ANALYSIS OF COLCHICINE EFFECT ON ANTHER CULTURE OF SUNFLOWER (*Helianthus annuus* L.)

Submitted by

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~ 2019 ~

AFFECTIONATELY DEDICATED TO

MY BELOVED FAMILY

AND

RESEARCH GUIDE

CANDIDATE'S DECLARATION

I hereby declare that this dissertation or part thereof has not been previously submitted by me for a degree of any other Institution or University

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C E R T I F I C A T E – I

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I also certify that the dissertation or part thereof has not been previously submitted by her for a degree of any university.

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Date : 30/05 /2019 **Research Guide**

C E R T I F I C A T E – I I

This is to certify that the dissertation entitled **"ANALYSIS OF COLCHICINE EFFECT ON ANTHER CULTURE OF SUNFLOWER (*Helianthus annuus* L.)"** submitted by **MISS. GUND PRATIKSHA RAJENDRA (Reg. No. 2017/BT/03/ML)** to the Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **AGRICULTURAL BIOTECHNOLOGY** has been approved by the student's advisory committee after oral examination in collaboration with the external examiner.

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ABBREVIATIONS

%	:	Percent
^{0}C	:	Degree celcius
BAP	:	6-Benzyl Amino Purine
bp	:	Base Pairs
CI	:	Chloroform : Isoamyl alcohol
cM	:	Centimorgan
Conc.	:	Concentration
DNA	:	Deoxyribose Nucleic Acid
dNTPs	:	Deoxy Nucleotide Triphosphates
EDTA	:	Ethylene Diamine Tetra Acetic Acid
et al.,	:	et alia (and other)
EtBr	:	Ethidium Bromide
ha	:	Hectare
Hr	:	Hour
IAA	:	Indole-3-Acetic Acid
IBA	:	3-Indole Butyric Acid
Kb	:	Kilo Base Pair
M	:	Molar
$MgCl_2$	:	Magnesium Chloride
Min	:	Minutes
ml	:	Milliliter
mM	:	Milli Molar
NAA	:	Naphthalene Acetic Acid
NaCl	:	Sodium Chloride
Ng	:	Nano Gram
PCR	:	Polymerase ChainReaction
pH	:	-log H^+ Ion Concentration
pmol	:	Picomol
RNase	:	Ribonuclease
Rpm	:	Revolutions Per Minute
SSR	:	Simple Sequence Repeat
TAE	:	Tris Acetate EDTA Buffer
Taq	:	*Thermusaqaticus*
TE	:	Tris EDTA
Tm	:	Temperature
U	:	Unit
UV	:	Ultra Violet
V	:	Volt
Viz.	:	Namely

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INTRODUCTION

CHAPTER I
INTRODUCTION

Sunflower (*Helianthus annuus* L.) belongs to the family *Asteraceae*. The chromosome number in sunflower is 2n=2x=34. Most species of the genus *Helianthus* are diploid (2n=34), tetraploid (2n=68) and hexaploid (2n=102) (Georgieva, 1976) and an estimated genome size of 2871-3189 Mbp with narrow genetic base. The sunflower plants are herbaceous, 1-6 m tall, usually with only a single, hair covered stem that may be more than an inch in diameter. Leaves are as long as 12 inches and are born on petioles arranged alternately on the stem. Sunflower has a typical head type of inflorescence. Ray florets are located around the margin of the head. The sunflower has peculiar response to light (Moghaddasi, 2011).

The name sunflower is derived from the flower head's shape. It is an annual plant grown as a crop for its edible oil. It is the world's second most important oil seed crop next to soybean. They grow best in fertile, moist, and well soaked soil. Sunflower was growing as an ornamental crop in India until 1920. Its cultivation has been popularized under oilseed mission and now it is being cultivated as one of the major oilseed crops beside peanut, soybean and mustard (Bayraktaroglu and Dagustu, 2011).

1.1 Origin of Sunflower

Sunflower is the third important major edible oil seed crop in the world after soybean and groundnut. The significant developments that took place in varietal front and the remarkable ability of the crop to adjust and grow successfully in different agro climatic conditions expanded sunflower production to all the continents. The genus *Helianthus* includes about 100 species, the majority of which are native to North America. The genus provides two food plants, *H. annuus*, the sunflower, and *H. tuberosus*, the Jerusalem artichoke. Several varieties of *H. annuus*, as well as other species of the genus, are sometimes cultivated as ornamentals. The common sunflower (*H. annuus* L.) is cultivated in every continent and is one of the four major annual crops grown for edible oil (Gielen, 1992). Sunflower probably originated in South-West United States and its seed was very early used for food by Indians. The cultivated sunflower is

native of America. It was taken to Spain from Central America before "grown by Indians for food and in New England for hair oil in 1615 (Moghaddasi, 2011).

1.2 Distribution and Productivity

It is cultivated as an oilseed crop in Argentina, China, France, Italy, Russia, South-Africa, Turkey, Uruguay, USA and Yugoslavia. According to United States Department of Agriculture (USDA), the Report of Global Agriculture Information Network India, 2018 overall sowing area in 2017-18 coverage during Kharif is about 3.35 lakh hector and production is 2.70 lakh MT against 190.31 lakh tons in 2016-2017. Among the world, Europe and America accounts 45% of total world's production followed by Russia (22%), Asian countries (10%) and others (13%). The largest sunflower producing state in India is Karnataka followed by Andhra Pradesh, Bihar, Maharashtra, Orissa and Tamil Nadu (www.nmoop.gov.in).

The sunflower area has crossed 20 million hectares, producing around 30 million tones of seed annually. In India sunflower becomes more popular oilseed crop due to high quality nutritional value, short duration crop, photo insensitivity, wide adaptability, drought and salt tolerance. Also it is being cultivated as cash crop at any season in India (www.wikipedia sunflower.com).

1.3 Biochemical Composition and Uses of Sunflower

Commercially available sunflower varieties contain oil range from 39% to 49% in the seed. Sunflower seed is highly nutritious containing 20% protein and 40-45% vegetable oil with a very high calorific values, cholesterol lowering factor constitute around 80-95% of total fatty acid, 60-70% linoleic acid with sufficient amount of calcium, iron and vitamin A, D, E and B complex. It supplies more Vitamin E than any other vegetable oil. Sunflower oil is also rich in polyunsaturated fatty acids and is advised for heart patient. It can also be used as substitute for mineral oil in various applications such as a fuel, lubricant, or oil for hydraulic system (Leland, 1996; Khalifa and Awad, 1997).

Sunflower oil is considered as premium oil because of its high level of unsaturated fatty acids and lack of linolenic acid. It is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels. The primary fatty acids in the oil are oleic and linoleic constitute typically 90% unsaturated fatty acids

(https://www.sunflowernsa.com). Sunflower oil is valued for its light taste, frying performance and health benefits. It is mainly used in salad dressings, cooking oil, margarine cooking, baby formula, lubrication, bio-fuel, hydrolic fluids, soaps and illumination. There are two types of sunflower oil available *viz*., linoleic and high oleic sunflower oil (Putnam *et al*., 1990).

Sunflower meal is rich in fiber, has a lower energy value and is lower in lysine but higher in methionine than soybean meal. Sunflower oil is a valued and healthy vegetable oil and sunflower seeds are considered as a healthy, tasty snack and nutritious ingredient in many foods. These seeds are used for human consumption and can also be used as birdfeed and as a high protein meal for livestock (Dedio, 2005). By products of the seed (seed cake) are a rich source of proteins (35%) and carbohydrates (18-20%) for animals and poultry feed (Ibrahim, 2012; Aminifar and Galavi, 2014).

In addition to these, sunflower seed have much industrial importance as its oil is used in the manufacturing soaps and cosmetics. Sunflower can be used as purple dye for textiles, body painting, varnishes, plastics and other decorations because of its good semidrying properties without color modification associated with oils high in linolenic acid (Putnam *et al*., 1990). Parts of the plant are used medicinally ranging from snakebite to other body ointments. The oil of the seed can be used on the skin and hair, while the dried stalk can be used as a building material (https://www.sunflowernsa.com). The use of sunflower oil (and other vegetable oils) as a pesticide carrier, and in the production of agrichemicals, surfactants, adhesives, plastics, fabric softeners, lubricants and coatings has been explored. The utility of these applications is usually contingent upon petrochemical feedstock prices (Putnam *et al*., 1990). The flowers are used as a yellow dye and the plant itself can be used for fodder, silage and as a green manure crop (Dedio, 2005).

1.4 Significance of Haploid in Agriculture

Haploids are plants with a gametophytic chromosome number and doubled haploids (DH) are haploids that have undergone chromosome duplication. The production of haploids and DHs provides a particularly attractive biotechnological tool and the development of haploid technology and a protocol to produce homozygous plants has had a significant impact on agricultural systems. Nowadays, these biotechnologies

represent an integral part of the breeding programmes of many agronomically important crops. There are several available methods to obtain haploids and DHs, of which *in vitro* anther or isolated microspore culture are the most effective and widely used (Germana, 2006). As an example, maize-induced chromosome elimination offers a very useful approach for rapid haploid plant production in bread wheat and durum wheat (Basu *et al*., 2011). Fairly recently, pearlmillet (*Pennisetum glaucum* L.) and *Tripsacum* species Pollen sources also served an identical role in haploid production in maize (Touraev *et al*., 2009).

Advantages of production of haploids and double haploids in plant breeding have been discussed by Kasha and Reinbergs (1980), Baenziger *et al*. (1984), Khush and Virmani (1996) and Raina (1997). Haploids offer two main advantages: (1) instant homozygosity through chromosome doubling and (2) ready detection of mutants without the interference of heterozygosity. Instant homozygosity in particular offers considerable opportunities for hastening breeding programs and in realizing certain crop improvement objectives that are otherwise too difficult and time consuming to pursue. Production of haploids and double haploids and utilization of such double haploids in breeding program has been very well elucidated in several agronomically important dicots like asparagus (Tsay, 1986), maize (Pescitelli *et al*., 1990a), wheat (Tuvesson *et al*., 1991), barley (Hoekstra *et al*.,1993), rice (Kim *et al*., 1993), petunia (Jain *et al*., 1996), brassica (Palmer *et al*., 1996), capsicum (Regner, 1996), potato (Veilleux, 1996) and tomato (Summers, 1997).

1.5 Anther Culture

Anther culture has been demonstrated to be an applicable technique for the development of doubled haploid. In some species, androgenetic doubled haploids have already been shown to be a useful tool for breeding. The potential of sunflower haplo-diploidization was first tested by Bohorova *et al*. (1980).

The production of homozygotes is important both for genetic studies and hybrid seed production in highly cross pollinated crops like sunflower (*Helianthus annuus* L.). Traditional breeding methods require a minimum of six generations for the development of near homozygous lines. Anther culture technology leading to production of homozygous diploids is a valuable tool in speeding up the progress of crop improvement.

However, success lies in the ability to manipulate anthers and microspores *in vitro* in a way that a wide array of genotypes responds with high regeneration frequency. Sunflower has been regenerated *in vitro* through organogenesis and embryogenesis (Greco *et al*., 1984; Patterson and Everett, 1985; Power, 1987; Espinasse *et al.*, 1989). As in other species, anther culture response of sunflower (*Helianthus* sp.) is strongly affected by physical, nutritional, physiological and genetic factors. By testing a number of different culture parameters, *i. e.* donor plant stages, culture media and conditions, appropriate schedule could be worked out for the successful regeneration of shoots at least for a number of sunflower lines and interspecific hybrids.

The advantage of this method is that there are thousands of microspores in each anther and numerous haploid plants can be obtained from a single anther. The main principle of anther culture is the prevention of pollen cell development, which normally results in formation of the male gamete. Instead, the immature pollen cells are induced to form embryos similar to somatic cells. Haploid plants have one set of chromosomes and therefore they are not fertile. At present, although anther culture is routinely used for producing haploid plants in practical breeding program, it is still necessary to improve basic techniques of the culture (Takashima *et al.*, 1995).

1.6 Effect of Mutagens on Anther Culture

Artificially induced mutations have been used more frequently to raise the frequency of mutations and variations, which can be amplified through chemical mutagens as for instance alkylating agents (ethyl methane sulfonate and methyl methane sulfonate), colchicine, sodium azide, as well as physical mutagens, such as ionizing radiations (Predieri, 2001). Chemical mutagens are more effective than physical ones. They enhance genetic variability in higher plants for successful breeding programs in vegetatively and sexually propagated plants (Dhanayanth and Reddy, 2000; Bhat *et al*., 2005). Chemical mutagenesis has been proved as fundamental in the improvement of crop plants. It is a simple technique used to create mutation in plants for improvement of potential agronomic traits, particularly for traits with a very low level of genetic variation (Szarejko and Forster, 2007). Chemical mutagens is one of the most important tools also used to study the nature and function of genes, which are the building blocks and basis of plant growth and development, there by producing raw materials for genetic

improvement of economic crops. Chemical mutagenesis is a coherent tool used in mutation breeding program for creating new alleles and is relatively cheap to perform and equally usable on a small and large scale (Laskar and Khan, 2014a).

Among chemical mutagens, colchicines treatment is one of the best tools of inducing and enhancing genetic variability in crops within a very short time span (Gnanamurthy *et al*., 2013). This chemical is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the orientation and structure of the mitotic fibers and spindle fiber (Khan and Goyal, 2009). Since chromosome segregation is driven by microtubules, colchicine is therefore applied to interfere with mitosis to induce polyploidy and mutations in plant cells. It is usually well tolerated in plant cells and mostly results in fruits and seeds that are larger, hardier and faster growing and more desirable (Ranney, 2006). For the above mentioned reasons, this type of genetic manipulation is frequently used in breeding plants to create genetic variability. Colchicine has been used for chromosome doubling to induce experimental polyploids since 1937 after Blakeslee and Avery, and Nebel (Burun *et al*., 2007). Plant losses may also be observed during colchicines application (Keles *et al*., 2015).

Several high yielding variants of crop plants have been developed using colchicines. The ability of this chemical to induce polyploids in crop species depend on the chemical concentration, duration of exposure and species of crop plant being investigated (Udensi *et al*., 2012a-c). So that, the main advantage of mutational breeding is the possibility of improving one or two quantitative characters without changing the rest of the genotypes and also, has a great potential and serve as a complementary approach in genetic improvement of crops (Mahandjiev *et al*., 2001).

Colchicine has been successfully applied during the first hours of anther and microspore culture, resulting in an increase of chromosome doubling in different species such as wheat (Barnabás *et al*., 1991; Hansen and Andersen, 1998), rice (Alemanno and Guiderdoni, 1994), *Brassica* (Möllers *et al*., 1994), tritordeum (Barcelo *et al*., 1994), tobacco (Takashima *et al.* 1995), maize (Saisingtong *et al*., 1996) and *Miscanthus* (Petersen *et al*., 2002). Colchicine can affect not only the percentage of doubling but also the whole androgenetic process. Colchicine has also been used to induce useful mutations in several economic ornamental plant species, such as *Datura*, *Portulaca*, *Petunia*,

Allium, and *Cucurbita*. The resulting mutants generally produce larger inflorescences, fruits, and pollen grains, and shorter stems (Pickens *et al*., 2006).

1.7 SSR Markers in Haploid Confirmation

Apart from the phenotypic traits, the mutagenic effects can be assessed more precisely using molecular markers. Molecular markers are considered essential tools in detecting genetic diversity among plant species (de Oliveira *et al*., 1996). Various molecular marker systems have been used, to detect molecular diversity. For instance, random amplified polymorphic DNA (RAPD) (Panwar *et al*., 2010), inter simple sequence repeat (Ansari *et al*., 2012), simple sequence repeat (SSR) (Panwar *et al*., 2010), and amplified fragment length polymorphism (AFLP) (Wang *et al*., 2005) markers. Shehata *et al*., (2009) used SSR markers to estimate the genetic distance in M_5 rice mutants. Sequence related amplified polymorphism (SRAP) is considered a simple and efficient tool with a higher through put scale and higher reproducibility than RAPDs, and is easier to perform than AFLPs.

SSRs are quite abundant in eukaryotic genomes, and are broadly used for genetic analysis because of their co-dominance, multi-allelic nature, reproducibility, extensive genome coverage and easy detection (Luro *et al*., 2008; Sharma *et al*., 2009). SSR molecular marker are helpful for confirmation of androgenetic trait of the haploid since haploid have only one band and diploid have two band (Couto *et al*., 2013). Triploid and tetraploid varieties could be identified from the SSR genotypes because some SSR loci generated 2 or more alleles for polyploids (Watanabe *et al*., 2008). Molecular markers based on genome sequences are more convenient and reliable for homozygous analysis.

Simple sequence repeats (SSRs) are microsatellites, are short sequences of nuclear DNA, consisting of tandemly repeated nucleotide units (1-5 nucleotides long). Identification of haploids mostly includes SSR molecular markers because they are stable and co-dominant and allow separation of homozygotes from heterozygotes (Belicuas *et al*., 2007).

SSRs are powerful DNA marker to easily differentiate between double haploid and diploid. SSR marker was successfully used for evaluation of genetic diversity in sunflower. SSR marker can be efficiently used to check the homozygosity or heterozygosity if there, with in lines. Allelic variation should be absent in doubled

haploid, which can be revealed by using multiple SSRs for multiple locus. Presence of multiple alleles (polymorphic) will confirm diploid plant out of unwanted anther walls (somatic diploids) during anther culture and presence of (monomorphic) band will confirm pollen generated haploid and DHs plants. SSR analysis shows the uniformity as well confirms the origin of DH lines (Zhang *et al*., 2005).

SSR markers are used to identify large numbers of spontaneous double haploids or homozygous plants at an early stage of development. Molecular markers would provide a fast, efficient, and cost-effective method for breeding programs. They are highly polymorphic, somatically stable, and inherited co-dominantly (Morgante and Olivieri, 1993), making the ideal markers for conforming origin and homozygosity. SSRs have been successfully used for identification of homozygous, spontaneous double haploids (Bouvier *et al*., 2002). Successful analysis through SSR marker for haploid/ploidy confirmation in various plants such as Maize (Couto *et al*., 2013), Pepper (keles *et al*. 2015), Coconut (Perera *et* al. 2008), have been studied.

1.8 Flow Cytometry Analysis for Ploidy Confirmation

The flow cytometer has of late become an essential piece of equipment for modern plant-breeding companies and in tissue culture of horticultural and medicinal plants because knowledge of plant DNA content is necessary in many breeding programmes/ technologies, e.g. those directly involving ploidy change in polyploid breeding. Polyploidization is usually accompanied by increased cell size, which leads to alterations in morphology that are favourable for horticultural plants (Sliwinska, 2018).

One of the simpler alternatives is determination of ploidy using flow cytometry, this to some extent has substituted the conventional chromosome counting and densitometry as the analysis besides being fast, precise and convenient can help in analysis of large number of samples within a short time. However, reliability of the results depends on the tissue being processed and the stains used as there is a possibility of nonspecific binding of fluorochrome, auto fluorescence from various pigments and interference of secondary metabolites with DNA stains leading to erroneous interpretations (Kallamadi and Mulpuri, 2016).

Recently, a rapid and exact method to determine nuclear DNA contents by flow cytometry (FCM) has been developed for various plant species, such as cloudberry

(Thiem and Sliwinska, 2003), lily (Nakano *et al*., 2006). FCM made it possible to identify the ploidy level of plants where the chromosomes are too small or too abundant, to be counted by microscopy.

Flow cytometry is widely used for haploid confirmation because of the ease and speed of both sample preparation and result acquisition (Loureiro and Santos, 2004), as it evaluates DNA content. Flow cytometry is proving to be a great tool to help determine the efficiency of the haploid-inducing method and the success of chromosomal duplication (Belicuas *et al*., 2007).

Flow-cytometry were considered and proved suitable method for identification of ploidy level of *Ocimum basilicum* in various stages of the plant development of these species. Flow cytometry as found to be the most efficient method for detecting induced changes in ploidy level. Flow cytometry was used as an efficient method for rapid detection of ploidy level in cocoyam (Omidbaigi *et al*., 2012).

Different scientists worked on different methods and concluded flow cytometry as the best accurate technique. Since, hundreds of plants are grown, flow cytometry for all of them is expensive and labour intensive (Arjunappa *et al.*, 2015). Successful analysis through Flow cytometry for haploid/ploidy confirmation in various plants such as Tobacco (Chowdhury, 1984), *Brassica napus* (Klima *et al.*, 2008), Pepper (Keles *et al*., 2015), Chilli pepper (Arjunappa *et al*., 2015) have been achieved.

