DOUBLE HAPLOIDS IN PEARL MILLET

MAJOR PROJECT REPORT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

NIHARIKA.T

08J21A2336

DEPARTMENT OF BIOTECHNOLOGY

JOGINPALLY B.R ENGINEERING COLLEGE

(AFFILIATED TO JNTUH, HYDERABAD, A.P)

YENKAPALLY, RANGAREDDY-500075.

2012

JOGINAPALLY B.R ENGINEERING COLLEGE
(Affiliated to Jawaharlal Nehru Technological University, Hyderabad, A.P)
Yenkapally, Moinabad mandal, RangaReddy dist-500 075

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

This is to certify that the project work entitled **"DOUBLE HAPLOIDS IN PEARL MILLET"** is a bonafide work carried at **International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru** by NIHARIKA.T, bearing the registered number 08J21A2336 in partial fulfillment of the requirements for the degree **BACHELOR OF TECHNOLOGY** in **BIOTEHNOLOGY** from **Joginapally B.R. Engineering College**, Yenkapally, affiliated to Jawaharlal Nehru Technological University, Hyderabad, during the academic year 2011-2012.

G.Srinivas
Assistant Professor
(Internal Guide)

Prof.G.Venkateshwarlu,
(Head of Dept.of Biotechnology)

Professor & Head
Department of Bio-Technology
J.B.R. Engineering College
Yenkapally (V), Moinabad (M)
R. R. District - 500 075

ICRISAT
Science with a human face
International Crops Research Institute for the Semi-Arid Tropics

Certificate

This is to certify that the dissertation entitled **Double Haploids in Pearl Millet** submitted by **Ms Niharika Tirumani**, had been carried our under my supervision. This work is towards the partial fulfillment of the requirements for her degree of Bachelor of Technology from **Joginapally BR. Engineering College, Moinabad**. This work is original and not has been submitted for other degree at any university.

Date : 30-4-2012

Nalini Mallikarjuna

Dr Nalini Mallikarjuna
Principal Scientist
Cell Biology

Headquarters: Patancheru 502 324, Andhra Pradesh, India
Tel +91 40 30713071 **Fax** +91 40 30713074 +91 40 30713075 **Email** icrisat@cgiar.org
Liaison Office: CG Centers Block, NASC Complex, Dev Prakash Shastri Marg, New Delhi 110 012, India
Tel +91 11 32472306 to 08 **Fax** +91 11 25841294

ICRISAT-Nairobi (Regional hub ESA)
PO Box 39063, Nairobi, Kenya
Tel +254 20 7224000
Fax +254 20 7224001
Email icrisat-nairobi@cgiar.org

ICRISAT-Bamako (Regional hub WCA)
BP 320
Bamako, Mali
Tel +223 20 223375
Fax +223 20 228683
Email icrisat-w-mali@cgiar.org

ICRISAT-Maputo
BP 1243
Maputo, Mozambique
Tel +258 21 461657
Fax +258 21 461658
Email icrisatmz@panintra.com

ICRISAT-Bulawayo
Matopos Research Station
PO Box 776
Bulawayo, Zimbabwe
Tel +263 383 311 to 15
Fax +263 383 307
Email icrisatzw@cgiar.org

ICRISAT-Lilongwe
Chitedze Agricultural Research Station
PO Box 1096
Lilongwe, Malawi
Tel +265 1 707297/71/67/87
Fax +265 1 707075
Email icrisat-malawi@cgiar.org

ICRISAT-Niamey
BP 12404
Niamey, Niger (via Paris)
Tel +227 20 722529, 20 722530
Fax +227 20 734329
Email icrisatn@cgiar.org

www.icrisat.org

Declaration

I hereby declare that this thesis is the result of work carried out by me in **"DOUBLE HAPLOIDS IN PEARL MILLET",** has been done under the supervision of **Dr. Nalini Mallikarjuna**, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru**.** This work is original and has not been submitted in part or full for any other degree of any other institution or University.

NIHARIKA.T

(08J21A2336)

Acknowledgment

My sincere thanks to ICRISAT for providing an opportunity to work in this esteemed organization.

