AND CONCLUSIONS

The present investigations entitled ‘‘Studies on genetic fidelity of tissue culture raised plants of strawberry cv. Ofra” are summarized as under:

5.1 Maintenance of *in-vitro* cultures of strawberry cv “Ofra”

- *In vitro* multiplying shoots for the last three years in the Department of biotechnology, UHF Nauni, Solan became the source plant material and explants namely runner tips and internodal segments for direct regeneration were excised.
- Small pieces of about 0.5-1.0 cm of runner tips and internodal segments of forementioned *in vitro* multiplying shoots of strawberry cv “ ofra” were cultured on MS medium supplemented with different concentrations and combination of plant growth regulator i.e 2.0 mg/l BA, 2.0 mg/l $GA_{3,}$ 30g/L of sucrose, 100mg/L Mesoinositol and 8g/L f the agar.
- Intially 45 flasks were cultured and monitored continuously for contamination, showed survival rate of 100% during first day.
- After one week, three flasks were found to be contaminated with bacterial infection showed survival rate of 93.33%. During 2^{nd}, 3^{rd} and 4^{rth} week, 12, 7, 8 flasks were found to be contaminated, showing 66.67%, 51.1% and 33.33% of survival rate, respectively.

5.2 ISOLATION OF GENOMIC DNA FOR MARKER BASED STUDIES

- Genomic DNA of 14 *in-vitro* raised plants and mother plant of strawberry cv ‘Ofra’ was isolated and then purified following RNase treatment and phenol-chloroform extraction.
- The quantity of isolated DNA was estimated spectrophotometrically. All the samples were found to contain DNA in the range of 14 µg/ml to 17 µg/ml.
- Quality of the DNA was assessed through agarose gel electrophoresis. Presence of single, compact and high molecular weight band indicated good quality of DNA.

- PCR protocol and concentrations of components were standardized for ISSR and RAPD amplification of genomic DNA of 14 *in-vitro* raised plants and mother plant.

5.3 GENETIC FIDELITY ANALYSIS AMONG *In-Vitro* RAISED PLANTS AND MOTHER PLANT OF STRAWBERRY cv 'Ofra' BY USING MOLECULAR MARKERS

- Genetic fidelity analysis amongst strawberry cv 'Ofra' was carried out using 20 ISSR and 15 RAPD primers.
- Total scorable bands obtained were 28 in ISSR and 16 with RAPD.
- PIC value was calculated for all primers, which showed highest value of 0.588 with RAPD primer CH6 and 0.749 with ISSR primer T20.
- Ptttern of molecular markers (Both ISSR, RAPD) was compared with that of mother plant. The pattern of 14 randomly selected *in-vitro* raised plants was found to be monomorphic. Hence denoting the genetic stable nature of micropropagated plants of about 3 years old cultures of strawberry cv 'Ofra'.

CONCLUSION

It is concluded that, in present study an efficient method for *In-vitro* propagation of strawberry through nodal and intermodal segment has been established. Further genetic stability has been established following ISSR and RAPD analysis, hence showing positive hopes toward tissue culture of this commercial cultivar of strawberry cv 'Ofra'.

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APPENDIX – I

Buffer

50X TAE buffer

Constituent	Quantity/ l
Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA (pH 8.0)	100ml

Make final volume 1 litre using ADW.

APPENDIX–II

6X Loading Dye

Constituent	Quantity
Bromophenol blue	0.16%
Xylene cyanol	0.16%
Glycerol	50%

Add ADW to make the final volume.

APPENDIX–III

Gel Electrophoresis

. Agarose gel 2%

4 gm agarose was added to 200 ml 1 X TAE buffer and dissolved. The molten gel was cooled to 40^{o}C and 5 µl of Ethidium bromide solution was added and mixed well. The molten gel was casted in a gel tray with comb containing 26 teeth to produce wells.

APPENDIX - IV

Sources of chemicals and instruments

1. Chemicals

Tris HCl, NaCl (molecular biology grade), chloroform, ethanol, CTAB, PVP, β-mercaptoethanol, isopropanol and disodium EDTA were obtained from SRL. dNTP mix, Taq polymerase and agarose were obtained from Bangalore Genei.

2. Instruments and other accessories

i) Instruments used in DNA extraction

Refrigerated Centrifuge	:	Eppendorf
Waterbath	:	Popular Traders
Deep freezer (-20°C)	:	Vestfrost
Deep freezer (-80°C)	:	New Brunswick Scientific Co. Inc.
Refrigerator	:	Godrej
pH meter	:	Systronics
Microfuge tubes	:	Axygen

ii) Instruments used in PCR based studies

PCR machine	:	Applied Biosystems
Laminar flow	:	Klenzaids
Weighing balance	:	Sartorius
Gel electrophoresis unit	:	Genei
UV transilluminator	:	Pharmacia
Gel Documentation System	:	SynGene
Microwave oven	:	Samsung
Thin walled PCR tubes	:	Axygen

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Admission Number	**:**	H-2014-43-M
Major Field	**:**	Biotechnology
Minor Field	**:**	Fruit Science
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ABSTRACT

The present investigations on strawberry (*Fragaria* × *annanasa*) was carried out with an objective to study the gene Genetic fidelity of tissue culture raised plants of strawberry by using RAPD and ISSR markers. Work has also been done to maintain *in vitro* cultures of strawberry by multiplying on MS medium supplemented with 2.0 mg/l BA, 2.0 mg/l GA_3, 30g/l of sucrose, 100 mg/l of mesoinositol and 8 g/l agar. A total of 20 ISSR Primers and 15 of RAPD primers were tried to generate ISSR and RAPD profile. RAPD and ISSR banding pattern of regenerated plants were compared with that of original mother plant. In case of ISSR 14 primers were found to be informative out of 20 primers whereas, in case of RAPD seven primers were found to be informative out of 15 primers. The amplified product was elctrophoresed and photographed using Gel documentation system and the banding pattern was found to be monomorphic. Hence denoting the genetic stable nature of micropropagated plants of about 3 years old cultures of strawberry cv 'Ofra.

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