Keeping in view the usefulness of cholchicine in inducing mutation and/or polyploidy, the present investigation entitled **"Analysis of colchicine effect on anther culture of Sunflower (*Helianthus annuus* L.)"** has been undertaken with the following objectives:

1. To study colchicine effect on anther culture
2. To analyse genetic variability in regenerants by SSR marker.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The haploid technology has now been adapted in different plant breeding programs across all the major continents as the most commonly used approach for rapid crop development for transferring genes of interest, chromosomal segments or even complete chromosomes by means of distant hybridization (Ceoloni and Jauhar, 2006; Baenziger and DePauw, 2009; Touraev *et al*., 2009).

2.1 Anther Culture in Sunflower

Among living organisms, plants are characterized by their capacity to with stand drastic changes in ploidy level; the most intriguing are being that of growing with haploid set of chromosomes. The haploid research is a continually expanding field ever since the discovery of first haploid plant in *Datura stramoni* by A. D. Bergner during 1921 (Hu Han and Yang Hon gyuan, 1986). Since, then haploids have been reported in more than 250 plant species. The discovery that culturing anther that contains microspores at an appropriate stage of development can yield androgenic haploids (Guha and Maheshwari, 1964; 1966) generated enthusiastic activity among crop improvement workers to exploit the techniques to enhance agricultural production. According to Rino Cella (1988) haploid science is one of the few life science disciplines which could carry on through the turm oil of the Great Cultural Revolution.

Although anther culture techniques have been successful, yet all material does not respond in the same way or to same degree. Significant variations can be seen from plant to plant or even within the same plant. Analysis of the parameters that underline these differences is needed to obtain a uniform and predictable response. The factors that are known to influence the success of anther culture, *viz*., donor plant genotype, donor plant physiology, stage of microspore development, pre-treatment, culture conditions and culture environment. For the last 30 years, the production of sunflower has been increased many folds due to the expansion of its cultivation in several parts of the world (Quresh *et al*., 1992). Anther cultures have attracted considerable attention as supplementary tool for the rapid production of haploid plants (Jain *et al*., 1996).

Plotnikov (1975) regenerated plantlets from cultured anther of *H. annuus* varieties on MS medium with IAA (0.8mg/l), Kinetin (2mg/l), IBA (1.5mg/l) or NAA (1.5 mg/l). However, plantlets did not survive to maturity. Similarly, Tzen and Lin (1975) obtained anther callus in *H. annuus* using Blaydes medium with1-2 mg/l NAA, 1-2 mg/l 24-D and 2-4 mg/l Kinetin but observed no regeneration.

Bohorova *et al*. (1980) have reported, immature anthers immediately before first mitosis are most suitable material for the induction of androgenesis in sunflower. Mix (1985) obtained anther callus from eight varieties of sunflower on MS+Thiamine HCl (0.4 mg/l), IAA (1 mg/l), NAA (2 mg/l), 2,4-D (0.1mg/l) and BAP (2 mg/l), and sucrose (60g), while regeneration medium consisted of MS+Thiamine HCl (0.1 mg/l), 2,4-D (0.25mg/l), Kinetin (1mg/l), GA_3(1mg/l) and sucrose (20 g), and obtained six plantlets out of which two were haploids.

Mezzarobba and Jonard (1986) identified sunflower anthers at late meiotic *i. e*., between the diad and tetrad stage as optimum for culturing. The temperature treatment to anthers up to 35^{o}C helped in better embryogenesis. They also noticed genotypic difference for embryogenesis and obtained two haploid and five diploid plants from anther culture.

Gurel *et al.* (1994) obtained 100% callusing on MS medium without hormones and MS medium containing kinetin + NAA (4mg/l +2mg/l) and maltose (30mg/l) instead of sucrose. Both division of uninucleate microspores and embryogenesis were achieved although in low rates with two of the four sunflower genotypes tested.

Thengane *et al*. (1994) reported a stimulatory effect of cold pretreatment on embryo induction in sunflower. The effect of cold treatment was found to be indirect. For preliminary experiments, anthers of uninucleate microspores were cultured on four types of basal media *viz*., Murashige and Skoog's MS, Gamborg's B5, Nitsch and Nitsch, and White, supplemented with 2, 4-D (1.0 mg/l) and BAP (0.5 mg/l) and 40 g/l sucrose. MS basal medium, being more responsive for embryo induction, was used for further experimentation. To optimize the culture requirement MS basal medium was supplemented with 2, 4-D (2-2.0 mg/l) and BAP (0.5-1.0 mg/l). The effect of cold pre-treatment, hormone regime and sucrose concentration was tested for embryogenic efficiency. Genotype had a significant effect on the capacity of embryo induction.

Addition of silver nitrate (2.5 mg/l), an ethylene inhibitor, stimulated embryo germination.

Coumans and Zhong (1995) studied the superiority of carbohydrate source for anther response in sunflower. Of the three tested sugars, sucrose proved to be better in promoting maximum callusing as well as embryoid formation. Maltose was completely inhibitory although it was reported to enhance the asymmetrical and symmetrical divisions in the isolated microspores.

Saji and Sujatha (1998) described protocol for high frequency callus induction and plant regeneration from sunflower (*Helianthus annuus* L.) anthers. Different variables using MS basal medium supplemented with 2.0 mg/l NAA and 1.0 mg/l BA were tested for their ability to enhance the frequency of anther callusing and subsequent embryogenesis. Of these, agar concentration, sucrose concentration, carbohydrate source had significant effect on callusing while differences due to incubation under dark vs. light conditions. However, all these factors exerted highly significant influence on embryogenesis when calli from the various media were transferred to medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA and achieved callusing as high as 100% and embryo formation at a frequency of 44%.

Vijaya *et al*. (2003) reported production of microspore derived embryos from cultured anthers is a well established technique for the isolation of homozygous lines in sunflower. Anthers of uninucleate microspores for wild *Helianthus* and interspecific hybrids were cultured on basal MS media with different hormonal combinations. The induction of callus was quick with high proliferation in the presence of 2,4-D, low cytokinin and auxin contents. Excess cytokinin and auxin had no significance in callus induction. Regeneration potential increased with the increased amount of casein hydrolysate and Benzyl amino purine. Kinetin had no specific influence on regeneration frequency of callus. Genotype had a significant effect on the capacity of callus induction and plantlet formation.

Alam*et al*. (2009) evaluated regeneration potentiality of anther of five varieties of *Brassica* species *viz.,* BARI Shariaha-7, Tori-7, Agrani, Daulat and Safal cultured *in vitro*. Different concentrations and combinations of growth regulators were supplemented in MS medium. The range of callus induction was 12.50-87.50%. Maximum callus

induction (75.00%) was observed on MS+2,4-D (4 mg/l)+BAP (1.0 mg/l). Among the genotypes, BARI Sharisha-7 showed the highest percentage of callus induction (60.42%). Among the treatments, highest percentage of shoot regeneration (75.00%) was observed on MS+4mg/l BAP+1.0mg/l NAA. BARI Sharisha-7 also showed the highest rate of plant regeneration (66.67%). Root induction was highest (75%) on half strength MS medium supplemented with IBA (1.0 mg/l) and NAA (0.5 mg/l). The plantlets with sufficient roots thus obtained were transferred successfully to plastic pots and subsequently to the field. BARI Sharisha-7 and Tori-7 survived easily in the pots as well as in the field but Safal was very poor in survivability both in the pots and in the field.

Sayem *et al*. (2010) analysed the performance of three different genotypes (BARI Sarisha-6, BARI Sarisha-8, and BARI Sarisha-11) in two different media *viz*., MS and B5 with different concentrations of phytohormone (2, 4-D) for callus induction from anthers of *Brassica* and subsequent plant regeneration in MS media with different concentrations of phytohormone (BAP and NAA). BARI Sarisha-8 showed better performance among genotypes and MS medium containing 0.5 mg/l 2, 4- D showed the best performance. In case of interaction, it was observed that genotype BARI Sarisha-8 produced the highest percentage of callus on both in MS 2, 4-D (0.5 mg/l) and B5 + 0.5 mg/l media. BARI Sarisha-11 also produced the highest percentage of callus in MS medium containing 2, 4-D (0.5 mg/l), which was statistically identical to BARI Sarisha-8. The interaction of genotype and media composition for days required for callus initiation indicated that there were significant variations among the genotypes. BARI Sarisha-8×135 + 2, 4-D (0.5 mg/l) took minimum time for days to callus initiation. On the other hand, BARI Sarisha-6×MS + 2, 4- D (0.1 mg/l), BARI Sarisha-11×MS+2, 4-D (0.1 mg/l) and BARI Sarisha-6 x B5 ± 0.1 mg/l 2,4-D took the maximum time for callus initiation, which were statistically identical.

According to Siddiqui (2014) *in vitro* production of doubled haploid (DH) plants through anther culture provides an efficient method for rapid production of homozygous lines. For this purpose, two different media MS and N6 supplemented with BAP (1mg/l) +2,4-D (1mg/l) +NAA (0.5mg/l) were evaluated for callus induction and green plant regeneration in rice. Results showed that the highest percentages of calli and green plants as a continuous process were obtained by N6 compared to MS media. The best response

to callus formation was observed in BR48 and minimum green plant obtained from OM576.

Miladinovic *et al*. (2016) reported optimal medium for shoot regeneration from cultivated sunflower anthers. Anthers of 74 cultivated sunflower genotypes in BC_2 generation of back crossing were surface sterilized and placed on four different MS medium based solid regeneration media. The appearance of organogenesis or somatic embryogenesis was observed and obtained data statistically analyzed. The experiment was set as completely randomised, with two factors. Callus, somatic embryo, shoots and root regeneration on the anthers of the tested genotypes was observed. Data were analysed by ANOVA and REML. Shoot regeneration (up to 9.39%) was observed in 48.65% tested genotypes. Variation of media composition proved to be essential for efficient sunflower anther culture. The use of appropriate statistical analysis was found to be of the great importance in setting anther culture experiments and optimization of the culture protocols.

Dogan *et al.* (2016) studied microspore culture of different hybrid sunflower cultivars and showed that androgenic methods can be used to obtain haploids of anther and microspores. In these methods, genetic potential of cultivars is very important for a successful culturing. The effects of different plant growth regulators and media composition on androgenic microspore culture were studied and found that different media compositions and culture conditions are necessary in order to develop haploid in sunflower.

2.3 Colchicine

The relatively high frequency of doubled haploids needed by plant breeders, requires the application of efficient techniques to induce doubling which must involve high doubling rates, low frequency of damage and minimum plant mortality less time consuming and easy to handle on a practical scale.

Colchicine is a toxic natural product and secondary metabolite extracted from plants of the genus *Colchicum* commonly known as *Autumn crocus*, wild saffron and naked lady (Folpini and Furfori, 1995). It is also known as “Meadow saffron”. It is known as “naked lady” due to the fact that flowers emerge from the ground long after the leaves have died back. It is present in most parts of the temperate areas of Europe, Asia

and America (Hartung, 1953). Colchicineis an alkaloid prepared from dried corms and seeds of *Colchicum autumnale.* The alkaloid colchicin among the Indian medicinal plants is contained in the corms of *Colchicum luteum* containe around 0.25% of colchicine and the seed contain about0.4% of colchicine. The *Iphigenia*, seeds contain as much as 0.51% colchicine. These plants are not available in sufficient quantities to warrant any commercial utilization. *Gloriosa superba* is another plant which also contains colchicine. A mixture of alkaloids consisting mainly of colchicine has been isolated from dried tubers of *G. superba*. Hence, *G. superba* acts as substitute plant of tropics to *Colchicum autumnale* for the alkaloid colchicine. It is antimitotic and act by blocking mitosis by preventing tubulin polymerization to microtubules. Colchicine disrupts mitosis by binding to tubulin, thus inhibiting the formation of microtubules and the polar migration of chromosomes, resulting in a cell with a doubled chromosome number. It has been used as an antiparasitic agent in ethnoveterinary use (Coassini and Poldini, 1988).

Colchicine is a mutagen that prevents formation of microtubules and which is usually used for doubling the chromosome number. Thus, it is routinely utilized in polyploid plant formation. Colchicine effectively functions as a “mitotic poison,” leading to noticeable mutagenic effects. Many reports highlight the mutagenic effects of colchicine on plant performance (Balkanjieva, 1980; Castro *et al*., 2003).

2.4Effect of Colchicine on Anther Culture

Wan *et al.* (1988) evaluated effect of colchicine treatment on embryogenic, haploid callus line of maize *(Zea mays* L.) derived through anther culture. Two colchicine levels (0.025% and 0.05%) and three treatment durations (24, 48, and 72 h) were used and compared to untreated controls. Chromosome counts and seed recovery from regenerated plants were determined. No doubled haploid plants were regenerated from calli without colchicine treatment. After treatment with colchicine for 24 h, the callus tissue regenerated about 50% doubled haploid plants. All of the plants regenerated from the calli treated with colchicine for 72 h were doubled haploids, except for a few tetraploid plants. No significant difference in chromosome doubling was observed between the two colchicine levels. Most of the doubled haploid plants produced viable pollen and a total of 107 of 136 doubled haploid plants produced from 1 to 256 seeds. Less extensive studies with two other genotypes gave similar results. These results

demonstrate that colchicine treatment of haploid callus tissue can be a very effective and relatively easy method of obtaining a high frequency of doubled haploid plants through anther culture.

Wong (1989) treated rice nodal segments from three flowering haploids for different lengths of time with 0.3%or 0.4% colchicine (dissolved in 2% DMSO) in an attempt to induce fertile seeds. A combination of higher colchicine concentration and longer hours of treatment reduced the survival rate of treated segments, but more fertile plants were transformed. Pooled data showed that, of the 842 segments used, 42.2% survived the treatment and sprouted, but only 31.9% were successfully established and grown to maturity. Among the 269 mature plants, 29.4% produced fertile seeds (panicles) with an average of 146.2 seeds per diploidized plant. He also noticed same results with application of colchicines in media for chromosome doubling and regeneration.

Barnabas *et al.* (1990) have applied different chromosome reduplication techniques to microspore-originated *Triticum aestivum* L. haploids. In addition to the conventional treatment (whole plant exposure to colchicine solution), spontaneously redoubled haploids were also examined. As an experimental treatment, different concentrations (0.01, 0.02, and 0.04%) of colchicine were added directly to the induction media. Colchicine did not affect the anther response or the plant regeneration capacity. The success and stability of genome redoubling was estimated on the basis of the fertility of the regenerated (R_0) plants and their progeny (R_1). Chromosome doubling produced by colchicine before the first microspore mitosis was significantly more efficient than the conventionally used techniques.

Alemanno *et al.* (1994) incubated rice anthers plating on a semisolid induction medium containing 250 or 500 mg/l colchicine for 24 or 48 hours followed by transfer to colchicine-free medium and standard anther culture procedures reported overall 1.5 to 2.5 fold increases in doubled haploid green plant productions compared to control anther cultures. The addition of colchicine had no detrimental effects on the different anther culture efficiency parameters, but in some treatments led to significant enhancement of anther callusing frequency or callus green plant regenerating ability. The most efficient treatment raised doubled haploid plant recovery from 31% to 65.5%. These results suggest that post-plating colchicine treatment of anthers, since it was found to improve

both anther culture efficiency and doubled haploid plant recovery frequency, could be integrated into rice doubled haploid plant production programmes.

Navarro-Alvarez *et al.* (1994) have found that addition of colchicine to wheat anther culture medium increased the doubled haploid plant production. Increasing the colchicine concentration reduced the number of embryoids produced from 77.4 to 29.9 embryoids/100 anthers but did not significantly affect the frequency of plant regeneration and increased the frequency of doubled haploid plants (19.0 to 72.3 DH plants/100 green plants).

Takashima *et al*. (1995) applied colchicine treatment directly to anthers excised from the flower bud of tobacco (*Nicotiana tabacum* L.). However, high frequencies of diploid plants were obtained among the plants derived from the anthers treated with colchicine. In particular, the highest diploidization rate (66.7%) was obtained in the 0.4% colchicine treatment for 8hrs. They concluded that colchicine application to anther before culture initiation is very simple method and can be an efficient and time saving technique for haploid breeding of tobacco.

Hensen and Andersen (1998) isolated microspores of two doubled haploid lines of wheat and treated with 0, 3, 10, 30, 1000 and 3000 μM of colchicine for 24 hours and 48 hours during microspore culture. Highest number of embryos regenerated from 24 hours colchicine treatments while highest frequencies of green plants and fertile plants were obtained from 48 hours colchicines treatment. The highest number of DH plants per spike resulted from treatment with colchicine concentrations of 300-1000 μM.

Zamani *et al.* (2000) studied the effect of colchicine added to induction medium for the production of doubled haploid plants after *in vitro* anther culture in one winter and two spring wheat genotypes. They reported that in case of winter wheat variety colchicine treatment resulted in 100% completely fertile plants.

Chen *et al*. (2002) investigated the effect of colchicine treatment on doubled haploid production efficiency and reported that MS media supplemented with 100 ml/l colchicine gave maximum chromosome doubling frequency (100%) while in case of colchicine treatment by immersing leaves and roots of plants in colchicine solutions (500 mg/l) yielded 98.2% doubling frequency.

Klistov and Artemeva (2004) used two methods for doubled haploid production in spring and winter wheat ecotypes and their hybrids obtained with *Aegilops speltoides* and *Agropyron erectus*. In the first method they utilized wheat x maize system in which emasculated wheat and hybrid heads were hand pollinated with maize pollen. Embryos were excised 12-14 days after pollination and cultured on B5 medium. The resulting seedlings were immersed in 0.1% colchicine solution, rinsed and transplanted in pots which were kept in green house. In second method, they used anther and microspore culture technique for doubled haploid production. Wheat and hybrid anthers were isolated immersed in P11 nutrient agar medium for 20-30 days. Embryo like structures was transplanted into medium with 0.5 mg/l Kinetin. They obtained four doubled haploid lines from *in vitro* culture and 102 doubled haploid lines through wheat x maize system.

Klíma *et al.* (2008) analyzed effect of microspore culture treatment with antimitotic agents colchicine, trifluralin and oryzalinon. The frequency of embryo formation, embryo development, plant regeneration and diploidization rate in three F_1 hybrids of winter rapeseed cultivars were compared. The ploidy level analysis of 1709 flowering microspore derived plants showed that *in vitro* applications of all antimitotic drugs increased the rate of doubled haploid (DH) plants significantly. The mean rate of DH plants from the trifluralin treatment was 85.7%, from colchicine 74.1% and 66.5% in the case of oryzalin, while only 42.3% in the untreated control variant whereas *in vivo* additional application of colchicine at the plantlet stage did not significantly increase the mean rate of DH plants (55.6%). Although there were no significant differences in diploidization efficiency between the *in vitro* applications of particular antimitotic agents. Trifluralin showed to be the most suitable because of its positive effect on embryo development and conversion into whole plants. In addition, the diploidization rate was sufficient and stable in all genotypes tested. The results indicate that the trifluralin treatment of microspore cultures could provide efficient chromosome doubling for the production of doubled haploid lines from winter oilseed rape breeding materials.

Burun *et. al.,* (2007) have reported colchicine effect on tobacco (*Nicotiana tabacum* cv. Karabalar 6265) at 3 different stages of anther culture. Before culture, anthers were treated with 0.4% aqueous solution of colchicine for 0, 2, 4, 6, 8, 10, and 12 hours. Culture response of anthers decreased as the treatment duration increased (except

12 hour) and the highest diploidization of 29.7% was obtained with 6 hours. When plantlets with 4 to 8 leaves immersed in 0.2% colchicine for 0, 7, 24 and 48 hours on a shaker, besides 4.3%, 42.3%, 37.8% and 33.3% doubled haploids, respectively, haploids, tetraploids, aneuploids, and mixedploids were also found among the treated plants. When chromosome doubling rate and viability are taken into consideration, among the 3 methods tested, plantlet treatment with 0.2% colchicine for 7 hours appeared to be more efficient with 42.3% dihaploids.