I would like to thank Dr.Nalini Mallikarjuna, for her guidance and support in wide crosses and cell biology laboratory, ICRISAT. I am eternally grateful to her.

I am thankful to Dr. DEEPAK JADHAV,Scientific officer, wide crosses and cell biology laboratory for his support.

I spcialy thank all students and members of the lab for providing constant support and the tips they shared from thir experiences.

I thank Prof G Venkateshwarulu, head of dept. of Biotechnology, Dr. N Suneetha, HOD-in-charge and Dr.Zehra Siddiqui, project coordinator for allowing me to do the project outside the college and for their support and encouragement.

I would like to thank my internal guide G.Srinivas, assistant professor for his support and valuable suggestions for the completion of my project. I would like to thank the other faculty at college for their support and suggestions.

I am sure that this is not the complete list. I thank one and all for their help.

CONTENTS

List of tables:

List of figures:

Abbreviations:

2-4D	2,4-Dichlorophenoxyacetic acid
NAA	Naphthaleneacetic acid
Kn	Kinetin
Zn	Zeatin
2ip	2 Isopentenyladanine
TDZ	Thidiazuran
BAP	Benzyl amino purine

ABSTRACT:

Anther culture is an important technique to generate double haploid plants. Doubled haploids would help to produce homozygous lines in single step which accelerates the breeding programmes. They are also useful for studying mutagenesis, dosage effects and interaction & linkage of genes. Pearl millet is a diploid (2n=14) plant and summer crop that originated from west Africa. It has become a challenge to develop a reproducible protocol for double haploids in pearl millet. For culture initiation it is necessary to select florets and anthers with appropriate developmental stage of microspore development. The optimal stage for pearl millet anther culture development has been found to be when the microspores are uninucleate with the nucleus in the center and when the spike is still enclosed in the flag leaf sheath. The growth of the donor plants and developmental stage of microspores have been important factors in the intiation of anther cultures. The present study aims to assess the effect of different factors such as growth hormones, media, pretreatments on the induction of androgenesis (division of microspores) and to develop a suitable protocol for producing embryoids of pearl millet in vitro using floret and anther culture.

Keywords: Anther culture, pearl millet, double haploids, embroids.

1.INTRODUCTION

The term ‘haploid’ refers to any organism, tissue or cell having the chromosomal constitution similar to the gametes of a given species. Haploid production is a very useful intermediary biotechnological tool for plant breeders and geneticists. Because of the single dose of each allele in haploids, they can be employed in several areas of basic research such as genetics, cytogenetic and mutation breeding. Haploids can be induced by either in situ or in vitro methods. Several methods have been developed for inducing haploids by artificial means (in situ) such as emasculation, delayed pollination, “bulbosum method” as in barley and wheat, and irradiation by gamma or X-rays. In vitro haploid production provides a rapid means of achieving homozygosity, thereby accelerating breeding as already in practice for many crops like rice and wheat.

Biotechnology offers great potential for creation, conservation, characterization and utilization of germplasm for breeding programs. Biotechnological tools such as genetic transformation (transgenic production), embryo rescue following wide hybridization, somoclonal variation (mutant selection), and anther and ovary culture (haploid production) can revolutionize crop improvement by helping to develop useful and novel cultivars with unique characteristics.

1.1 Applications of doubled haploids in crop improvement

“Haploid” refers to any organism, tissue or cell having the chromosomal constitution similar to the gametes of a given species (chase, 1947). Because of the single dose of each allele in haploids, they can be employed in several areas of basic research in genetics and cytogenetic. Lines derived from haploids provide a rapid means of achieving homozygosity, and accelerate breeding of new varieties. For example,

doubled haploid (DH) plants are useful for developing inbred lines (Fouroughi-Wehr and Wenzel, 1990). They also facilitate selection of recessive mutations at the cellular level (Grafe et al, 1986; Marion-Poll et al, 1988). *In vitro* haploid production has a great potential for mutation studies in crop plants since it permits screening of several thousand individuals at unicellular stage. In addition, homozygous DH lines can be usefully deployed to elucidate the behavior of traits controlled by recessive alleles.