Islam (2010) compared the effect of direct and indirect colchicine treatmentto anthers and isolated microspores of wheat. For first experiment 100 mg/l colchicine added separately with three different basal media and found in all cases more or less fertile plants production compared to control (without colchicine). Colchicine application on anther culture medium showed significant increase in embryo formation and green plant regeneration. The direct treatment of colchicine to isolated microspore culture decreased three-four fold embryoids induction but improved fertile plant regeneration. Application of colchicine for both cases in anther and microspore culture increased the average chromosome doubling frequency (84.94%) compared to the control (55.26%) where colchicine was not added. For the second experiment, different concentrations of colchicine (50, 100, 150 mg/l for three days) added in AMC medium and found reduced number of embryos and regenerated green plants, while the regeneration of albino plants was stimulated. When colchicine concentration increased the number of embryos decreased significantly, while the doubling index increased which was the main target of this study. In this case average frequency of diploid plants were increased (81.73%) compared with control (72.40%) among the three treatments. This finding has increased the knowledge about the benefit of colchicine application and optimized its concentration on isolated microspore for improving doubled haploids production in wheat microspore culture.

Premvaranon *et al.* (2011) cultured anthers of KDML 105×SPR 1 (*Indica*× *Indica*) on Linsmaier and Skoog (LS) medium, which contained nutrients, growth regulators for haploid generation. The supplementation of 0.2 g/l colchicine and 100 μM 2,4-D was the most efficient in LS media. Over 70% of viable double haploid ELS (Embryo like structure) were produced in 8 weeks and subcultured only twice compared

with conventional anther which takes more than 12 weeks. This new technique can therefore be applied to rice in order in shorten time to produce higher number of double haploid plantlets.

Wurschum *et al.* (2012) have investigated the applicability of an *in vitro* approach for chromosome doubling based on microspore culture. Results showed pronounced increase in the proportion of doubled haploid triticale plants compared to the spontaneous doubling rate, but also compared to the doubling obtained by the standard *in vivo* approach. In addition, the frequency of plants surviving from culture medium to maturity is also much higher for the *in vitro* approach. Colchicine concentrations of 1 mM for 24 hours or 0.3 mM applied for 48 or 72 hours during the first hours of microspore culture performed best.

Rukmini *et al.* (2013) reported that application of anther culture technique for improvement of *indica* rice is a formidable task as they are known to be recalcitrant to culture unlike *japonica* rices. An effort was made to assess the influence of cold pretreatment and phytohormones on the anther culture response of Rajalaxmi (CRHR 5) and Ajay (CRHR 7), two elite and popular *indica* rice hybrids. Cold pretreatment for 7-9 days at 10^{o}C was found to have a positive influence on the callus induction frequency and a ratio of 1:4 for 2,4-D and NAA and 1:3:1 ratio of Kinetin: BAP: NAA ratio proved to be optimal for callus induction and green plant regeneration respectively.

Keles *et al*. (2015) compared the rate of spontaneous doubled haploid in seven green pepper genotypes. Murashige and Skoog (MS) nutrient medium with NAA (4 mg/l) and BAP (0.5 mg/l), 0.25% activated charcoal, 30 mg/l sucrose, and 15mg/l silver nitrate ($AgNO_3$) was used. Ploidy levels of plants obtained through anther culture were detected using both flow cytometry and simple sequence repeats (SSR) markers. The results showed that different spontaneous doubled haploid rates were obtained from different pepper types are haploid plants undergoing spontaneous chromosome doubling. This was followed by charleston and capia types with 31.9% and 30.4% doubling, respectively. Green pepper type gave the lowest spontaneous doubled haploid rate with 22.2% doubling. The results will be useful both for future work on haploid in pepper and for breeding programs.

Arjunappa *et al.* (2015) assessed the ploidy levels of androgenic plants of chilli pepper (*Capsicum annuum* L.) obtained through direct embryogenesis, by chromosome counting, chloroplast counting and flow cytometry methods. These results revealed that all the techniques were successful in assessing the ploidy levels. Besides chromosome count and chloroplast count techniques could be employed effectively as flow cytometry. Twelve chromosomes and 8 chloroplasts were observed in haploid plants, where as 24 chromosomes and 16-20 chloroplasts in double haploid plants. Plants which were haploids and those which did not undergo spontaneous doubling were treated with 0.5% colchicine and tested for doubling by using flow cytometry, result obtained as 45% spontaneous double haploids, 51% haploids, 2% trihaploid and 2% aneuploid plants.

Olszewska *et al*. (2015) used selected interspecific hybrids (905 × 'Sono') F_1, (905 × 'Mino') F_1, (405 × 'Luba') F_1, (405 × 'Sono') F_1 of pepper as plant material for the induction of androgenesis. The apical parts of 106 androgenic haploids were placed for six days on the MS medium containing colchicine at the concentration equal 400 mg dm^{-3}. The largest groups of regenerants were mixoploids 29-55% and haploids 20-50%. For the studied genotypes an effective diploidization at the level of 17-27% was observed. Micropropagation was performed on the 23 haploids obtained and the received micro cuttings (in the number of 73) underwent again a six day and a nine day treatment with colchicine on the MS medium containing 200 mg dm^{-3} of colchicine. The effectiveness of the regeneration after the second colchicine treatment was significantly reduced. Single diploid plants appeared among the regenerants (905 × 'Sono') F_1 and (405 × 'Luba') F_1.

Miao *et al.,* (2016) examined colchicine concentration and duration for improving induction of polyploidy. The combinations of three materials (shoot tips, pre-germinated seeds and grin), five colchicine concentrations (100, 200, 500, 1,000 and 2,000 mg/l) and three treatment durations (12, 24 and 48 hrs) were tested in *Dendranthema indicum* var. *aromaticum*. A total of 7 tetraploids and 301 chimeras determined by chromosome number analysis. The treatment of grin seeds with 1,000 mg/l colchicine for 24 h (14.5%) and shoot tips with 1,000 mg/l colchicine for 7 d (40%) were suitable for induction of chromosome doubling.

Pusadkar and Jha (2018) employed *in-vitro* colchicine treatment on callus obtained from anther culture of indica rice. S x R cross was used for colchicine treatment after callus induction from anther culture treatment of 100 and 500 mg/l of colchicine for 48 and 72 hours and then shifted to regenerative media. The experiment was performed along with control (without colchicine treatment) in which green callus percentage and overall plant regeneration percentage increases by 1 to 2.5 fold in treated calluses compared to control. The treatment containing 100 mg/l of colchicine in regeneration media followed by 48 hrs of incubation has given highest green callusing percentage of 23.13% as control having only 9.16% of green calli induction in S x R cross.

2.4 Haploid Confirmation by using Molecular Markers

SSRs are powerful DNA marker to easily differentiate between double haploid and diploid.SSR marker can be efficiently used to check the homozygosity or heterozygosity if there, with in lines. Allelic variation should be absent in doubled haploid, which can be revealed by using multiple SSRs for multiple locus. Presence of multiple alleles (polymorphic) will confirm diploid plant out of unwanted anther walls (somatic diploids) during anther culture and presence of monomorphic band will confirm pollen generated haploid and DHs plants. SSR analysis shows the uniformity as well confirms the origin of DH lines.

The SSR molecular markers are used to confirm the androgenetic trait of the haploid. SSR markers have been widely used in ploidy analysis and chromosome duplication (Belicuas *et al*., 2007; Barret *et al*., 2008; Perera *et al*., 2008; Zhang *et al*., 2008; Diao *et al*., 2009; Li *et al*., 2009; Mayor and Bernardo, 2009; Kebede *et al*., 2011; Prigge *et al*., 2012).

The single multi-allelic self incompatibility gene has been used in apple by Verdoodt *et al*. (1998) to discriminate homozygous from heterozygous individuals obtained by anther culture as well as by parthenogenesis *in situ*. Microsatellites markers can be utilized to assess homozygosis and to confirm the gamatic origin of calli and plantlets (Germana, 2006).

Perera *et al.* (2008) used anther culture to obtain dihaploid (DH) coconut plants and determined their ploidy level by flow cytometric analysis. Simple sequence repeat (SSR) marker analysis was conducted to identify the homozygous diploid individuals.

Ploidy analysis showed that 50% of the tested plantlets were haploid and 50% were diploid. Polymorphic fragments of the mother palm and their segregation patterns in anther-derived plantlets were used to determine the origin of the diploid plantlets. Using a diagnostic SSR marker (CNZ43), all the diploid plantlets tested were identified as being derived from microspores (*i.e*. were homozygous) and were thus candidates for use in coconut breeding programs.

According to Cao *et al*. (2010) homozygous genotypes are valuable for genetic and genomic studies in higher plants. Anthers of sweet orange at the uninucleate stage were induced and two embryogenic calli were obtained that further regenerated to embryoids (2/400). Plantlets obtained after transferring the embryoids to a shoot regeneration medium, but were short lived. Ploidy analysis *via* both flow cytometry and chromosome counting verified that these two lines were diploids. Additionally, 43 simple sequence repeat (SSR) markers which showed to be heterozygous in the Valencia sweet orange donor line confirmed homozygosity and doubled haploids in the anther derived lines. Furthermore, analysis of the doubled haploids *via* cleaved amplified polymorphic sequence (CAPS) markers and target region sequencing confirmed the allelic state of two genes (LCYE and LCYB) involved in the carotenoids biosynthesis of sweet oranges.

Malik *et al*. (2011) investigated the effects of temperature pre-treatment, thidiazuron, naphthalene acetic acid, and 6-Benzylaminopurine on *in vitro* gynogenic plant production from un-pollinated melon (*Cucumis melo* L.) ovaries. Simple sequence repeat (SSR) marker analysis was conducted to identify the homozygous diploid individuals. The temperature pre-treatment (4°C) for 4 days increased embryo formation frequency (63.3%) significantly. Addition of thidiazuron (0.04 and0.02 mg/l) in the induction medium significantly increased the number of responding ovaries (46.6%, 65.83%), respectively. The maximum number of plantlet regeneration (22.5%) was achieved by culturing the ovary derived embryos on Murashigue and Skoog medium (MS medium) supplemented with 0.6 mg/l 6-Benzylaminopurine. Spontaneous doubled haploids originated directly through embryogenesis were subjected to genetic analysis using SSR molecular marker with 23 primers pair for homozygosity. SSR markers with microsatellite CMGA172 confirmed that the alleles in the parental material were also present in the gynogenic plantlets, but amplified only two alleles as compared to four

alleles of the heterozygous parent material at same locus. Therefore these regenerated plantlets were considered homozygous and produced through a process of gametophytic embryogenesis.

Grewal *et al*. (2011) analyzed 3000 doubled haploid (DH) lines through anther culture of 28 crosses involving *indica* and *japonica* rice (*Oryza sativa* L.) cultivars. Cultivars *indica* showed low anther culture ability (1.2% callus induction) whereas japonica cultivars had 20-fold higher (28.1%) anther culture ability. A set of 121 and 124 DH lines generated from the japonica cultivar (IR69428) × *indica* variety (IR64) was used for phenotypic and molecular analysis. Significant variation was observed among DH lines for agronomic traits including Zn content. However, the phenotypic variance within each DH line was comparable with the mean phenotypic variance of the parents, suggesting no variation within DH line(s). A set of 209 simple sequence repeat (SSR) markers was selected to construct a linkage map with total genetic distance of 2148.8 cM. Simple sequence repeat analysis showed 1:1 ratio of *indica* and *japonica* alleles. Of the 209 markers, 21 showed distorted segregation and these markers are randomly located over 12 chromosomes. Homozygosity was detected for all the marker loci in 124 DH lines and 28 were heterozygote. Results showed that *indica* cultivars are recalcitrant and genes for anther culture ability are partially dominant. Molecular and phenotypic trait analysis of 12 DH lines showed that the origin of DHs is from pollen and these 121 DH lines are thus a valuable genetic resource in mapping quantitative trait loci (QTL) for grain Zn content and other agronomic traits. Interestingly, some of the DH lines had *indica* traits and high (28.3 mg kg/l) grain Zn content in polished rice.

Battistelli *et al*. (2013) reported usefulness of SSR markers to confirm duplicated plants, as confirmed by flow cytometry. The specimens with only bands from the hybrid donor were considered to be doubled haploids from haploid seeds, and plants with bands from the inducer and respective hybrid were diploids resulting from the cross. Band pattern of haploid duplicates confirmed by the markers.

Drumeva *et al*. (2014) studied the doubled haploid origin of diploid plants developed by the method of gamma induced parthenogenesis from hybrid Albena. Two co-dominant loci were characterized in the progeny of the analyzed diploid plants. Fertility restorer line 937 R was used as a pollen source. Pollen was irradiated with

gamma rays at dose 700 Gy. In both investigated loci, the allele specific for the pollen source was not observed in the analyzed lines, which is evidence that the pollen did not participate with its own genetic material in the formation and development processes of these plants. In parallel, SSR analysis showed that the progeny of the investigated plants was homozygotic in both loci. The data suggest doubled haploid origin of the diploid plants from hybrid Albena developed as a result from the use of irradiated pollen in combination with embryo culture.

Ribeiro *et al*. (2018) identified maize haploid plants and compared the efficiency in identification of maize haploid plants using the R1-nj morphological marker, plant vigor, flow cytometry, chromosome counting, and microsatellite molecular markers under tropical conditions. Molecular markers and flow cytometry are more efficient in classifying plant ploidy level. Seeds obtained in the crosses involving the GNZ9501 commercial hybrid were evaluated for the haploid induction rate (HIR) according to the R1-nj marker and were considered as putative haploids or putative diploids. Subsequently, all putative haploid seeds were sown in pots in the greenhouse to be evaluated and confirmed as haploids. After germination, plants were evaluated considering plant vigor, amount of DNA according to flow cytometry, chromosome number, and band profile with the use of SSR molecular markers. Putative haploids and putative diploids that were confirmed to be true were categorized as true positives (TP) and true negatives (TN). Subsequently, the TPs were subjected to chromosomal duplication. Putative haploids according to the R1-nj were subjected to molecular marker analysis using microsatellite markers (SSR). The polymorphic primer allowed differentiation of homozygous, haploid, and diploid individuals. The gymnogenetic inheritance of the haploid inducer progenies could be confirmed through molecular analyses because the haploids, due to their homozygous nature, showed only one band in the gel, which originated from the hybrid genitor used as the female parent. Plants identified as diploids by flow cytometry also showed two bands using SSR. There were 65 true positives representing 0.46% HIR or 25% of the total putative haploids characterized by the R1-nj marker.

2.5Flow Cytometry Technique for Ploidy Analysis

Flow-cytometry (FCM) would appear to offer advantages in plant breeding and might be used especially are very rapid and easy marker for ploidy manipulation such as polyploidisation by colchicine (Costich *et al*., 1993).

Couto *et al*. (2012) identified haploids by flow cytometry and correlated the nuclear DNA content to the morphological and morphometric traits of the seeds that gave rise to them. In addition, molecular markers were used to confirm the androgenetic nature of the haploid. The seeds obtained were derived from the cross between the inbred line W23 and the commercial hybrid P30F90. Among these seeds, a group was selected, putative haploids, whose embryo was white and the pericarp purplish. This group, consisting of 330 seeds, was characterized based on seed morphology, seed morphometry and nuclear DNA content. Flow cytometry analyses identified four haploids, and all of them were small size plants and had brittle leaves. The weight, length, thickness and width of the haploid seeds were very variable indicating that morphometric traits do not constitute reliable data for visual selection of haploid seeds. Based on results, the inbred line W23 induced haploid maize even under tropical conditions. Microsatellite molecular markers (SSR) proved to be efficient, confirming the androgenetic trait of the haploids.

Ghanavati and Nematpajooh (2012) determined ploidy levels in annual species of *Onobrychis* genus by flow cytometry. Three middle leaves of greenhouse grown plants were randomly selected for chloroplast counts in 10 pairs of stomata guard cells of the lower surface of leaf. For determining ploidy levels, the usual method was counting chromosomes which determined in the metaphase cells of root tip meristems with having minimum of 10 metaphase plate mitosis for each species. All of the samples were also studied, to determine ploidy levels using flow cytometry, and to find the mode and ratio for each of them. The results indicated that numbers of chloroplast stomata guard cells and flow cytometry in the tetraploid samples were approximately twice the numbers in the diploid samples. Ploidy levels were highly correlated ($r = 0.97$) with mean numbers of chloroplasts in stomata guard cells, and the ploidy levels were highly correlated ($r = 0.93$) with modes of the flow cytometry. The mean numbers of chloroplasts in stomata guard cells were highly correlated ($r = 0.95$) with modes of flow cytometry in all the studied populations. Therefore, these observations determine that counting stomata guard cells in

chloroplasts and flow cytometry can be recommended as a fast and simple method for determining ploidy levels in annual species of *Onobrychis* genus.

Hooghvorst *et al*. (2018) in order to improve the anther culture protocol, applied three growth regulator combinations and four colchicine treatments (150, 150, 300 and 300 mg/l) to microspores of two japonica rice genotypes (NRVC980385 and H28) in induction medium. In addition, a post anther culture procedure using colchicine or oryzalin was tested to induce double haploid plantlets from haploid plantlets. A cold pre-treatment of microspores for 9 days at 10°C increased callus induction 50 fold in the NRCV980385 genotype. For both genotypes, 2 mg/l 2, 4-D and 1 mg/1 kinetin on colchicine free induction medium gave the best culture responses. The culture ability of both genotypes changed on colchicine supplemented induction media. A high genotype dependency was recorded for callus induction, callus regenerating green plantlets and regeneration of green double haploid plantlets. Colchicine at 300 mg/1 for 48 h enhanced callus induction 100-fold in H28. Colchicine supplemented media clearly improved green double haploid plantlet regeneration. The post-anther culture treatment of haploid plantlets at 500 mg/1 of colchicine permitted fertile double haploid plantlets to be generated. Finally, an enhanced medium throughput flow cytometry protocol for rice was tested to analyse all the plantlets from anther and post anther culture. Plantlets showing only double haploid ploidy were observed at the lower antimitotic concentrations (250 mg/1 of colchicine and1.25 mg/1 of oryzalin). Within the plantlets that changed his chromosome content, mixiploids, including double haploid ploidy, were the majority.

MATERIAL AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present study entitled **"Analysis of colchicine effect on anther culture of Sunflower (*Helianthus annuus* L.)"** was carried out at the Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur (Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani) during the year 2017-2019. The details of laboratory procedure followed during this research work are described in this chapter.

3.1 Experimental Materials

3.1.1 Plant material

The experimental material included in present study consists of sunflower genotype SS-2038. The detail of characteristic features and the nature of sunflower genotype is given in Table no. 1.

Table no. 1: Sunflower Genotype used for Anther Culture.

Sr. no.	Name of the genotype	Test weight	Volume weight	Oil%
1	SS-2038	7.00	35.18	35-40

3.2 Experimental Methods

3.2.1 Preparation of Stock Solutions of Media

The media used in the present study was MS medium (Murashige and Skoog, 1962). The composition of the media is given in the Table No.2. The stock solutions of major and minor nutrients, vitamins and hormones *viz*., NAA, BAP (1mg/ml) were prepared, properly labelled and stored at 4^{o}C.

Table No. 2: Chemical Composition of MS Media

Constituent	Concentration of stock solution (mg/l)	Volume of stock per litre of medium (ml)
Stock Solution I		
NH_4NO_3	33000	**50**
KNO_3	38000	
$CaCl_2.2H_2O$	8800	
$MgSO_4.7H_2O$	7400	
KH_2PO_4	3400	
Stock Solution II		
KI	166	**5**
H_3BO_3	1240	
$MnSO_4.4H_2O$	4460	
$ZnSO_4.7H_2O$	1720	
$Na_2MoO_4.2H_2O$	50	
$CuSO_4.5H_2O$	5	
$COCl_2.6H_2O$	5	
Stock Solution III		
$FeSO_4.7H_2O$	5560	**5**
$Na_2EDTA.2H_2O$	7460	
Stock Solution IV		
Myo-Inositol	20000	**5**
Nicotinic acid	100	
PyridoxinHCl	100	
ThiaminHCl	100	
Glycine	400	
Carbon Source		
Sucrose		30gm/litre
Agar agar		8g/litre

Note: Dissolve ferrous sulphate ($FeSO_4.7H_2O$) and Disodium EDTA ($Na_2EDTA.2H_2O$) separately. Dissolve Disodium EDTA into warm sterile water and then add ferrous sulphate to the same solution with continuous stirring.

3.2.2 Isolation of Explant

Capitula was harvested prior to opening of ray florets when most of the microspores was at the mid to late uninucleate stage of development and the optimal pollen stage was determined from anther morphology.

3.2.3 Preparation of Explant

The flower buds of 1.5 to 2.0 cm in diameter with cream colored anthers possessing mostly uninucleate microspores were used. The flower buds were surface sterilized by immersing in 70% ethanol and 0.1% Mercuric chloride for 2 minute each, then washed with double distilled water for 3 times to remove traces of chemicals and dried on blotting paper.

3.2.4 Inoculation of Anthers

The anthers which were at the mid to late uniuncleate stage were excised. All extraneous materials like papillae were removed. The white conical ovary portion at the bottom and the corolla joint at the top were cut with the help of a sharp scalpel to squeeze out the bundle of syngenesious anthers. Anthers were dissected from disc florets and inoculated onto solid media. In each bottle four anthers were inoculated, as the development of disc florets were centripetal and anthers of different florets were likely to contain microspores at different stages of development. Whenever possible, anthers from different florets were randomly distributed among bottles of various media compositions to minimise variation in response to microspore development. The excised anthers were treated directly with different concentrations of colchicine, and indirectly into the media.

Immediately after inoculation, the anthers were incubated in the dark at 28^{o}C until macroscopic globular structures were seen.

3.2.5 Colchicine Treatment

3.2.5.1 Preparation of Colchicine Stock Solution

A stock solution of colchicine was prepared using different concentration of colchicine. 0.1, 0.5, 1, 1.5 and 2g colchicine were dissolved in 100 ml distilled water, sterilized using syringe filter (0.45 µm) and stored at 4^{o}C (Leblance *et al.,* 1995).

3.2.5.2 Colchicine Treatment to Anthers

Anther culture was attempted in SS-2038 sunflower genotype. Anthers were treated with 0.4% colchicines at various time intervals such as 0, 2, 4, 6, 8, and 10 hrs (Burun and Emiroglu, 2008). After the standardization of time, anthers were treated with different concentration of colchicine as described in Table no. 3.

Table No. 3: Colchicine Treatment to Sunflower Anther.

Treatment No.	Concentration Tested (%)
1	0.1
2	0.5
3	1
4	1.5
5	2

3.2.6 Callus Induction

The colchicine treated and untreated anthers were inoculated on MS media supplemented with 2 mg/l NAA and 1 mg/l BAP for callus induction (Patil *et al.*, 2018). The bottles were incubated at 28^{o}C in dark condition. After inoculation, the callus induction and development was carefully observed in the inoculated anthers.

3.2.7 Subculture of Calli

After two weeks of inoculation browning was observed and hence it was necessary to subculture. After 28 days of inoculation, fresh calli were subcultured on the same media containing the same level of growth hormone.

3.2.8 Colchicine Treatment to the Callus in Medium

Four levels of colchicine treatment, 0.5%, 1.0%, 1.5% and 2% were applied to callus in indirect treatment. Embryogenic Calli were divided into small pieces (0.5-1 cm) and the pieces were placed in tubes containing 15ml agar solidified MS medium supplemented with colchicine. After 72 hours of incubation at 28^{o}C in the dark, the calli were removed and subcultured twice (at 1week and 2 week intervals) on MS media supplemented with 0.5mg/l BAP (Table no. 4).

Table No. 4: Concentration of Colchicine in MS Medium

Treatment No.	Concentration Tested (%)
1	0.5
2	1
3	1.5
4	2

3.2.9 Statistical Analysis

The data were analysed in completely randomized factorial design (CRD) to find out the significance of different colchicine treatments, hormone combination and their interactions with three replications. All the data were recorded in percentage.

3.2.9.1 Callus Induction Frequency

$$\text{Frequency of callus induction} = \frac{\text{No. of explants induced callus}}{\text{Total No. of explants inoculated}} \times 100$$

3.2.9.2 Abbott's Formula

$$\text{Corrected per cent mortality} = \frac{T - C}{100 - C} \times 100$$

Where,

T - Per cent mortality in treatment

C - Per cent mortality in control

Then the data were subjected to probit analysis by Finney (1971) using computer software Polo Plus 1.0 (LeOra software) to obtain the value of median lethal time (LT_{50}) and lethal concentration (LC_{50}) for each treatment.

3.3 Haploid Confirmation by Using Molecular Markers

3.3.1 DNA Extraction

Reagents and Solutions for DNA Isolation

1) **Extraction Buffer**

To prepare extraction buffer stock of following solutions were prepared and then subsequent extraction buffer was prepared.

1. **CTAB** (10%): 10gm CTAB was dissolved in sterile double distilled water and volume made to 100ml.
2. **NaCl** (5M): For preparation of 5M stock solution, 23.4gm Sodium chloride was dissolved in sterile double distilled water and final volume made up to 100ml.
3. **Tris-HCl** (1M) (pH 8.0): For preparation of 1M stock, 15.76gm Tris-HCl was dissolved in sterile doubled distilled water and pH was adjusted at 8.0 by adding 0.1N HCl and final volume made to 100ml.
4. **EDTA** (0.5M) (pH8.0): EDTA (0.5M) was prepared by adding14.61 gm of EDTA in double distilled water and pH was adjusted to 8.0 by adding pellets of NaOH and final volume made up to 100ml.

After preparation of stock, all the stock solutions except CTAB were autoclaved. Extraction buffer was prepared by taking following composition from stock solutions.

Table No. 5: Composition of 2% Extraction Buffer

Sr. No.	Components	Stock	Quantity	Final Concentration
1	CTAB	10%	20ml	2%
2	NaCl	5.0 M	28ml	1.4M
3	EDTA (pH 8.0)	0.5 M	4ml	20mM
4	Tris-HCl (pH 8.0)	1.0 M	10ml	100mM
5	β-Mercaptoethanol	-	200µl	0.2%
6	Double distilled water	-	31 ml	-
	Total volume		**100 ml**	

2. Chloroform: **Isoamyl alcohol:** mixture was prepared in the ratio of 24:1 (v/v)

3. TE Buffer (pH 8.0)

10mM Tris HCl	:	0.121 gm
1mM EDTA	:	0.03 gm
Double distilled water	:	90 ml
Final volume	:	100 ml

4. RNase A stock (10 mg/ml)

1M Tris HCl (pH)	:	10 µl
4M NaCl	:	3 µl
RNase A	:	10 mg

Final volume was made up to 1ml with double distilled water and heated at 100^{o}C for 15 min in water bath. The solution was allowed to cool slowly at room temperature and stored at 4^{o}C.

6. Ethanol (70%)

Absolute alcohol	:	70 ml
Distilled water	:	30 ml

3.3.2 Extraction of Genomic DNA

1. About 0.5 gm of young fresh leaf tissue/callus was used for DNA extraction.
2. The leaf tissue was ground in 2ml of preheated 2% CTAB extraction buffer.
3. The mixture was transferred to the micro centrifuge tube, mixed well and incubated at 65^{o}C for 45 minutes in a hot water bath.
4. After incubation, the mixture was subjected to centrifugation at 10,000 rpm for 10 min at 4^{o}C.
5. The upper aqueous phase was collected in fresh micro centrifuge tube and equal volume of Chloroform: Isoamyl alcohol was added and mixed well by inverting the tubes and again centrifuged at 10,000 rpm for 10 minutes at 4^{o}C.
6. The colourless aqueous supernatant was collected from the centrifuged tubes.
7. To the collected supernatant, equal volume of chilled iso-propanol was added, mixed well and the DNA was allowed to precipitate overnight at -20^{o}C.
8. The DNA was pelleted by centrifugation at 10,000 rpm for 10min at 4^{o}C. The collected pellet was washed with 70% alcohol to remove any impurities remaining in the pellet.
9. The collected pellet was air dried and dissolved in 50µl TE buffer.
10. After proper dissolving, DNase free RNase was added to remove RNA by incubating at 37^{o}C for 30 to 45 minutes.
11. The DNA was precipitated in the aqueous phase by adding 2 volumes of absolute ethanol, kept at -20^{o}C for at least 30 minutes and pelleted at 10,000 rpm.

12. The DNA pellet was washed twice with 70% ethanol, air dried and dissolved in required quantity of TE buffer. The isolated DNA sample was stored at -20^{o}C for long term preservation.

3.3.3 Agarose Gel Electrophoresis

The qualitative analysis of genomic DNA was performed by Agarose gel electrophoresis. Agarose gel (0.8%) was prepared by dissolving 0.8 g of Agarose in 100 ml 1X TAE buffer. After cooling the solution to about 45^{o}C, 5µl Ethidium bromide (10mg/ml) was added. 5µl of DNA was mixed with 1µl of 6X gel loading dye and loaded. The isolated DNA samples were resolved on 0.8% Agarose gel at 5V/cm for 1 hour.

3.3.4 Reagents for Agarose Gel Electrophoresis

1. 50X TAE Buffer

Tris base	: 242 g
Glacial acetic acid	: 57.1 ml
EDTA	: 37.2 g
Distilled water	: 1000 ml

2. 6X Gel Loading Dye

3. Ethidium Bromide (10 mg/ml)

Ten mg Ethidium bromide was dissolved in 10 ml distilled water. Tube wrapped in aluminium foil and stored at 4^{o}C.

3.3.5 Determination of Quantity and Quality of Isolated DNA

The quantity and quality of isolated DNA was determined by using spectrophotometer. The instrument was set to a blank with 50µl distilled water. 1µl DNA sample was added to 49 µl distilled water in a cuvette and quantity and quality was determined by recording the absorbance ratio of A_{260}/A_{280}. The ratio higher than 2.0 indicate the impurity of protein and less than 1.8 indicate RNA contamination in sample. The amount of DNA (µg/ml) was calculated by the following formula,

$$\text{DNA (µg/ml)} = \frac{A_{260} \text{ X } 50 \text{ X Dilution factor}}{1000}$$

3.3.6 Dilution of DNA Sample

A part of DNA sample was diluted with appropriate quantity of sterile nuclease free water to yield a working concentration of 50 ng/µl for SSR markers analysis. These DNA samples were stored at -20^{o}C for PCR amplification.

3.4 SSR Analysis

3.4.1 Selection of Primers

PCR reaction was performed with DNA preparations by using 28 pairs of SSR markers (Eurofins Genomics, India). The SSR primers specific for high oil content, high oleic acid content, test weight, high yield traits depending on the sunflower genotype used in present study were selected for analysis of homozygosity in anther culture. The details of SSR primers are given in Table no. 6.

Table no. 6: List of SSR Primers Used for Haploid Confirmation of Sunflower Genotype.

Sr. No.	Primers	Sequence (5' to 3')	No. of bases
1	ORS-5	F- AACATCTGGAGCAGCAAATTCAG	23
		R- CTGCTGCCCACCATACTG	18
2	ORS-662	F- CGGGTTGGATATGGAGTCAA	20
		R- CCTTTACAAACGAAGCACAATTC	23
3	ORS-536	F- GAAATAGGAGGGGATCTTACCG	22
		R- GCGGAGAGAAAGACGAAGAG	20
4	ORS-243	F- GGGATGACGTGCGTTTGG	18
		R- ACCACCATTTCTACCGTTTCTC	22
5	ORS-552	F- CCATCCCTTCCCTCTCTTTC	20
		R- GTGGCTGGAATCTCATCACC	20
6	ORS-388	F- AACCTAAACCCGTCCCAAAC	20
		R- TGTTGTCCTAACTGGGCTCAC	21
7	ORS-578	F- CTCTCAATCCCTAAAGTCCCCT	22
		R- TGGTGGATGTGGTTGTTGAT	20
8	ORS-811	F- CCTTCTCCTCAATCTTTGGCTA	22
		R- AGGAATGAAATGGGTGTGTGT	21
9	ORS-337	F- TTGGTTCATTCATCCTTGGTC	21
		R- GGGTTGGTGGTTAATTCGTC	20
10	ORS-780	F- TGATTACAACCCTAATTCGCATAC	24
		R- GATACTGGTGGGACAGATGTTG	22
11	ORS-1068	F- AATTTGTCGACGGTGACGATAG	22
		R- TTTTGTCATTTCATTACCCAAGG	23

12	ORS-149	F- GCTCTCTATCTCCCTTGACTCG	22
		R- TGCTCTAAGATCTCAGGCGTGC	22
13	ORS-58	F- TGTACCAAGGGTCGTTGTCA	20
		R- CGACCCCGAGTTTTGTTG	18
14	ORS-488	F- CCCATTCACTCCTGTTTCCA	20
		R- CTCCGGTGAGGATTTGGATT	20
15	ORS-598	F- CCAAATGTGAGGTGGGAGAA	20
		R- ATAGTCCCTGACGTGGATGG	20
16	ORS-1159	F- TTTCGTGATGGTGATTGATGATT	23
		R- CAGCAACTCTGACCGTTTCATTA	23
17	ORS-959	F- CCGCTAAGTATAAACCGCCTATT	23
		R- CGTCCTCTTCGCATCAATCTTAT	23
18	ORS-807	F- CCGATATTTTGACCGATATTTTGC	24
		R- TCTCACCCTTCATCTCCTTCC	21
19	ORS-484	F- CCCTGGAGTGTATGTCCGTTA	21
		R- ATCCGTCTGCTGCCTAATCC	20
20	ORS-160	F- TCCCTTCCTTTCATCGTCTGCT	22
		R- TGGCAATTTGCCAAGGACC	19
21	ORS-16	F- GAGGAAATAAATCTCCGATTCA	22
		R- GCAAGGACTGCAATTTAGGG	20
22	ORS-880	F- AAGTAGCTTTGCTTTCCTTCGTC	23
		R- CGAAACGCGGATTATTGTCTTAT	23
23	ORS-928	F- CATGGTTATTTTGGTTTGGGTTT	23
		R- GCTATTATCATGTCCTTGTCCTTTT	25
24	ORS-920	F- CGTTGGACGAAGAACTTGATTT	22
		R- ACTTCCGTTTGTTCCGAGCTT	21
25	ORS-154	F- GCACCTTTGGTGAGGAGATA	20
		R- TGCATCAGTAGCTATTGTCTAT	22
26	ORS-1265	F- GGGTTTAGCAAATAATAGGCACA	23
		R- ACCCTTGGAGTTTAGGGATCA	21
27	ORS-423	F- TCATATGGAGGGATCTGTTGG	21
		R- AAGCAACCATAATGCATCAGAA	22
28	ORS-323	F- CGGGAAACTAGGATCAGAGG	20
		R- GCCGGAGGATTAGAGGAGTT	20

3.4.2 Components Used for SSR Reaction Mixture

A reaction mixture of a final volume of 25µl was prepared by using different concentrations of genomic DNA, dNTPs, SSR primers, $MgCl_2$ and *Taq* DNA polymerase to obtain an optimum reaction mixture for complete amplification of the genomic DNA is given in Table No. 7.

Table no. 7: PCR Components, their Stock Solutions, Final Concentration and Volume in One Reaction.

Sr. No.	Components	Stock solutions	Final concentration	Volume for one reaction (µl)
1	10X PCR buffer	10X	1X	2.5
2	DNTPs	10 mM	0.2 mM	0.25
3	*Taq* DNA polymerase	3 U/µl	1.0 U/µl	0.33
4	$MgCl_2$	25 Mm	1.7 mM	1.70
5	Forward Primer	100 pmol/µl	25 pmol	0.25
6	Reverse Primer	100 pmol/µl	25 pmol	0.25
7	Genomic DNA	50 ng/µl	50 ng	1.0
8	Nuclease free water	-	-	18.75
			Total volume	**25.00 µl**

3.4.3 Method

PCR reaction mixture was prepared with the above mentioned components and divided equally (each of 24µl) into PCR tubes. One µl DNA was added by changing tips every time to avoid contamination. PCR tubes were then placed in thermal cycler (SensoQuest Labcycler, Germany) for amplification of genomic DNA.

3.4.4 Optimization of PCR Condition for SSR Analysis

The optimization of PCR condition was done by changing annealing temperatures of primers. Different annealing temperatures (T_m±4°C) were tried depending upon melting temperature (T_m) of primers with the help of gradient PCR conditions. Number of bands and amplification frequency at different temperature were taken into consideration for the selection of annealing temperature in SSR-PCR

Table no. 8: Cyclic Parameters of Thermal Cycler for SSR Analysis.

Step	Temp (°C)	Time	Cycles	Function
1	94	5 min	1	Initial denaturation
2	94	30 sec	40	Denaturation
3	T* (Opt)	50 sec		Annealing
4	72	1.00 min		Extension
5	72	10 min	1	Final extension
6	4	∞	1	Hold

T*(opt) - Annealing temperature optimized for each primer

3.4.5 Resolution of Amplified Product

The amplified products were resolved on 1.5% Agarose gel in 1X TAE buffer at 100V for 2 hours. After electrophoresis, the gel was analyzed for observation of banding pattern and photographed in a Gel Documentation System (Alpha-Innotech, USA).

3.4.6 Data Scoring and Analysis

The amplified products generated from SSR PCR reaction were resolved on Agarose gel. The amplicons which distinguishes haploid and donor parents lines were observed and scored. The images were scored for presence (1), absence (0), and missing and doubtful case scored as 9. Band size was determined by using software AlphaEaseFC 4.0 with reference to100bp DNA ladder (Banglore Genei, India) respectively for SSR products.

Data analysis was performed using NTSYS-pc (Numerical Taxonomy System, Version 2.02i) (Rohlf, 1998). The SIMQUAL programme was used to calculate the Jaccard's coefficient. Dendrogram was constructed using Unweighted Paired Group Method for Arithmetic Mean (UPGMA) based on Jaccard's similarity coefficient.

3.5 Sample Preparation for Flow Cytometry Analysis

The ploidy level of calli derived from colchicines treated anthers and colchicine treated calli at various concentration *viz*., 0.1%, 0.5%, 1%, 1.5% and 2% concentrations were detected using a flow cytometer. Flow cytometry protocol involved directly staining the DNA content for cell cycle analysis in a 5µl solution of Propidium iodide in each DNA sample and incubated for 10 -15 min. at room temperature. Volume in each sample made up to 1ml by adding wash solution. Later the each sample was filtered through 30µm nylon mesh and the samples were analysed and results documented. The fluorescence measured is correlated with the DNA content of the stained nuclei. For each peak detected, an index can be calculated by using flow cytometry results, therefore the comparison of the index gives the relationship between the DNA contents in the stained nuclei that produces those peaks (Leblanc *et al*., 1995).

RESULTS AND DISCUSSION

CHAPTER-IV
RESULTS AND DISCUSSION

It has long been realized that anther culture could be of great value for studies of breeding cycle and fundamental genetics of higher plants because they are affected by problems of dominance and segregation (Kimber and Riley, 1963; Melchers, 1972). Development of homozygous diploid pure lines or dihaploids is achieved through anther culture in a single step which is formidable exercise in conventional breeding. In depth study of interplay of various factors controlling or regulating the process of anther callusing through *in vitro* was made in the present investigation in sunflower.

In vitro androgenesis *via* anther culture is most preferred techniques for obtaining haploids plants. The production of haploids provides a particularly attractive biotechnological tool to produce homozygous plants which help the plants breeder to develop new variety within a short span of time. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols. In anther culture, some time the anther walls, which is diploid, can regenerated into a diploid plant. These diploids are needs to be detected and rejected before development of DHs. To differentiate between diploid and DH plants morphological characters and molecular marker can be used. SSR marker are used to differentiate DHs from diploid were selected.

4.1 Anther Culture Development in Sunflower

Anther culture was attempted in SS-2038 sunflower genotype. Buds containing anthers with pollen grains at mid to late uninucleate stage were surface sterilized and treated with colchicine and cultured on MS medium (Plate No.1) supplemented with growth regulators. The experimental results from the present investigations pertaining to anther culture, cultural factors and production of haploids and/or double haploid are presented.

The experiment was conducted with five different colchicine treatments. Each treatment consisted of three replications RI, RII and RIII has been checked for sunflower genotype for best callus regeneration. Data were collected on anther survival percentage

and days to callus induction. The observations were statistically analysed using Completely Randomized factorial Design (CRD).

4.1.1 Effect of Colchicine Treatments on Sunflower Anther

4.1.1.1 Treatment of Anthers before culture

Anther culture response after treatment of anther with colchicine decreased with both the increase in the colchicine concentration and soaking duration. When the anthers were treated with 0.4% colchicine for 2, 4, 6, 8 and 10 hrs the response were 98.61, 73.61, 15.2, 13.75, 8.82% respectively, while the response of untreated control was 100% (Table no.9).

The colchicine treated anthers were black in colour, at long durations (10 hrs). The anthers become more blackish in colour and the callus which is reproduced from anther was also black in colour, compact in nature and small in size, whereas control anthers were green in colour and the callus which was reproduced from anther was also green in colour, fragile and larger in size.