A new field of haploid usage is in genome characterization and genetic linkage mapping particularly for QTL analysis. DH populations are an important tool to obtain reproducible DNA polymorphism in barley (Heun et al, 1991) maize (Murigneux et al, 1993) and rice (McMouch et al, 1991; Xu et al, 1994). DH populations are also used effectively for comparative mapping and to find out the male and female recombination frequencies. Perhaps the most exciting application of haploidy in cereals is for genetic transformation. Since microspores or egg cells are both single and haploid, they are attractive targets for transformation (Ziauddin et al 1990; Jahne et al. 1994). They allow the integration of foreign genes into a haploid genome, which after subsequent diploidization develops into a completely homozygous plant. Delivery of DNA into such homozygous genomes would omit the recovery of transformants with lethal mutants while simultaneously avoiding formation of chimerical regenerates.

1.2 Methods of producing doubled haploids

Haploids may arise spontaneously (spontaneous parthenogenesis, Polyembryony, spontaneous androgenesis), but their frequency is too low (less than 10") to be of any use in practical breeding. First spontaneous haploid was found in Datura (Blakeslee et al, 1922) followed by that in Nicotiana (Clausen and Mann, 1924), Triticum compactum (Gaines and

Aase, 1926), Oryza sativa (Morinaga and Fukushima, 1931), Zea mays (Randolph, 1932), and Hodeum vulgare (Johanson, 1934).

Haploids can be induced by two methods: in situ or in vitro. Much effort has been made to induce haploid sporophytes of crop plants by various physical, chemical and biological stimulants or via in vitro culture. Several methods have been developed for inducing haploids by artificial means (in situ). These are emasculation, delayed pollination, bulbosum method and irradiation by gamma or X-rays.

Parthenogenesis following interspecific hybridization with irradiated pollen has yielded haploids in most of the dicotyledonous species like tobacco and sugar beet (Horlow et al, 1996). Systems that induce parthenogenesis include alien cytoplasm substitution as in wheat, use of indeterminate gametophyte gene as in maize (Lin, 1978) or haploid initiator gene as in barley (Hagberg and Hagberg, 1980).

Wide hybridization followed by chromosome elimination of one of the Partners by interspecific and intergeneric hybridization was successfully used for haploid production in cereals. A large number of barley haploids were obtained by crossing Hordeum vulgare (cultivated species) with Hordeum bulbosum (wild species, "bulbosum" method). Irrespective of the direction of the cross, the haploids recovered were of the vulgare genome (Symko, 1969; Kasha and Kao, 1970; Subrahmanyam and Kasha, 1973; Matrk and Mahu, 1994). By making intergeneric crosses between wheat plants used as female parent and maize plants as pollinators, haploid plants were obtained by following embryo rescue method (Laurie and Bennett, 1988).

There are limitations in case of each of the above-mentioned techniques. The bulbosum method works satisfactorily in barley and to some extent in wheat. Following the report of Guha and Maheswari (1964) on direct development of embryos from microspores of Datura anthers in vitro, several efforts were made to produce haploids by in vitro culture of

male or female gametes. During last 30 years, haploid plants or tissues have been obtained through anther or ovary culture from hundreds of species including members of Graminae (Sopory and Munshi, 1996; Keller and Korzun, 1996). The frequency of androgenesis was successfully increased in a number of cereal crops, and new DH cultivars of wheat (de Buyser et al, 81), barley and maize have been released. Use of anther culture has contributed more than 100 new cultivars of rice in China (Meifang, 1992) and over 42 japonica rice cultivars in Korea. Exploitation of gametoclonal vanation has resulted in the development and release of a rice cultivar Dama in Hungary (Heszky et al, 1991). Up to 1991, 21 improved wheat cultivars were developed in China using anther culture and released into production. Currently many wheat breeding Programs combine anther culture with conventional breeding as a routine method for wheat improvement in China and in Europe.