When anthers were treated with colchicine before culture for 0 to 10 hrs duration, the rate of callus producing anthers tended to decrease as the duration increased. In the control group 100% of the anthers produce calli, but 2 h treatment reduced the anther culture response to 98.61; treatments for longer durations resulted in lower rates, ranging between 73.61% to 8.82% (Table no.9). Takashima *et al*. (1995) also reported decrease in culture response with the increase in soaking duration and obtained the highest diploidization rate (66.7%) in the 0.4% colchicine treatment for 8 hours for tobacco anther. Burun *et al.* (2007) also reported a decrease in culture response with the increase in soaking duration for tobacco anther and resulted in highest diploidization rate (29.7%) in the 0.4% colchicine treatment for 6 hours.

The Lethal time of this treatment is LT50=4.8303 and LT95=9.717 (Table no.10). Therefore, for further experimentation sunflower anther were treated with various concentrations of colchicine for 4.8 hours duration.

Table No.9: Culture response of anthers treated with 0.4% colchicine for various soaking durations.

Treatment	No. of inoculated anthers			No. of survived Anthers and produced callus			Average	Percentage (%)
	RI	RII	RIII	RI	RII	RIII		
Control	24	24	24	24	24	24	24	100
2hrs	24	24	24	24	24	23	23.66	98.61
4hrs	24	24	24	18	17	18	17.66	73.61
6hrs	24	24	24	5	4	2	3.66	15.2
8hrs	24	24	24	4	4	2	3.33	13.75
10hrs	24	24	24	2	4	0	2.00	8.82
Coefficient of Variation = 9.513 CD(0.01) = 2.940 CD(0.05) = 2.097								

Table No. 10: Effect of soaking time on survival of anthers treated with 0.4% Colchicine.

Treatment	No. of inoculated Anther	No. of killed Anther	% Mortality	Corrected Mortality
Control	72	0	0	0
2hrs	72	1	1.39	1.4
4hrs	72	19	26.39	26.4
6hrs	72	61	84.72	84.7
8hrs	72	62	86.11	86.1
10hrs	72	66	91.67	91.7
LT 50= 4.8303 LT 95= 9.717				

Table No. 11: Culture response of anthers at various concentrations of colchicine.

Treatment (%)	No. of inoculated anthers			No. of survived anthers			Average	Survival Percentage	Callus Induction Frequency
	RI	RII	RIII	RI	RII	RIII			
Control	20	20	20	20	18	15	17.667	95.0	88.33
0.1	20	20	20	6	4	4	4.667	25.0	23.33
0.5	20	20	20	5	4	3	4.000	22.5	20
1	20	20	20	4	3	3	3.333	17.5	16.66
1.5	20	20	20	4	1	2	2.333	12.5	11.66
2	20	20	20	3	0	2	1.667	10.0	8.33
Coefficient of Variation = 26.897 CD(0.01) = 3.765 CD(0.05) = 2.685									

Table No. 12: Effect of different concentrations of colchicine on survival of anthers.

Treatment (%)	No. of inoculated Anther	No. of killed Anther	% Mortality	Corrected Mortality
Control	60	7	11.67	0
0.1	60	46	76.67	73.6
0.5	60	48	80.00	77.4
1	60	50	83.33	81.1
1.5	60	53	88.33	86.8
2	60	55	91.67	90.6
LC 50= 0.0052 LC 95= 29.524				

Table No. 13: Days required for callus induction in different concentrations of colchicine.

Treatment (%)	RI	RII	RIII	Total	Mean
Control	11	10	10	31	10.33
0.1	10	12	11	33	11.0
0.5	11	10	11	32	10.66
1	13	12	12	37	12.33
1.5	12	13	12	37	12.33
2	14	13	13	40	13.33
SE	0.479				
CD@	1.173				

CHAPTER-IV
RESULTS AND DISCUSSION

It has long been realized that anther culture could be of great value for studies of breeding cycle and fundamental genetics of higher plants because they are affected by problems of dominance and segregation (Kimber and Riley, 1963; Melchers, 1972). Development of homozygous diploid pure lines or dihaploids is achieved through anther culture in a single step which is formidable exercise in conventional breeding. In depth study of interplay of various factors controlling or regulating the process of anther callusing through *in vitro* was made in the present investigation in sunflower.

In vitro androgenesis *via* anther culture is most preferred techniques for obtaining haploids plants. The production of haploids provides a particularly attractive biotechnological tool to produce homozygous plants which help the plants breeder to develop new variety within a short span of time. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols. In anther culture, some time the anther walls, which is diploid, can regenerated into a diploid plant. These diploids are needs to be detected and rejected before development of DHs. To differentiate between diploid and DH plants morphological characters and molecular marker can be used. SSR marker are used to differentiate DHs from diploid were selected.

4.1 Anther Culture Development in Sunflower

Anther culture was attempted in SS-2038 sunflower genotype. Buds containing anthers with pollen grains at mid to late uninucleate stage were surface sterilized and treated with colchicine and cultured on MS medium (Plate No.1) supplemented with growth regulators. The experimental results from the present investigations pertaining to anther culture, cultural factors and production of haploids and/or double haploid are presented.

The experiment was conducted with five different colchicine treatments. Each treatment consisted of three replications RI, RII and RIII has been checked for sunflower genotype for best callus regeneration. Data were collected on anther survival percentage

and days to callus induction. The observations were statistically analysed using Completely Randomized factorial Design (CRD).

4.1.1 Effect of Colchicine Treatments on Sunflower Anther

4.1.1.1 Treatment of Anthers before culture

Anther culture response after treatment of anther with colchicine decreased with both the increase in the colchicine concentration and soaking duration. When the anthers were treated with 0.4% colchicine for 2, 4, 6, 8 and 10 hrs the response were 98.61, 73.61, 15.2, 13.75, 8.82% respectively, while the response of untreated control was 100% (Table no.9).

The colchicine treated anthers were black in colour, at long durations (10 hrs). The anthers become more blackish in colour and the callus which is reproduced from anther was also black in colour, compact in nature and small in size, whereas control anthers were green in colour and the callus which was reproduced from anther was also green in colour, fragile and larger in size.

When anthers were treated with colchicine before culture for 0 to 10 hrs duration, the rate of callus producing anthers tended to decrease as the duration increased. In the control group 100% of the anthers produce calli, but 2 h treatment reduced the anther culture response to 98.61; treatments for longer durations resulted in lower rates, ranging between 73.61% to 8.82% (Table no.9). Takashima *et al*. (1995) also reported decrease in culture response with the increase in soaking duration and obtained the highest diploidization rate (66.7%) in the 0.4% colchicine treatment for 8 hours for tobacco anther. Burun *et al.* (2007) also reported a decrease in culture response with the increase in soaking duration for tobacco anther and resulted in highest diploidization rate (29.7%) in the 0.4% colchicine treatment for 6 hours.

The Lethal time of this treatment is LT50=4.8303 and LT95=9.717 (Table no.10). Therefore, for further experimentation sunflower anther were treated with various concentrations of colchicine for 4.8 hours duration.

Table No.9: Culture response of anthers treated with 0.4% colchicine for various soaking durations.

Treatment	No. of inoculated anthers			No. of survived Anthers and produced callus			Average	Percentage (%)
	RI	RII	RIII	RI	RII	RIII		
Control	24	24	24	24	24	24	24	100
2hrs	24	24	24	24	24	23	23.66	98.61
4hrs	24	24	24	18	17	18	17.66	73.61
6hrs	24	24	24	5	4	2	3.66	15.2
8hrs	24	24	24	4	4	2	3.33	13.75
10hrs	24	24	24	2	4	0	2.00	8.82
Coefficient of Variation = 9.513 CD(0.01) = 2.940 CD(0.05) = 2.097								

Table No. 10: Effect of soaking time on survival of anthers treated with 0.4% Colchicine.

Treatment	No. of inoculated Anther	No. of killed Anther	% Mortality	Corrected Mortality
Control	72	0	0	0
2hrs	72	1	1.39	1.4
4hrs	72	19	26.39	26.4
6hrs	72	61	84.72	84.7
8hrs	72	62	86.11	86.1
10hrs	72	66	91.67	91.7
LT 50= 4.8303 LT 95= 9.717				

Table No. 11: Culture response of anthers at various concentrations of colchicine.

Treatment (%)	No. of inoculated anthers			No. of survived anthers			Average	Survival Percentage	Callus Induction Frequency
	RI	RII	RIII	RI	RII	RIII			
Control	20	20	20	20	18	15	17.667	95.0	88.33
0.1	20	20	20	6	4	4	4.667	25.0	23.33
0.5	20	20	20	5	4	3	4.000	22.5	20
1	20	20	20	4	3	3	3.333	17.5	16.66
1.5	20	20	20	4	1	2	2.333	12.5	11.66
2	20	20	20	3	0	2	1.667	10.0	8.33
Coefficient of Variation = 26.897 CD(0.01) = 3.765 CD(0.05) = 2.685									

Table No. 12: Effect of different concentrations of colchicine on survival of anthers.

Treatment (%)	No. of inoculated Anther	No. of killed Anther	% Mortality	Corrected Mortality
Control	60	7	11.67	0
0.1	60	46	76.67	73.6
0.5	60	48	80.00	77.4
1	60	50	83.33	81.1
1.5	60	53	88.33	86.8
2	60	55	91.67	90.6
LC 50= 0.0052 LC 95= 29.524				

Table No. 13: Days required for callus induction in different concentrations of colchicine.

Treatment (%)	RI	RII	RIII	Total	Mean
Control	11	10	10	31	10.33
0.1	10	12	11	33	11.0
0.5	11	10	11	32	10.66
1	13	12	12	37	12.33
1.5	12	13	12	37	12.33
2	14	13	13	40	13.33
SE	0.479				
CD@	1.173				

Plate No. 1: Anthers placed on MS medium

Plate No. 2: Swelling of anthers

Plate No. 3: Callus induction (Control)

Plate No. 4: Callus induction from anthers treated with 0.1% colchicine

Plate No. 5: Callus induction from anthers treated with 0.5% colchicine

Plate No. 6: Callus induction from anthers treated with 1% colchicine

Plate No. 7: Callus induction from anthers treated with 1.5% colchicine

Plate No. 8: Callus induction from anthers treated with 2% colchicine

After standardization of time, anthers were treated with different concentrations of colchicine *i.e.*, 0.1, 0.5, 1, 1.5 and 2% for 4.8 hours. The cultured anthers responses in survival percentage were 25, 22.5, 17.5, 12.5 and 10% respectively for these treatments whereas the response of untreated control was 95%. The cultured anthers responses in callus induction frequency were 23.33, 20.00, 16.66, 11.66, and 8.33 respectively for these treatments whereas the response of untreated control was 88.33 (Table no. 11).

The colchicine treated anthers were black in colour, at higher concentrations (2%) the anthers become more blackish in colour and the callus which is reproduced from anther were also black in colour, compact in nature as well as small in size (Plate no. 4, 5, 6, 7. 8). The control anthers were green in colour and the calluses which were reproduced from anther was also green in colour, larger in size and fragile (Plate no. 3).

Similarly Islam (2010) also reported three-four fold decreased embryoids induction on direct treatment of colchicine to isolated microspore of wheat. Direct treatment of isolated microspores with different concentrations of colchicines *viz*., 50, 100, 150 mg/l for 3 d was performed in an attempt to estimate an optimal concentration for the compound during treatment of the microspore. Analysis of variance showed clear difference between the untreated control and average of all treatments, for embryo formation, green plant regeneration and albino plant frequency and observed negative effect on direct treatment of microspores with colchicine the subsequent formation of embryos. Frequencies of green plants regenerated were affected negatively of the treatment while formation of albinos was stimulated.

According to the Abbott's formula, the Lethal concentration of this treatment LC50=0.0052 (Table no.12). The corrected mortality obtained in 0.1% colchicine concentration is 73.6 which is higher than LC50=0.0052 and LC95=29.524. This suggests that the lower concentration of colchicine should be tested for analyzing its effect on the sunflower anther.

The difference in number of days required for callus induction in various treatments of colchicine was also observed (Table no. 13). The anthers treated with 2.0% colchicine concentration required more days (13.33) for callus induction as compared to 0.1% (11) and control (10.33).

4.1.1.2 Colchicine Treatment to Callus Through Medium

The callus was inoculated into colchicine containing medium at different concentrations *viz.*, 0.5, 1, 1.5 and 2% of for 72 hours and then after shifted to colchicine free medium. The culture response of callus after treatment with colchicine through medium decreased with the increase in colchicine concentrations. The response of survival percentage of callus treated with different concentration of colchicine 0.5, 1, 1.5 and 2% were 82.5, 72.5, 62.5 and 47.5% respectively, and the response of untreated control was 95%. The response of callus regeneration percentage of treated calli were 86.66, 76.66, 66.66 and 55% respectively, and the response of untreated control was 96.66% (Table no. 14).

The calli proliferated on the colchicine containing medium were brown/green in colour in 0.5, 1, 1.5%, and in 2% colchicine concentration. In the present study, rate of calli browing increases with increase in the concentration of colchicines in the media (Plate no. 10, 11, 12, 13). In control 45 calli were green and 13 callus were brown in colour. Size of this calli was large and fragile in nature (Plate no. 9). In 0.5% colchicines treated calli contained 24 green and 28 brown callus and in 2% colchicines treated calli contained 18 green and 28 brown calli with small size and compact in nature. This shows that the colchicines had direct effect on the cell and causes necrosis of the cell and death.

However, Pusadkar and Jha (2018) reported positive effect of colchicines on greening calli cultured on colchicines containing media. They made crosses of rice varieties and used S x R cross for colchicine treatment to anther culture derived callus in media supplemented with 100 and 500 mg/l of colchicine for 48 and 72 hours and then shifted to regenerative media. The experiment was performed along with control (without colchicine treatment) in which green callus percentage and overall plant regeneration percentage increased by 1 to 2.5 fold in treated calli compared to control. The treatment containing 100 mg/l of colchicine in regeneration media followed by 48 hrs of incubation has given highest green callusing percentage of 23.13% as control having only 9.16 % of green calli induction in S x R cross. The addition of colchicine had no detrimental effects on the different anther culture efficiency parameters. The highest numbers of green 23% calli were achieved in cross (SX R) while the lowest plant regeneration 3.1% obtained in the cross (SRP). Similar results were reported by Alemanno (1994) by plating rice

Table No. 14: Culture response of callus at various concentrations of colchicine (in medium).

Treatment	No. of callus Inoculated			No. of callus survived			Average	Survival Percentage	Callus regeneration %	No. of callus observed	
	RI	RII	RIII	RI	RII	RIII				Green	Brown
Control	20	20	20	20	18	18	18.667	95	96.66	45	13
0.5%	20	20	20	19	17	16	17.333	82.5	86.66	24	28
1%	20	20	20	17	15	14	15.333	72.5	76.66	18	28
1.5%	20	20	20	15	13	12	13.333	62.5	66.66	15	25
2%	20	20	20	14	10	9	11.000	47.5	55	10	23
Coefficient of Variation = 11.572 CD(0.01) = 4.531 CD(0.05) = 3.186											

Table No. 15: Effect of different concentration of colchicine on survival of callus (in medium)

Treatment	No. of callus inoculated	No. of callus killed	% Mortality	Corrected Mortality
Control	60	2	3.33	0
0.5	60	8	13.33	10.3
1	60	14	23.33	20.7
1.5	60	20	33.33	31.0
2	60	27	45.00	43.1
LC 50= 2.6714 LC 95= 21.9306				

Plate No. 9: Callus regeneration on without colchicine containing MS medium

Plate No. 10: Callus induction by supplying 0.5% colchicine in medium

Plate No. 11: Callus induction by supplying 1% colchicine in medium

Plate No. 12: Callus induction by supplying 1.5% colchicine in medium

Plate No. 13: Callus induction by supplying 0.5% colchicine in medium

anthers on a semisolid induction medium containing 250 or 500 mg/l colchicine for 24 or 48 hrs of incubations followed by transfer to colchicine-free medium that of standard anther culture procedures resulted in overall 1.5 to 2.5 fold increase in doubled haploid green plant productions compared to control anther cultures.

Also Navarro-Alvarez *et al.* (1994) recorded the effect of colchicine added to induction medium for the production of fertile doubled haploid plants after *in-vitro* anther culture in wheat. Anther cultures of the three genotypes (Mv Szigma, Acheloos, Vergina) were treated with 0.03% colchicine for 3 days at the beginning of microspore induction. They do not found significant effect of on anther response and embryoid production of the genotypes examined.

According to the Abbott's formula, the Lethal concentration of this treatment LC50= 2.6714 (Table no.15). The corrected mortality obtained at highest tested colchicine concentration 2.0% is 43.1 which is lower than LC50= 2.6714. This suggests that the higher concentration of colchicine should be tested for analyzing its effect on the sunflower anther in media.

It is obvious from present investigation that calli survival and regeneration is more in colchicines treatment in media as compared to direct colchicne treatment. Similar result recorded by Islam, (2010) in wheat the colchicine applied to the anthers compared with direct treatment on the isolated microspores. For first experiment 100 mg/l colchicine added separately with three different basal media and found in all cases more or less fertile plants production compared to control. The direct treatment of colchicine to isolated microspore culture decreased. for the different concentrations of colchicine (50, 100, 150 mg/l for three days) added in AMC medium. In this case average frequency of diploid plants were increased (81.73%) compared with control (72.40%) among the three treatments. This finding has increased the knowledge about the benefit of colchicine application and optimized its concentration on isolated microspore for improving doubled haploids production in wheat microspore culture.

4.2 Haploid Confirmation and Genetic Variability Study

4.2.1 DNA Isolation and Quality Analysis

The high molecular weight genomic DNA was extracted from callus of sunflower genotype by Cetyl Trimethyl Ammonium Bromide (CTAB) DNA extraction method given by Doyle and Doyle (1987) with some modifications. This method yielded qualitatively as well as quantitatively pure genomic DNA.

The quantification of extracted DNA was done by measuring absorbance at 260 nm wavelengths. Purity of DNA was checked by reading absorbance ratio of $A_{260/280}$. Also the quantitative and qualitative analysis was done by resolving DNA on 0.8% Agarose gel. The concentrations of all DNA samples were ranged between 900-1700 ng/µl. Working samples were prepared by diluting with sterile nuclease free water to obtain final concentrations of 50ng/µl for SSR analysis.

4.3 Confirmation of Haploid by Using Microsatellite (SSR) Marker

4.3.1 Optimization of PCR Components for SSR Markers

PCR reaction mixture for SSR was optimized as 1X *Taq* polymerase buffer 25mM $MgCl_2$, 3.0 U *Taq* DNA polymerase, 10mM dNTPs respectively. A working concentration of genomic DNA 50ng/µl and primer concentration of 25 pmol for SSR markers analysis resulted in good amplification. The total reaction volume of 25µl contained, 2.5µl *Taq* polymerase buffer, 0.25µl dNTPs, 1.7µl $MgCl_2$, 0.25µl each primer (forward and reverse), 0.33µl *Taq* DNA polymerase, 1µl of DNA (50ng/µl) and 18.75µl sterile nuclease free water (Table no.11).

Table No. 16: PCR Components and Stock Solutions for SSR Analysis.

Sr. No.	Components	Stock solutions	Final concentration	Volume for one reaction (µl)
1	10X PCR buffer	10X	1X	2.5
2	dNTPs	10 mM	0.2 mM	0.25
3	*Taq* DNA polymerase	3 U/µl	1.0 U/µl	0.33
4	$MgCl_2$	25 mM	1.7 mM	1.70
5	Forward Primer	100 pmol/ µl	25 pmol	0.25
6	Reverse Primer	100 pmol/ µl	25 pmol	0.25
7	Genomic DNA	50 ng/µl	50 ng	1.0
8	Nuclease free water	-	-	18.75
			Total volume	**25.00 µl**

4.3.2 Optimization of PCR Cyclic Parameters for SSR Analysis

The specific allelic patterns were obtained by fifteen SSR markers that could confirm identity of haploid plant. The annealing temperatures for SSR primers were optimized by using different degrees of temperatures in gradient PCR conditions, applying annealing temperature 2-5^{o}C below the melting temperature of the SSR primer (T_m). The list of SSR primers and their optimized annealing temperature is listed in (Table no.17).