1.3 Pearl millet as the candidate crop for study

Pearl millet is a diploid (2n = 2x = 14) C4 species known by the scientific name ***Pennisetum glaucum*** (L) ***R.*** Br. Its common names are bulrush or cattail millet, or ***bairn*** (Hindi). It is a summer crop, and originated from West Africa. Pearl millet is the sixth most important cereal in the world and widely cultivated in the semi-arid tropics as a major staple food crop. If pulse is the poor man's meat, millet is the poor man's bread. It is equal or even superior to rice and maize in protein and oil content. It is cultivated on about 26 million hectares in semi-arid tropics of the Africa and the Indian sub-continent for food, fodder, feed and fuel. It is also grown on a small scale as a high quality forage crop in the United States, Australia, South America and southern Africa (Ananad Kumar and Andrews, 1993). It is a hardy cereal suited to areas with low and erratic rainfall, and can grow in soils that are too infertile or sandy for sorghum or maize cultivation.

World population is going to become 9 billion by the end of 2020.

Malthusian population predictions continue to alarm agricultural researchers. There is shrinkage of both agricultural land and agricultural workforce because of urbanization and industrialization. Successful agronomic policies have to seek new technologies that will allow us to produce more and better food by fewer people on less land. Because of its exceptional ability to tolerate drought, pearl millet may extend food grain production into regions too arid for sorghum.

1.4 Why doubled haploids in pearl millet?

Most of the current breeding in pearl millet is aimed at maximum Exploitation of hybrid vigor for both grain and forage yields. The diverse germplasm in pearl millet is useful to create usable genetic variability and to broaden the genetic basis of cultivars. It is ideally suited for exploitation of heterosis because of its high level of heterozygosity and susceptibility to inbreeding depression. Inbreeding of popular parental lines of pearl millet *(TI86, T23DA,* and *T23DB)* led to a sudden decrease in vigor and exposed recessive types of chlorophyll deficiencies and sterility. A marked inbreeding depression was observed for several agronomic traits and led to meiotic irregularities including a decline in chiasma frequency. Heterozygote's produced by crossing inbred lines show significant increase in chiasma frequency. In addition, favorable agronomic traits like higher photosynthetic efficiency and faster grain filling are observed in hybrids when compared to their parental lines. Promising progeny lines from such hybrid populations can be selected and genetic fixation of useful sets of traits in the recombinants can be achieved quickly using *in vitro* haploid production technique.

1.5 Why in *vitro* cultures for haploid production?

Pearl millet being a cross-pollinated crop is highly heterozygous and heterogeneous in nature. Genetic studies of this crop necessitate the

development of homozygous inbreds. Out of different methods to induce haploidy, *in vitro* culture is most popular. Haploids obtained from *in vitro* culture of gametophytes (either male or female) of hybrid plants can be doubled through various means. This kind of production of haploids from F_1 hybrids leads to the fixation of gene combinations which otherwise may not be easily possible to isolate from a segregating population through conventional means. Doubled haploid lines carrying desirable genes from parental lines can be a very useful source for developing superior hybrids or synthetics.

Powell et al (1975) discovered two naturally occurring haplo-haplo twin seedlings (2n = 2x = 7) of pearl millet and studied until they set seed before describing their haploid characteristics. Manga and Pantulu (1969) described meiotic chromosome irregularities in the pollen mother cells of the naturally occurring haploids. However, the rate of such spontaneous haploid occurrence was usually very low (in the case of Tift 23A, it is 1 in 10000). Until now, very limited work is carried out on in vitro production of haploids in pearl millet (Dang Ha and Pernes, 1982; Nitsch, 1982; Choi et al, 1989). Therefore, the present study was in vitro androgenesis by anther and floret culture.

1.6 Specific objectives of thesis

- The overall goal of my study is to develop a suitable protocol for producing doubled haploids of pearl millet in vitro using suitable explants such as anthers and florets.
- To determine different factors affecting anther culture.
- To maximize callus induction.
- To characterize the doubled haploids produced and to verify their haploid origin.

2.Review of literature

Haploids can be produced from *in vitro* cultured meiotic spores of either male or female origin. Induction of haploids through anther or pollen culture is termed as androgenesis and that from unpollinated ovary culture is called gynogenesis. Efforts to develop protocols for *in vitro* haploid production were initiated in cereals over 32 years ago (Niizeki and Ono, 1968). As most attempts have been only on major cereals, success is limited to few cereals like rice, maize, wheat and barley.