The sunflower genotypes were amplified by SSR markers in a 96 well thermal cycler (Senso quest Lab cycler, Germany) programmed for 35 cycles as follows: initial denaturation at 94^{o}C for 5 min. further denaturation at 94^{o}C for 30 sec, annealing at T (opt) for 50 sec., extension at 72^{o}C for 1 min. and final extension for 10 min at 72^{o}C followed by pause/hold at 4^{o}C (Table no.7).

Table no. 17: Optimized Annealing Temperatures (T opt.) of SSR Markers.

Sr. No.	Primer ID	Sequence	Annealing Temperature (^{o}C)
1	ORS-05	F-AACATCTGGAGCAGCAAATTCAG	58
		R-CTGCTGCCCACCATACTG	
2	ORS-662	F-CGGGTTGGATATGGAGTCAA	57
		R-CCTTTACAAACGAAGCACAATTC	
3	ORS-1265	F-GGGTTTAGCAAATAATAGGCACA	57
		R-ACCCTTGGAGTTTAGGGATCA	
4	ORS-552	F- CCATCCCTTCCCTCTCTTTC	57
		R- GTGGCTGGAATCTCATCACC	
5	ORS-488	F- CCCATTCACTCCTGTTTCCA	58
		R- CTCCGGTGAGGATTTGGATT	
6	ORS-243	F- GGGATGACGTGCGTTTGG	57
		R- ACCACCATTTCTACCGTTTCTC	
7	ORS-1159	F- TTTCGTGATGGTGATTGATGATT	59
		R- CAGCAACTCTGACCGTTTCATTA	
8	ORS-928	F- CATGGTTATTTTGGTTTGGGTTT	57
		R-GCTATTATCATGTCCTTGTCCTTTT	
9	ORS-58	F- TGTACCAAGGGTCGTTGTCA	58
		R- CGACCCCGAGTTTTGTTG	
10	ORS-337	F- TTGGTTCATTCATCCTTGGTC	58
		R- GGGTTGGTGGTTAATTCGTC	
11	ORS-959	F- CCGCTAAGTATAAACCGCCTATT	57
		R- CGTCCTCTTCGCATCAATCTTAT	

12	ORS-154	F- GCACCTTTGGTGAGGAGATA	57
		R- TGCATCAGTAGCTATTGTCTAT	
13	ORS-780	F- TGATTACAACCCTAATTCGCATAC	57
		R- GATACTGGTGGGACAGATGTTG	
14	ORS-536	F- GAAATAGGAGGGGATCTTACCG	58
		R- GCGGAGAGAAAGACGAAGAG	
15	ORS-423	F- TCATATGGAGGGATCTGTTGG	57
		R- AAGCAACCATAATGCATCAGAA	

4.3.3 Haploid Confirmation Based on SSR Fingerprint Profile Analysis

Compared with cytological and isozyme analysis, molecular markers based on genome sequences are more convenient and reliable for homozygous analysis. SSRs are quite abundant in eukaryotic genomes, and are broadly used for genetic analysis because of their co-dominance, multi-allelic nature, reproducibility, extensive genome coverage and easy detection (Luro *et al*., 2008).

Ploidy analysis *via* both flow cytometry and chromosome counting verified that these two lines were diploids. Additionally, 43 simple sequence repeat (SSR) markers which showed to be heterozygous in the Valencia sweet orange donor line confirmed homozygosity and doubled haploids in the anther derived lines (Cao *et al*., 2010).

SSR analysis would be very useful in breeding for rapid and early verification of haploid in large population. SSR analysis has been successfully used for haploid and parentage identification of other crop plants. In maize, SSR analysis allowed the identification of the haploid plant (Battistelli *et al*., 2013).

Different types of markers have been designated as per convenience by authors (Belicuas *et al*., 2007; Couto *et al.*, 2012; Battistelli *et al*., 2013 and Keles *et al*., 2015). SSR markers have been widely used in ploidy analysis and chromosome duplication by various scientists (Belicuas *et al*., 2007; Barret *et al*., 2008; Perera *et al*., 2008; Zhang *et al*., 2008; Diao *et al*., 2009; Li., L. 2009; Mayor and Bernardo, 2009; Kebede *et al*., 2011; Prigge *et al*., 2012) for haploid confirmation. The haploid confirmation discussed here under was done by comparing banding patterns of haploids with donor plant similar findings were reported for analysis and identification of haploids by using SSR markers by Couto *et al.* (2012) in maize. For haploid analysis, the total of two types of banding patterns was observed in the donor plant and haploids.

The SSR molecular markers were used to confirm the androgenic trait of the haploid. In present study total 28 SSR primers were screened, among these fifteen SSR primers *viz*., ORS-05, ORS-662, ORS-1265, ORS-552, ORS-488, ORS-243, ORS-1159, ORS-928, ORS-58, ORS-337, ORS-959, ORS-154, ORS-780 ORS-423 and ORS-536 showed polymorphism and were used to confirm the haploid identification of regenerated calli with donor parent line. Rest of 13 primers did not shown amplification.

Both regenerated lines and the anther donor parent were analysed with various SSR markers. All fifteen SSR primers were trait specific (high oil content, high oleic content, test weight, high yield) and amplification results shown the presence of these specific traits in the regenerated calli and donor plant. The results showed that the regenerated calli in all treatments (direct colchicine treatment to anthers and colchicine treatment in media) had homozygous profiles with a single band while the donor plant had two bands that indicated allelic sites (Plate no. 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28). The fifteen SSR markers showed 100% homozygosity in callus. This confirms the androgenetic origin of regenerated calli.

Also, in the present investigation the presence of novel alleles in the SSR profiling was not observed in any colchicine treatments (in direct treatment to anther and media supplement), suggesting no any changes in the DNA sequences of the calli.

The fifteen SSR markers *viz.*, ORS-05, ORS-662, ORS-1265, ORS-552, ORS-488, ORS-243, ORS-1159, ORS-928, ORS-58, ORS-337, ORS-959, ORS-154, ORS-780 ORS-423 and ORS-536 can be used for confirmation of haploid in sunflower anther culture.

Table No. 18: Haploid Confirmation by SSR Markers.

Sr. no.	Primer code	Alleles present in diploid plant (bp)	Allele present in calli (bp)	Nature of regenerated calli
Direct colchicines treatment to anther				
1	ORS-05	200, 300	300	Confirmed haploid
2	ORS-662	230, 314	314	Confirmed haploid
3	ORS-1265	180, 222	222	Confirmed haploid
4	ORS-536	270, 300	300	Confirmed haploid
5	ORS-552	250, 450	450	Confirmed haploid

6	ORS-488	100, 200	200	Confirmed haploid
7	ORS-243	130, 220	220	Confirmed haploid
8	ORS-1159	130, 270	270	Confirmed haploid
9	ORS-928	140, 230	230	Confirmed haploid
10	ORS-58	180, 300	300	Confirmed haploid
11	ORS-337	120, 220	220	Confirmed haploid
12	ORS-959	150, 280	280	Confirmed haploid
13	ORS-154	100, 170	170	Confirmed haploid
14	ORS-780	100, 180	180	Confirmed haploid
15	ORS-423	120, 220	220	Confirmed haploid
Colchicine treatment to calli in media				
1	ORS-05	200,300	300	Confirmed haploid
2	ORS-1265	180,222	222	Confirmed haploid

Similar findings was reported by Parera *et al*. (2014) for abiotic stress (temperature) where they used SSR marker assay to assess its feasibility for identifying the origin of anther-derived calli of cassava. This assay was carried out with a set of eight anther-derived calli lines obtained from the heat pretreatment at 38°C. The SSRs marker (SSRY 19, SSRY 21, SSRY 63, SSRY 82, SSRY 164, and SSRY 182) showed the distinguishable heterozygous pattern of the donor plant with two alleles. These biallelic diagnostic SSRs were used to assess the eight anther-derived callus lines. The SSR analysis (i.e., SSRY 21, SSRY 63, and SSRY 182) showed a distinguishable monoallelic distribution when compared to the mother plant, which strongly suggests a potential haploid origin of the anther-derived calli. Also, the presence of novel alleles in the SSR profiling suggesting changes in the DNA sequence was observed.

Belicuas *et al*. (2007) identified four haploids among 462 plants obtained from the cross between the line W23 and the hybrid BRS1010 of sunflower. Two polymorphic primers were used on the parents, mmc0022 and mmc0081. The four haploids had the same size bands as the male parent; in other words, they were characterized as androgenetic haploids.

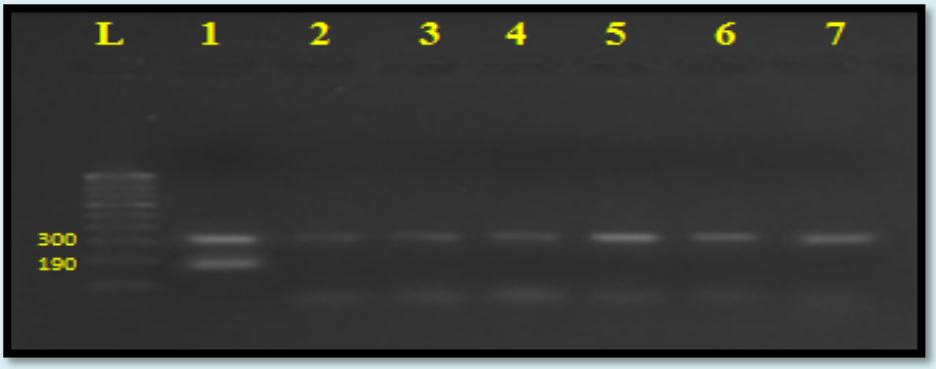


Plate No. 14: Banding profile of haploids and donor plant obtained by Primer ORS-05.

Plate No. 15: Banding profile of haploids and donor plant obtained by Primer ORS-662.

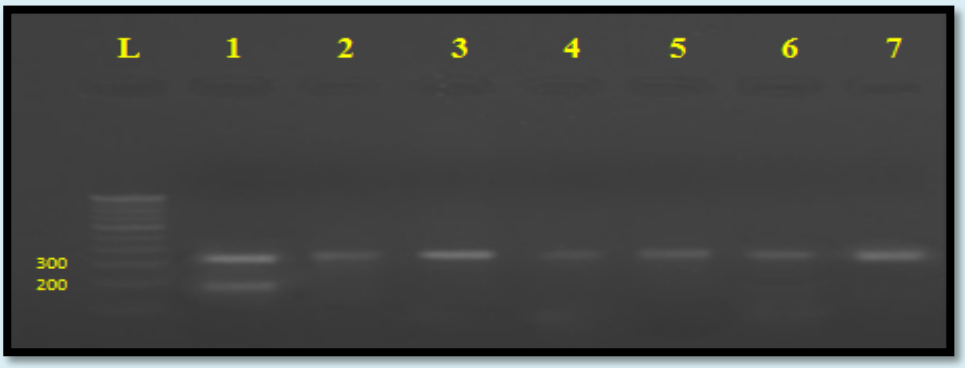


Plate No. 16: Banding profile of haploids and donor plant obtained by Primer ORS-1265.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, 6 and 7: Callus regenerated from 0.1%, 0.5%, 1%, 1.5% and 2% colchicine treated anthers (haploids).
L: 100 bp ladder

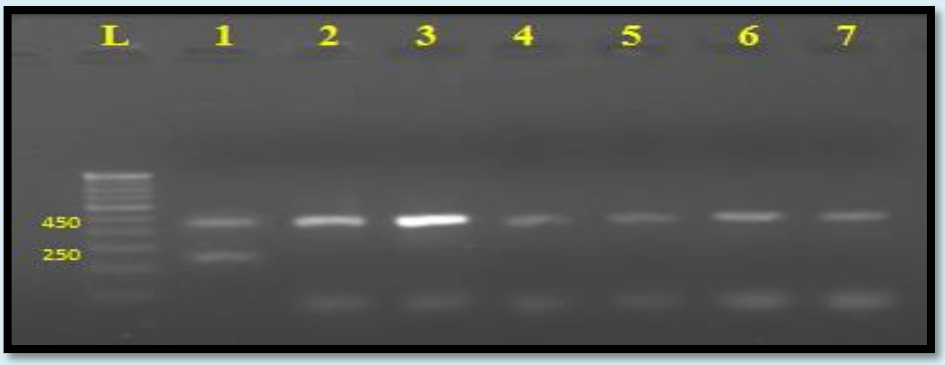


Plate No. 17: Banding profile of haploids and donor plant obtained by Primer ORS-536.

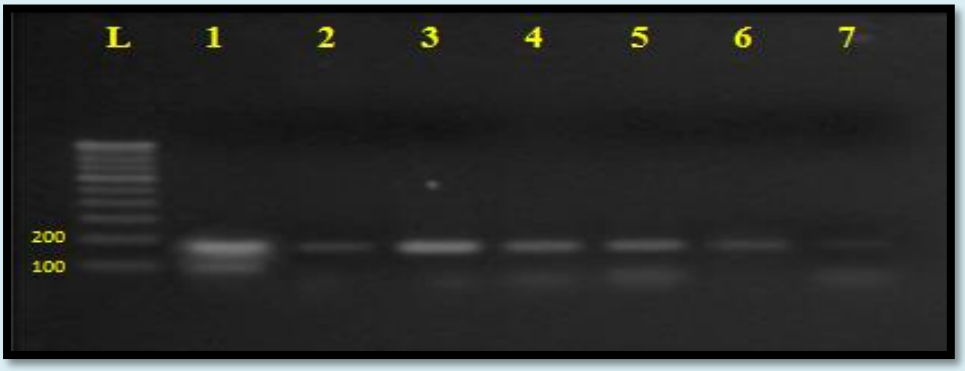


Plate No. 18: Banding profile of haploids and donor plant obtained by Primer ORS-552.

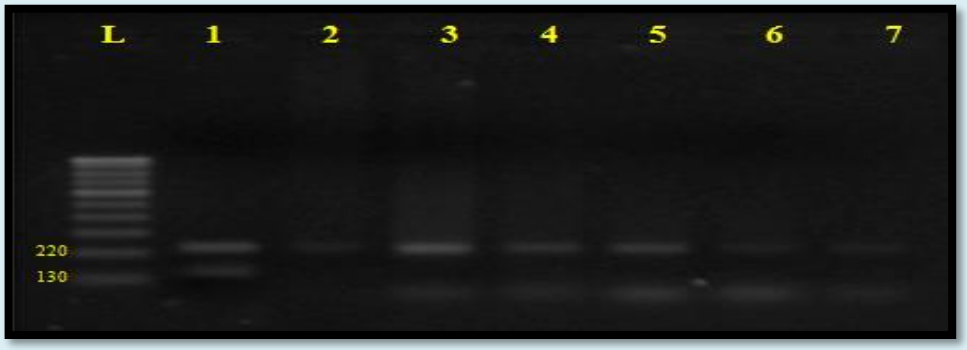


Plate No. 19: Banding profile of haploids and donor plant obtained by Primer ORS-488.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, 6 and 7: Callus regenerated from 0.1, 0.5, 1, 1.5 and 2% colchicine treated anthers (haploids).
L: 100 bp ladder

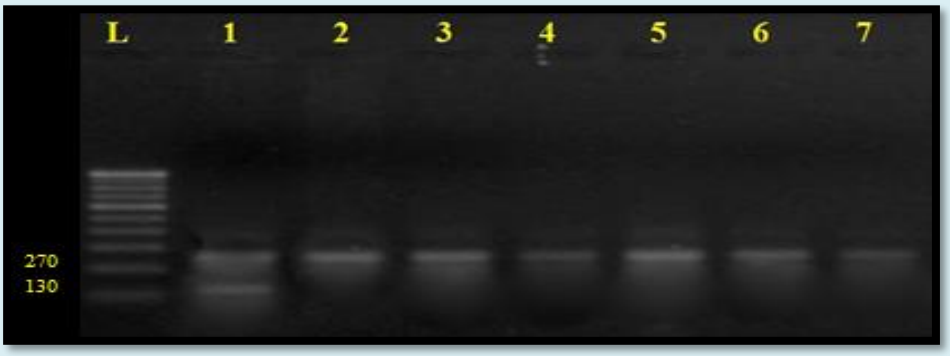


Plate No. 20: Banding profile of haploids and donor plant obtained by Primer ORS-243.

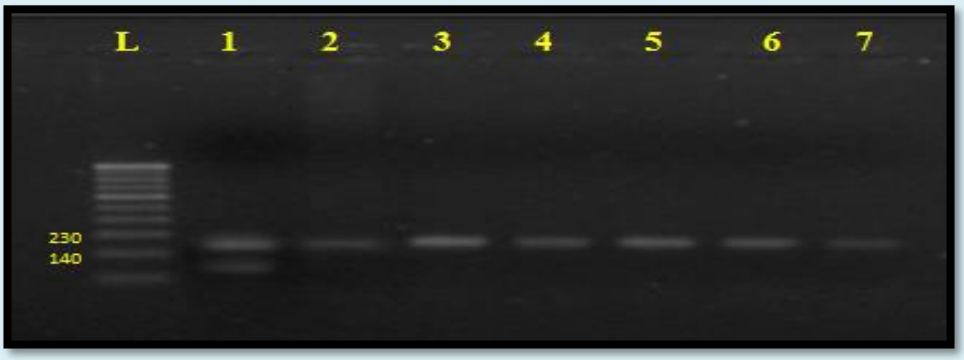


Plate No. 21: Banding profile of haploids and donor plant obtained by Primer ORS-1159.

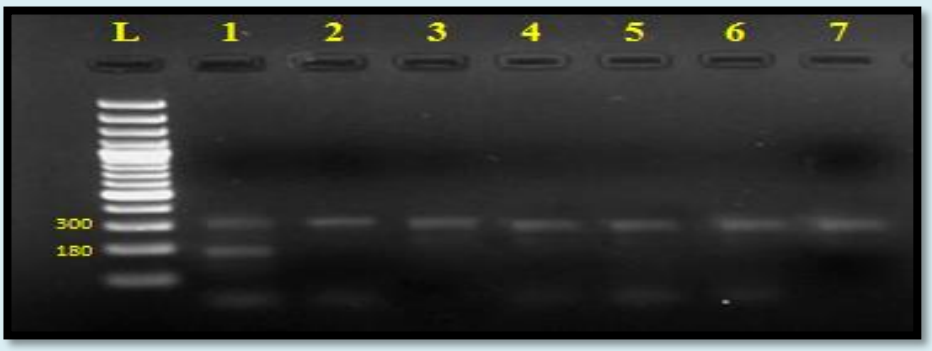


Plate No. 22: Banding profile of haploids and donor plant obtained by Primer ORS-928.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, 6 and 7: Callus regenerated from 0.1, 0.5, 1, 1.5 and 2% colchicine treated anthers (haploids).
L: 100 bp ladder

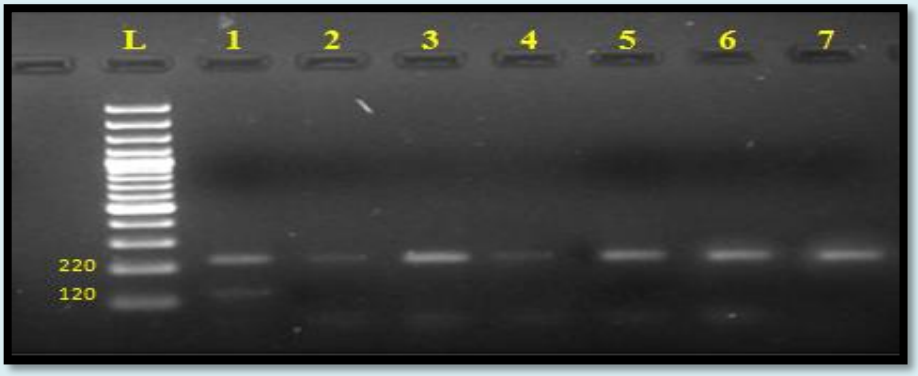


Plate No. 23: Banding profile of haploids and donor plant obtained by Primer ORS-58.

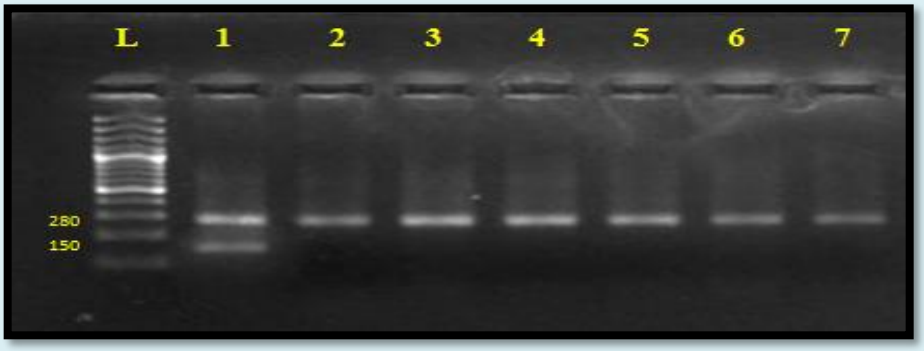


Plate No. 24: Banding profile of haploids and donor plant obtained by Primer ORS-337.

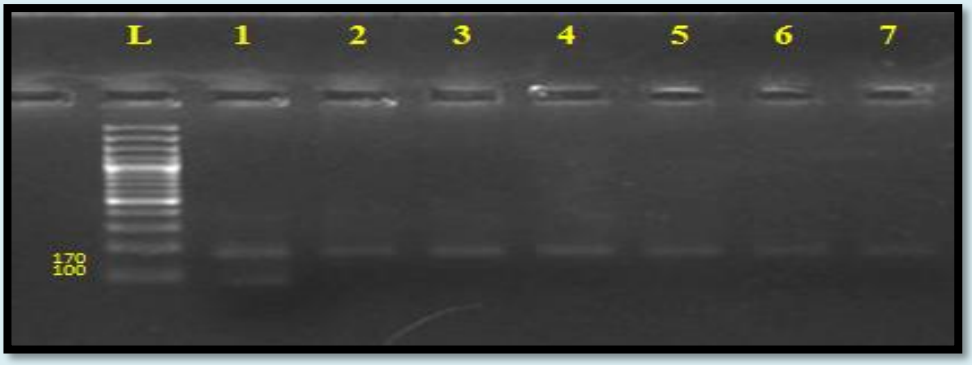


Plate No. 25: Banding profile of haploids and donor plant obtained by Primer ORS-959.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, 6 and 7: Callus regenerated from 0.1, 0.5, 1, 1.5 and 2% colchicine treated anthers (haploids).
L: 100 bp ladder

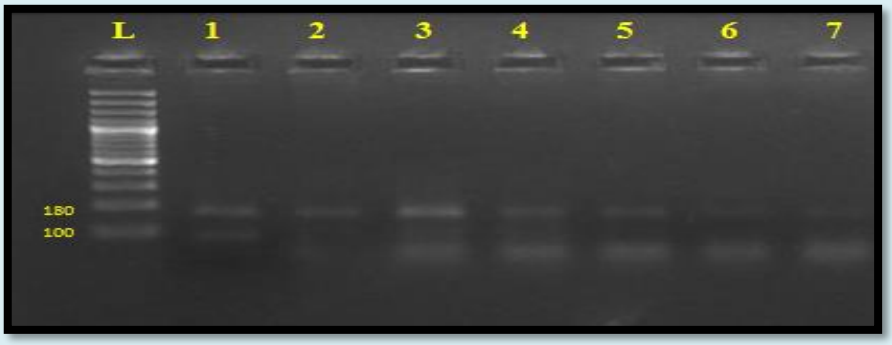


Plate No. 26: Banding profile of haploids and donor plant obtained by Primer ORS-154.

Plate No. 27: Banding profile of haploids and donor plant obtained by Primer ORS-780.

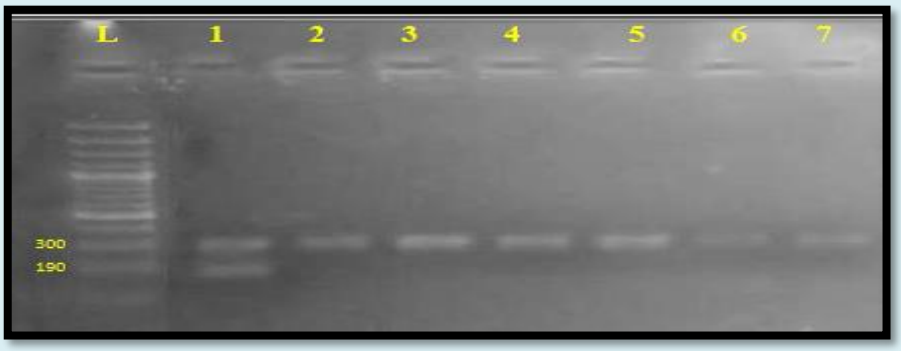


Plate No. 28: Banding profile of haploids and donor plant obtained by Primer ORS-423.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, 6 and 7: Callus regenerated from 0.1, 0.5, 1, 1.5 and 2% colchicine treated anthers (haploids).
L: 100 bp ladder

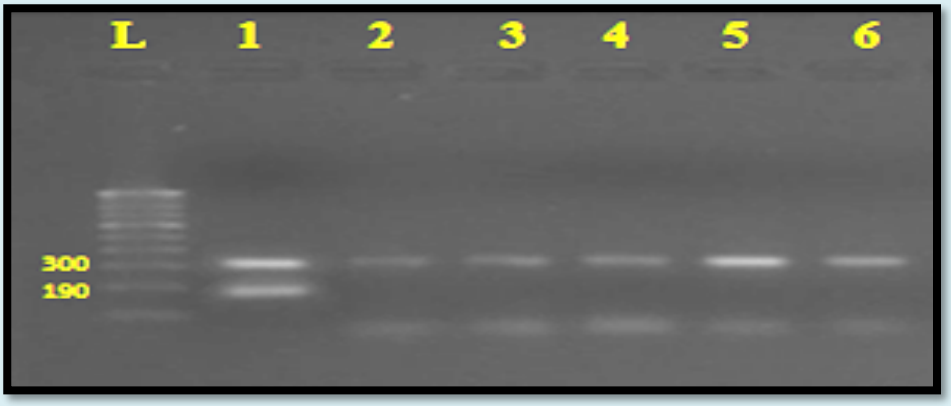


Plate No. 29: Banding profile of haploids and donor plant obtained by Primer ORS-05.

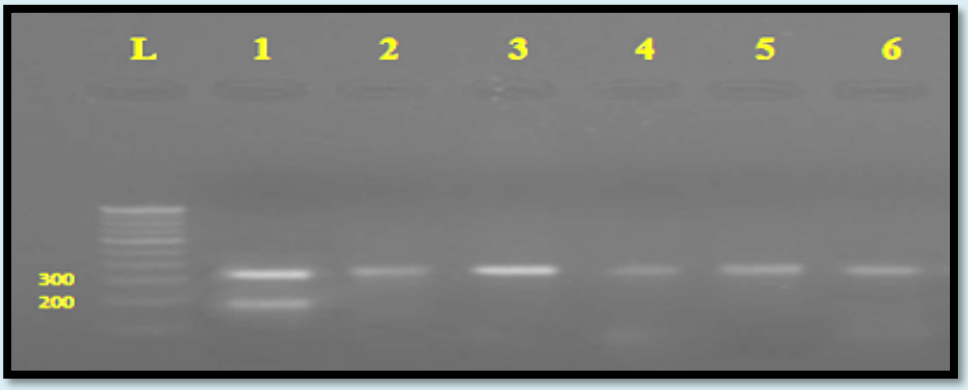


Plate No. 30: Banding profile of haploids and donor plant obtained by Primer ORS-1265.

1: Donor plant (diploid).
2: Control callus (haploid)
3, 4, 5, and 6: Callus treated with different concentreation 0.5, 1, 1.5 and 2% of colchicine in medium (haploids).
L: 100 bp ladder

Microsatellites have been also employed to characterize regenerants obtained from citrus anther culture (Germana *et al*. 2000a, b; Germana and Chiancone, 2003) and to assess homozygosity in apple (Kenis and Keulemans, 2000) and pear (Bouvier *et al*., 2002). The single multi-allelic self-incompatibility gene has been used in apple by Verdoodt *et al*. (1998) to discriminate homozygous from heterozygous individuals obtained by anther culture as well as by parthenogenesis *in situ*.

In present study allelic results shown that, extra unique alleles were not found in the regenerated calli at a locus other than diagnostic heterozygous allele present in donar sunflower genotypes indicates absence of somaclonal variation in the regenerated calli. Krutovsky *et al*. (2014) studied and found only one locus (UAKLly6) was heterozygous for the maternal tree no. 30 and therefore could serve as a diagnostic locus for haploid cell lines (CLs). At this locus, none of the CLs contained both maternal alleles, so presumably they all were haploid. However, all CLs contained unique alleles at 3-7 loci, which were absent in the maternal tree, and were likely to have a mutational origin indicating a very high rate of somaclonal variation.

The molecular analysis using microsatellite markers showed this markers usefulness as authentic technology in demarcation of haploids and their donor parental lines and also the somaclonal variation arises due to spontaneous and /or induced muataion in haploids. The identified set of SSR markers will be useful for the confirmation of sunflower haploids and will facilitate for sunflower breeding programmes.

4.3.4 Clustering of Haploids and Donor Parental Line on the Basis of SSR Analysis

The similarity index can be used to measure the relatedness of samples (Nybom and Hall, 1991; Welsh *et al*., 1991). The genetic similarity was retrieved from microsatellite (SSR) data using Jaccard's coefficient. The similarity matrix was generated by using scored data of SSR fingerprint and used to depict dendrogram through UPGMA cluster analysis by using software NTSYS-pc Version 2.02i (Rohlf, 1998).

Two major clusters were found to represent unique grouping of 12 members (eleven haploids and one donor parent) at 50% similarity value. These clusters *viz*., Cluster-I, Cluster-II comprised of 7 and 5 colchicine treatments respectively, clearly

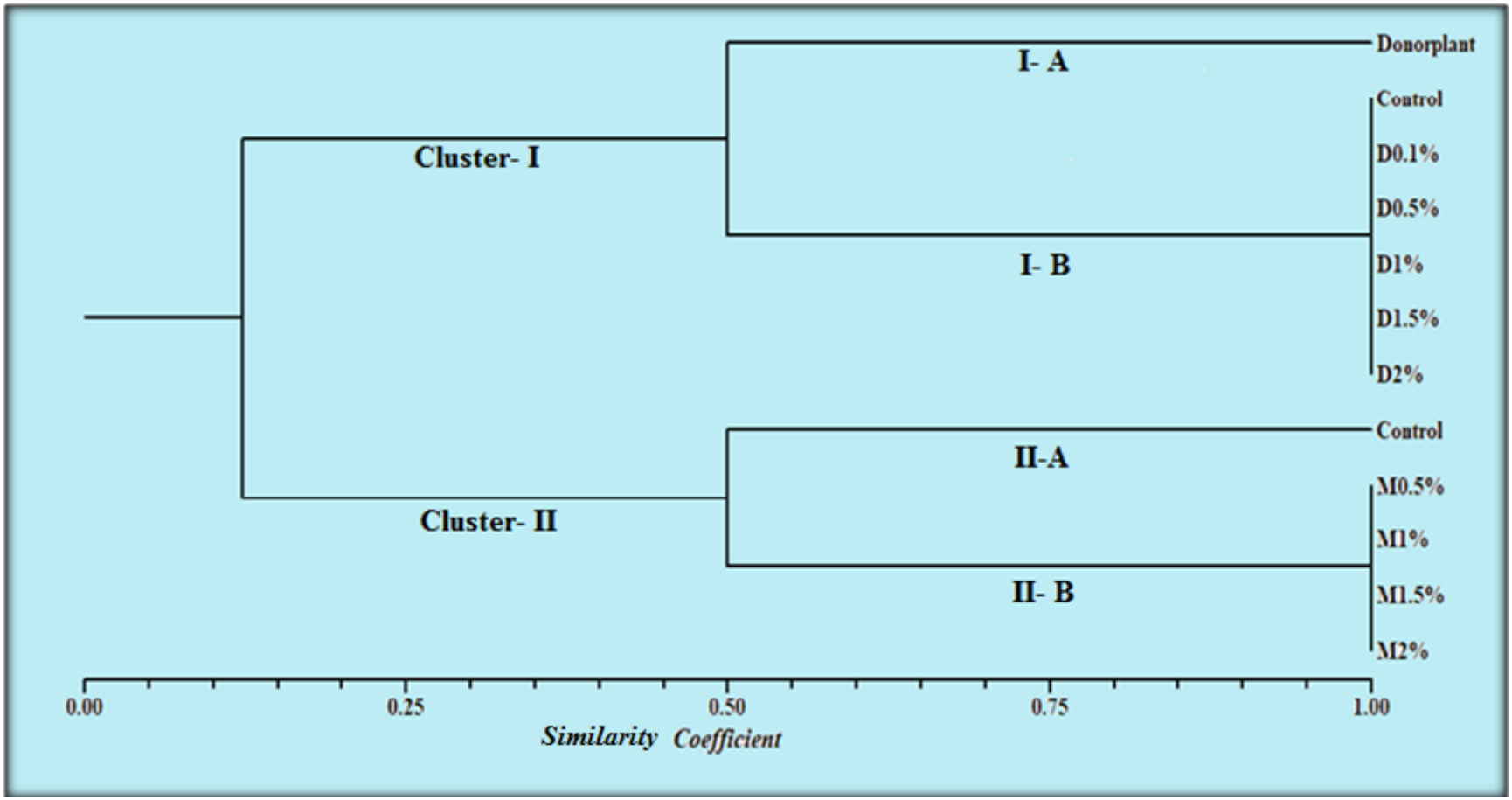


Fig.1. Dendrogram constructed using UPGMA analysis demonstrating relationship among 11 haploid and their donor parent based on Jaccard's similarity coefficient value obtained by SSR data.

Table no. 19 Similarity matrix generated by using SSR Fingerprint

	Donor plant	Control	D 0.1%	D 0.5%	D 1%	D 1.5%	D 2%	Control	M 0.5%	M 1%	M 1.5%	M 2%
Donor plant	1.00											
Control	0.50	1.00										
D 0.1%	0.50	1.00	1.00									
D 0.5%	0.50	1.00	1.00	1.00								
D 1%	0.50	1.00	1.00	1.00	1.00							
D 1.5%	0.50	1.00	1.00	1.00	1.00	1.00						
D 2%	0.50	1.00	1.00	1.00	1.00	1.00	1.00					
Control	0.13	0.11	0.11	0.11	0.11	0.11	0.11	1.00				
M 0.5%	0.06	0.13	0.13	0.13	0.13	0.13	0.13	0.50	1.00			
M 1%	0.06	0.13	0.13	0.13	0.13	0.13	0.13	0.50	1.00	1.00		
M 1.5%	0.06	0.13	0.13	0.13	0.13	0.13	0.13	0.50	1.00	1.00	1.00	
M 2%	0.06	0.13	0.13	0.13	0.13	0.13	0.13	0.50	1.00	1.00	1.00	1.00

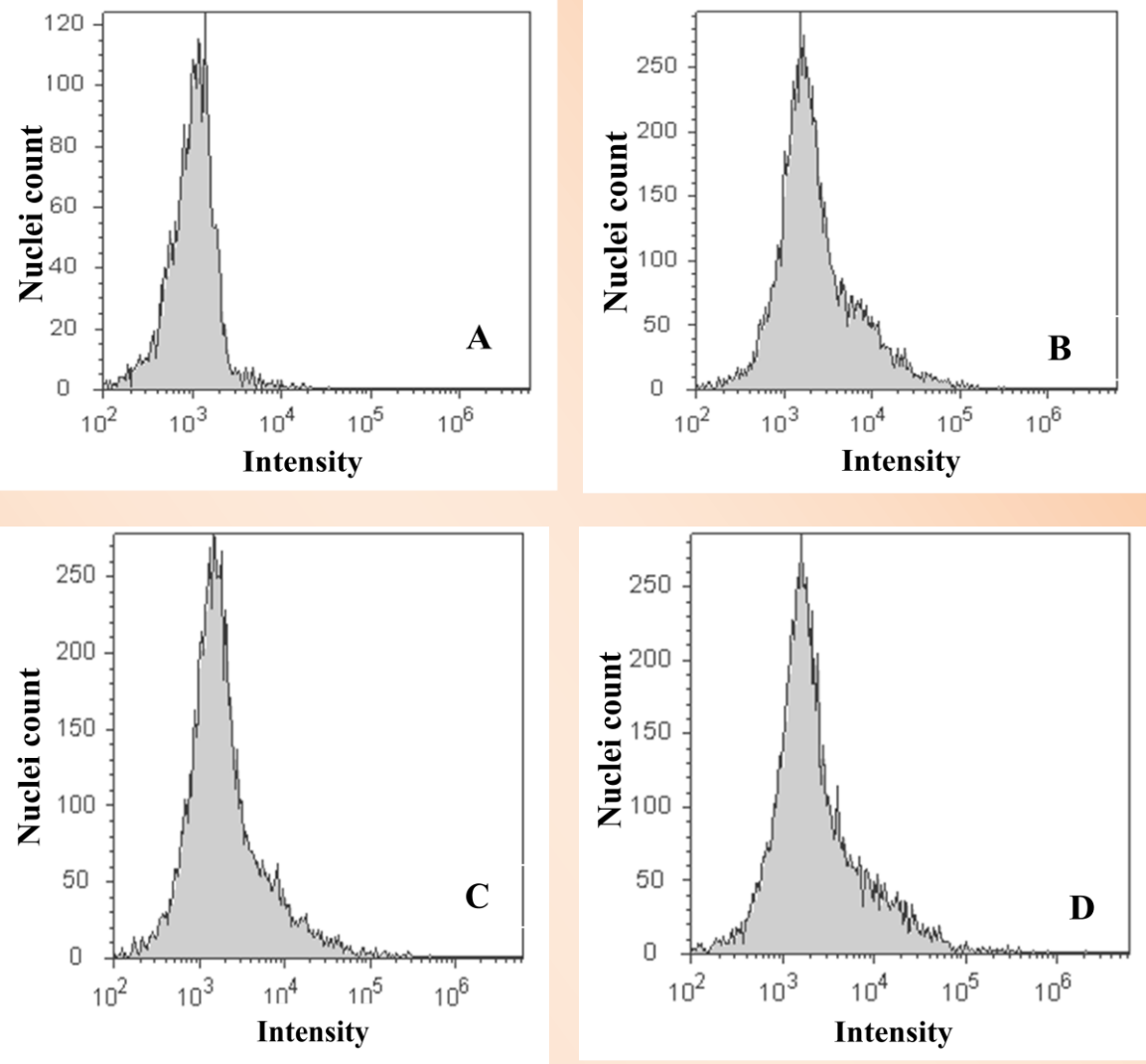
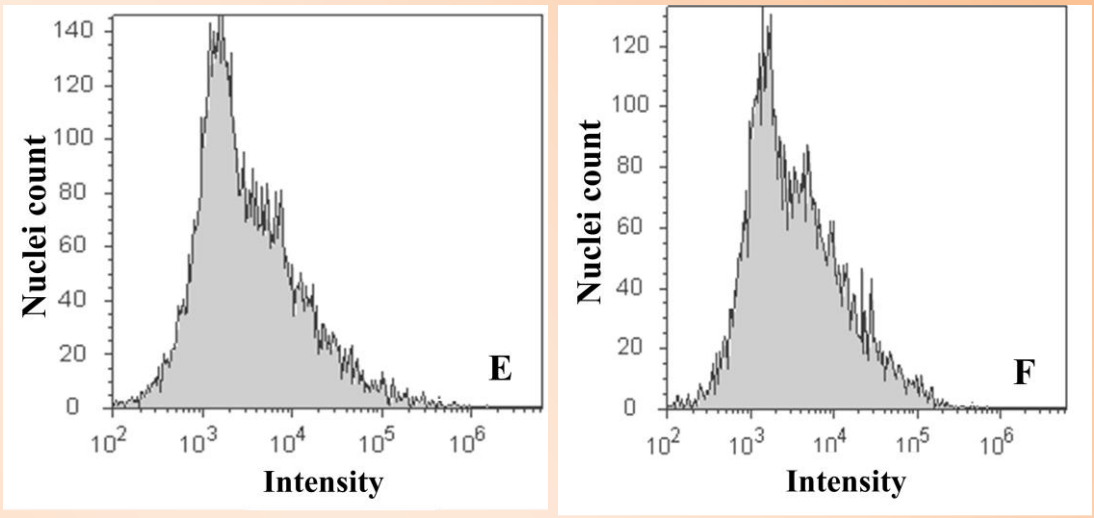


Fig No. 2: Flow cytometric histograms of relative fluorescence obtained after simultaneous analysis of nuclei isolated from A) Sunflower leaf DNA and direct colchicine treatment to anthers: B) Control, C) 0.1%, D) 0.5%. Vertical axis = Number of nuclei read; Horizontal axis= Intensity of relative fluorescence.



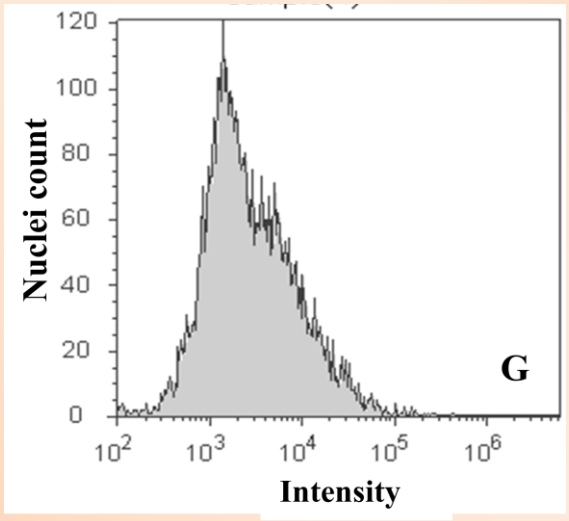
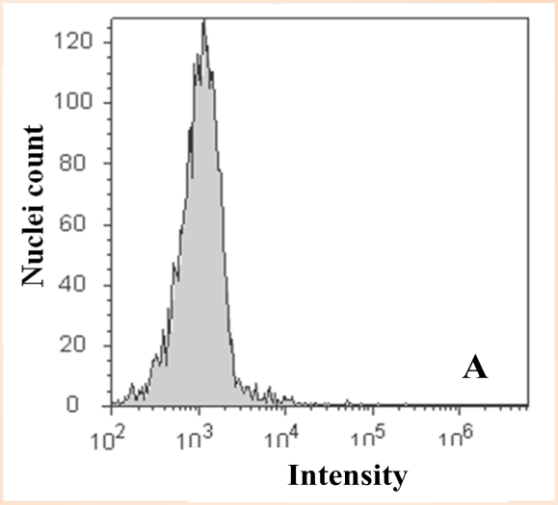
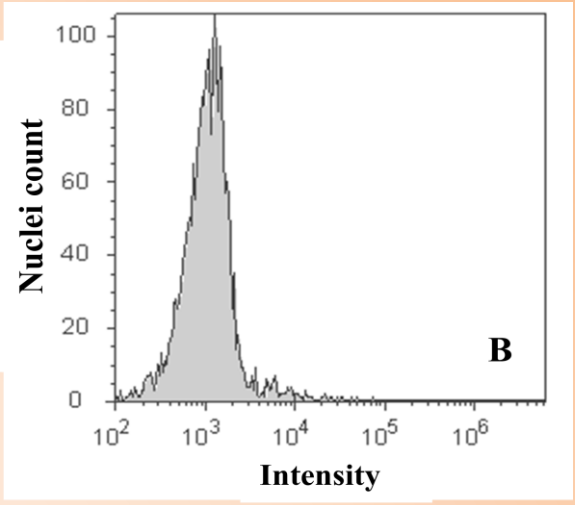
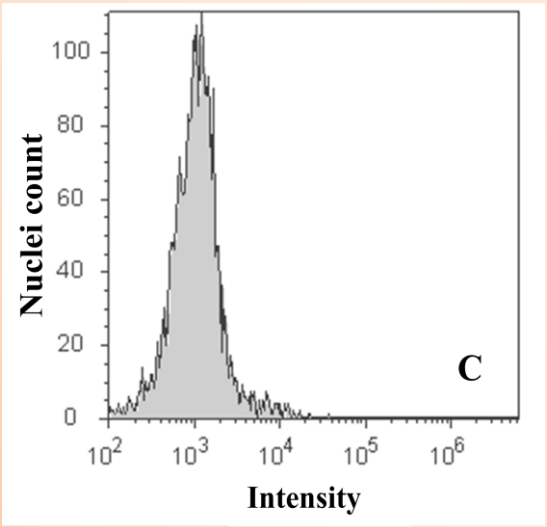
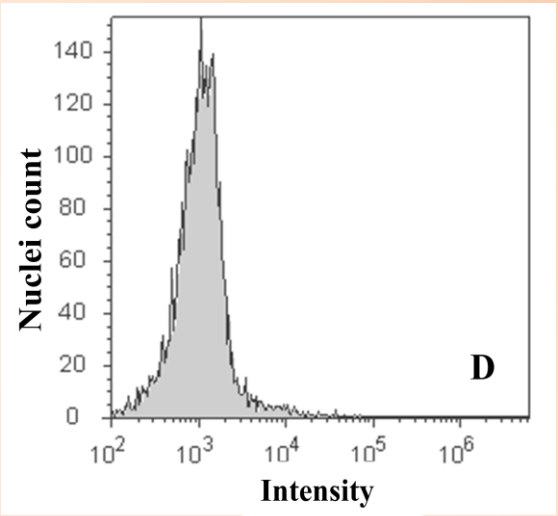


Fig. No. 3: Flow cytometric histograms of relative fluorescence obtained after simultaneous analysis of nuclei isolated from direct colchicine treatment to anthers: E) 1%, F) 1.5%, G) 2%. Vertical axis = Number of nuclei read; Horizontal axis= Intensity of relative fluorescence.









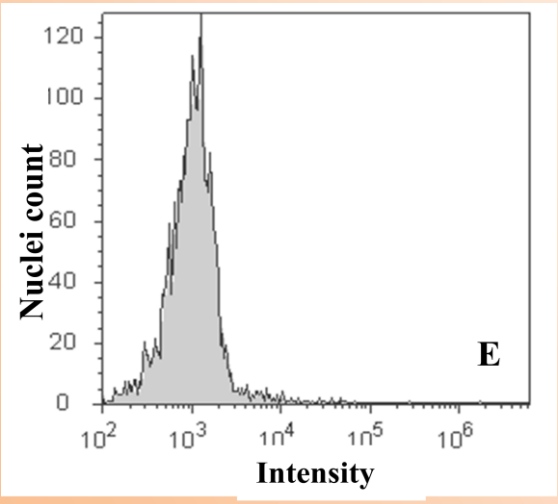


Fig No. 4: Flow cytometric histograms of relative fluorescence obtained after simultaneous analysis of nuclei isolated from indirect colchicine treatment to callus in media: A) Control, B) 0.5%, C) 1% D) 1.5% E) 2%. Vertical axis = Number of nuclei read; Horizontal axis= Intensity of relative fluorescence.

Cluster I divided into two subclusters *i.e.* subcluster IA and subcluster IB. subcluster IA had only donor parent line and subcluster IB consists of 6 haploids of calli derived from anthers directly treated with 0.1, 0.5, 1, 1.5, 2% colchicines and control.

Cluster II divided into into two subclusters *i.e.* subcluster IIA and subcluster IIB. subcluster had control calli line and subcluster IIB concomprised of 5 haploids which includes calli regenerated from colchicines supplemented in media and control.

Observing the dendrogram produced by SSR analysis data shows that both clusters showing 100% similarity due to use of trait specific primers. The SSR markers used in the present study were trait specific for high oil content, test weight and high oleic acid content, they grouped in one cluster.

So, these regenerated calli at various colchicine treatments showing trait specificity thereby we conclude that these are true haploids having androgenetic origin.

4.4 Flow Cytometry Analysis

The DNA contents of the calli derived from direct and indirect colchicine treatment and the diploid sunflower from leaf tissue were similar when estimated using flow cytometry (Fig. no. 2, 3, 4). However, peaks from treated calli and sunflower did not generally overlap perfectly. Therefore, a peak at about twice the index of the peak corresponding to the treated calli was expected in cases of chromosome doubling.

The changes in the ploidy level have been observed in calli derived from direct and indirect colchicine treatments. Histograms from FCM analysis revealed that the DNA content of nuclei had one peak in donor plant as well as in all direct and indirect colchicine treatment along with their control. These results suggest that FCM analysis could determine the ploidy level of sunflower, as diploid or double haploid.

Moyne *et al.* (1993) established embryogenic calli from leaf explants. Flow cytometry of isolated nuclei was used to determine ploidy level of calli and embryogenic calli suspension. In calli, the majority of cells were tetraploid as were the explants from which they had originated. On the contrary, in suspensions aneuploid and polyploidy cells were frequent.

Leblanc *et al*. (1995) established collection of embryogenic deploid calli of *Tripsacum* and treated with colchicine to induce chromosome doubling. Section containing duplicated cells in calli were identified using flow cytometry and ploidy level

was determined in the regenerated plantlets. Tetraploid plant from several origins were obtained in contrast to wild polyploidy plants, which show apomictic development.

Similarly, in asparagus, the ploidy levels of embryogenic callus lines and regenerated shoots were determined by FCM to verify their stability in long-term culture (Limanton-Grevet *et al.*, 2000). Shiga *et al.* (2009) determined ploidy levels of *in vitro* shoots produced through anther culture of asparagus by flow cytometry (FCM). Of the 110 anther-derived shoots, there were 83 diploids, 21 tetraploids, 3 octaploids, and 3 mixoploids, but no haploids. This result suggestes that polyploids might develop by the fusion of nuclei during pollen-mother cell division before the tetrad stage.

4.4.1 Confirmation of Ploidy Level of Calli

In SSR analysis all calli derived from direct and indirect colchicine treatments were homozygous giving single allel whereas donor plant was heterozygous with two allels. Therefore, according to the SSR markers and FCM results calli derived from direct and indirect colchicine treatments are double haploids. The double haploids are also obtained in the control callus in both the cases, indicates spontaneous dihaploidization of the chromosomes.

Similarly, Islam (2010) also obtained 83.73% double haploid plants in colchicine treatment (50, 100, 150 mg/l for three days) as compared to 72.40% in control in wheat microspore culture.

Keles *et al.* (2015) obtained statistically highest average spontaneous doubled haploidy rate from bell pepper type with 53.4% followed by charleston and capia types (charleston: 31.9%, capia: 30.4%). The lowest result was observed in green pepper type with 22.2%. Spontaneous doubled haploidy rates of the tested genotypes of capia (23.8%, 27.3%, 27.3%, 30.3%, 30.4%,33.3%, 33.3%, and 37.5%) and bell (52.8%, 52.9%, 52.9%, 60%, and 61.7%) pepper types were found to be more stable than the charleston (14.3%, 20%, 20%, 38.7%, 42.9%, 42.9%, and 44.4%) and green (8.3%, 9.1%, 20%, 23.5%, 25%, 25%, and 44.4%) pepper types. The spontaneous doubled haploid plants were tested using flow cytometry and an SSR locus to determining whether the plants were doubled haploid or not. These tests showed that all doubled haploid plants were homozygous.

Spontaneous doubling has also been reported in other plant species. In a review study conducted by Kim *et al*. (2007), spontaneous doubled haploidy in different species

such as in rice (Cho and Zapata, 1990), barley (Hoekstra *et al*., 1993), and wheat (Kim and Baenziger, 2005) were reported. Spontaneous diploidy rate of *Brassica rapa* ssp. *chinensis* plants obtained through microspore culture was found to be over 70% by Gu *et al*. (2003). In a study carried out by Vanous (2011) in maize, spontaneous doubled haploidy rates for the male inflorescence varied between 2.8% and 46% and were found to be highly genotype specific.

SUMMARY AND CONCLUSION

CHAPTER-V

SUMMARY AND CONCLUSION

5.1 Summary

Haploid plants are of great importance in plant breeding because they help to shorten the breeding process. However, since these plants have half the normal chromosome number, they must be made diploid. Doubling of chromosome of haploid plants using chemicals or spontaneously is called dihaploidizition. Some chemicals such as colchicines are used for chromosome doubling (Keles *et al*., 2015).

5.1.1 Effect of Colchicine Treatments on Sunflower Anther

Capitula was harvested prior to opening of ray florets when most of the microspores was at the mid to late uninucleate stage of development and the optimal pollen stage was determined from anther morphology. The flower buds of 1.5 to 2.0 cm in diameter with cream colored anthers possessing mostly uninucleate microspores were used. The flower buds were surface sterilized by immersing in 70% ethanol and 0.1% Mercuric chloride for 2 minute each then wash with double distilled water for 3 times to remove traces of chemicals and dried on blotting paper.

5.1.1.1 Treatment of Anthers Before Culture

When the anthers were treated with 0.4% colchicine for different hours i.e. 2, 4, 6, 8 and 10 hrs the response were 98.61,73.61,15.2, 13.75, 8.82% respectively, while the response of untreated control was 100% and gave maximum response for callus induction at 4.8 hrs. Colchicine treated anthers were black colour as compared to control anthers. The Lethal time of this treatment is LT50=4.8303 and LT95= 9.717.

After standardization of time, anthers were treated with 0.1, 0.5, 1, 1.5 and 2% colchicines for 4.8 hours. Colchicine treated anthers were black colour as compared to control anthers. The cultured anthers responses in survival percentage were 25, 22.5, 17.5, 12.5 and 10% respectively for these treatments whereas the response of untreated control was 95%. The callus induction frequency were 23.33, 20, 16.66, 11.66, and 8.33 respectively for these treatments whereas 88.33 for untreated control. The number of days required for callus induction in colchicine treated anther was 11, 10.66, 12.33, 12.33, 13.33 respectively and 10.33 days required for untreated control. The Lethal concentration of this treatment LC50= 0.0052. The corrected mortality

obtained in 0.1% colchicine concentration is 73.6 which is higher than LC50=0.0052. This suggests that the lower concentration of colchicine should be tested for analysing its effect on the sunflower anther.

5.1.1.2 Colchicine Treatment to Callus Through Medium

The callus was inoculated into colchicine containing medium at different concentrations *viz.*, 0.5, 1, 1.5 and 2% for 72 hours and then shifted to colchicine free medium. The survival percentage of treated callus were 82.5, 72.5, 62.5 and 47.5% respectively, and the response of untreated control was 95%. The callus regeneration percentage of control and treated callus was 96.66, 86.66, 76.66, 66.66 and 55% respectively. The corrected mortality obtained at highest tested colchicine concentration 2.0% is 43.1 which is lower than LC50= 2.67. Colchicine treated calli were brown colour as compared to control green calli.

It is obvious from present investigation that calli survival and regeneration is more in colchicines treatment in media as compared to direct colchicine treatment.

5.1.2 Molecular Characterization of Callus for Haploid Confirmation

Sunflower genotype SS-2038 and calli were subjected to the molecular analysis. DNA was extracted from donor plant and regenerated callus in direct and indirect colchicine treatment by Doyle and Doyle (1987) protocol with some modifications and yielded 900-1700 ng/µl DNA.

In present study *in vitro* colchicine treated callus of sunflower genotype was selected for SSR marker analysis. A co-dominant SSR marker was used to separate haploid and diploid plants. The extracted DNA was subjected to PCR amplification by using 28 SSR primers, of these 15 SSR primers used to confirm the androgenic trait of the haploid calli lines of sunflower genotype.

The results obtained from SSR fingerprinting were found to be useful for identification of haploids and parentage confirmation. Both regenerated lines and the anther donor parent were analysed with various SSR markers. The results showed that both the regenerated haploid lines had homozygous profiles with a single band while the donor parent had two bands that indicated allelic sites. The banding patterns of the ORS-05(300), ORS-662(314), ORS-1265(222), ORS-552(450), ORS-488(200), ORS-243(220), ORS-1159(270), ORS-928(230), ORS-58(300), ORS-337(220), ORS-959(280), ORS-154(170), ORS-780(180), ORS-423(220) and ORS-536(300) appeared in the antheral calli lines and the donor plant line. The 15 SSR markers

serves diagnostic locus to haploid and showed 100% homozygosity in calli, confirming the haploid nature of regenerated calli.

The fifteen SSR markers, identified in present study *viz.,* ORS-05, ORS-662, ORS-1265, ORS-552, ORS-488, ORS-243, ORS-1159, ORS-928, ORS-58, ORS-337, ORS-959, ORS-154, ORS-780 ORS-423 and ORS-536 can be used for confirmation of haploid in sunflower anther culture.

The similarity matrix was generated by using scored data of SSR fingerprint and used to depict dendrogram. Two major clusters were found to represent unique grouping of 12 members (eleven haploids and one donor parent) at 50% similarity value. These clusters *viz*., Cluster-I, Cluster-II comprised of 7 and 5 colchicine treatments respectively, clearly separating haploids from diploid donar plant.

In present study allelic results shown that, extra unique alleles were not found in the regenerated calli (in direct treatment to anther and media supplement) at a locus other than diagnostic heterozygous allele present in donor sunflower genotype indicates absence of somaclonal variation in the regenerated calli, suggesting no any changes in the DNA sequence of the calli.

As per SSR analysis all calli derived from direct and indirect colchicine treatments were homozygous giving single allel whereas donor plant was heterozygous with two allels. Therefore, according to the SSR markers and FCM results calli derived from direct and indirect colchicine treatments are double haploids. The double haploids are also obtained in the control callus in both the cases, indicates spontaneous dihaploidization of the chromosomes.

From the present study, it is evident that, the molecular tools, SSRs are useful and effective for the confirmation of the androgenic trait of the haploid.

5.2 Conclusion

This information may be important for understanding the correlation of haploid trait with molecular markers and development of new approaches for selective breeding of sunflower. The present investigation reveals following conclusions,

1. The colchicines both in direct and indirect treatment had great impact on anther survival and callus induction frequency, but the concentration need to be optimise further.
2. Calli survival and regeneration in indirect colchicine treatment is more as compared to direct colchicine treatment.

3. The SSR fingerprinting data revealed that the sunflower genotype (SS-2038) produced haploids in the study are the true haploids, confirmed producing both regenerated calli and donor plant specific banding pattern.
4. The haploid specific bands generated in SSR analysis by primers ORS-05(300), ORS-662(314), ORS-1265(222), ORS-552(450), ORS-488(200), ORS-243(220), ORS-1159(270), ORS-928(230), ORS-58(300), ORS-337(220), ORS-959(280), ORS-154(170), ORS-780(180), ORS-423(220) and ORS-536(300) can be used for the identification of haploids of sunflower. These markers may become a useful tool for identification of androgenic trait of the haploid and double haploids in sunflower.
5. Calli obtained from direct and indirect colchicine treatments are double haploids.
6. SSR markers and FCM analysis can be used to determine the ploidy level of sunflower, as diploid/haploid or double haploid.

Also, tissue culture and molecular studies results obtained during the present study are useful for planning of effective breeding of sunflower. Further research is needed to determine the best combinations on the molecular basis for the development of sunflower haploids and double haploids for crop improvement programme.

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ABSTRACT

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Analysis of Colchicine Effect on Anther Culture of Sunflower (*Helianthus annuus* L.)

Sunflower (*Helianthus annuus* L.), is a multi-purpose crop employed for diverse uses such as dye production, edible oil extraction and for medicinal applications. Production of microspore-derived embryos from cultured anthers is now well established technique for isolation of homozygous lines in sunflower. In this present study different concentrations of colchicine 0.1, 0.5, 1, 1.5 and 2% were used to develop haploid and increase ploidy level of sunflower by direct treatment to anther and treatment to callus in medium.

In direct colchicine treated anthers response in survival percentage were 25, 22.5, 17.5, 12.5and 10%, respectively; whereas, the response of untreated control was 95%. The callus induction frequency was 23.33, 20, 16.66, 11.66 and 8.33, respectively and 88.33 for untreated control. In indirect colchicine treatment, survival percentages of treated callus were 82.5, 72.5, 62.5and47.5% respectively and 95% of untreated control. The callus regeneration percentage of control and treated callus was 96.66, 86.66, 76.66, 66.66 and 55%, respectively.

Ploidy level of regenerated callus and its donor plant were confirmed by SSR marker and flow cytometry. Out of 28 SSR markers, 15 markers *viz*., ORS-05, ORS-662, ORS-1265, ORS-552, ORS-488, ORS-243, ORS-1159, ORS-928, ORS-58, ORS-337, ORS-959, ORS-154, ORS-780, ORS-423 and ORS-536 were found useful for the identification of haploids in sunflower. Both the regenerated haploid lines had homozygous profiles with a single band, while the donor plant had two bands that indicated allelic sites. The fifteen SSR markers showed 100% homozygosity.

According to SSR markers and Flow cytometry results, calli derived from direct and indirect colchicine treatments are double haploids. The double haploids are also obtained in the control callus in both the cases, indicates spontaneous dihaploidization of the chromosomes.

Key words: Sunflower, anther culture, colchicine, SSR, flow cytometry, ploidy.

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