After initial research on androgenesis and gynogenesis in higher plants for production of doubled haploid plants, anther culture caught the attention of most researchers, especially after its success in *Datura* (Guha and Maheswari, 1964). Subsequent failure to trigger androgenesis in several recalcitrant species (Keller, 1990) revived an interest in gynogenic pathways. Thus, gynogenesis was explored in dicotyledonous species like onion and sugar beet (Campion and Auemonti, 1988; Van geyt et al. 1987; Ferrant and Bouharmont. 1994). In cereals, all of the three methods - anther culture, ovary culture, and isolated microspore culture have been applied for *in vitro* haploid regeneration. Each method has its own advantages and disadvantages.

Protocols for *in vitro* haploid production are continuously being refined in major cereals listed above. However, in the case of pearl millet, apart from the analysis of spontaneous haploids (Powell et al, 1975), only a few publications are available on in *vitro* studies of haploid production (Dang Ha et al, 82; Nitsch et al. 1982).

2.1 Anther culture

Dang Ha and Pernes (1982) were the first to report successful in vitro androgenic haploids in pearl millet lines Tifl23 D2B and a F1 hybrid.

In the same year, Nitsch (1982) reported haploid regeneration from in vitro cultures of anthers. Vasil and Haydu (1981) carried out studies on somatic embryogenesis of a related species *Pennisetum purpureum* and obtained diploids from in vitro culture of anthers. However, all the regenerated plants were from the cells derived from the anther wall. Nalini (1987) examined the suitability of different culture media for pearl millet anther culture and obtained multicellular microspores on MS media, but regeneration of whole plants was not achieved. Thus there was no established, suitable protocol for in vitro haploid production in pearl millet. Further efforts are demonstrated by only one report (Choi et al, 1989), which focused on two genotypes for refining the technique of androgenesis and regeneration. Keeping in view of these limited reports on androgenic induction and regeneration, in pearl millet, (a) improvement of culture conditions, (b) modifications of induction and regeneration media, and (c) screening and selection of best responding genotype must be considered for improving the techniques.

2.2 Technique for anther culture

Various workers modified the original technique of anther culture developed by Guha and Maheswari (1984; 1966). It has been applied with varying degrees of success in obtaining haploids in many plants including crop species (Chu, 1982; Maheswari et al, 1982; Sopory and Munshi, 1996). In *vitro* androgenic ability depends on the frequency of atypical pollen that deviates from its normal gametophytic pathway. Though a small fraction of such atypical pollen is observed in vivo, this type of pollen never contains more than four cells or nuclei. On the other hand, by optimizing various factors that influence androgenesis, frequency of such atypical pollen can be increased in vitro. Competence for pollen embryo induction depends on different treatments during preculture period.

Further growth of such induced embryos is dependent on various in vitro conditions and the culture media used.

2.2.1 Direct anther culture

In this method, the excised flower buds are surface-sterilized and the anthers are removed. They are subsequently cultured either on solid or liquid medium. Depending on the incubation conditions and plant species (for review, Sopory and Munshi, 1996), the developing microspores produced either embryos or calli that emerged through anther lobes. Subsequently these calli were transferred onto the regeneration medium to recover haploids.

2.2.2 Float anther culture

Sunderland and Roberts (1977) introduced the technique of floating anthers on a liquid medium in petridishes. The anthers dehisce and the developing pollen embryos are shed into the medium. These shed pollen embryos are subsequently transferred to the differentiation medium for plantlet regeneration. Float anther culture has been successfully used in many species (Xu et al, 1981; Chen and Chen, 1983).

2.3Factors influencing anther culture

Various factors effect androgenesis at physical, physiological, chemical and genetic levels (for reviews Maheswari et al, 1980; 1982; Bajaj, 1983, Hebrle- Bors, 1985; Sopory and Munshi, 1996). Protocols were improved during the last two decades in other cereals like rice, maize and barley where even microspore derived transgenic plants were regenerated (Jahne et al, 1994). These improvements in the technique of anther culture usually involved - screening of different genotypes for

initial induction and further use of selected ones; physiological status and growth conditions (temperature and light intensity) of the donor plants; developmental stage of the explant and in vitro culture conditions. All these are critical for inducing best response from anthers..

2.3.1Genotype of the donor plant

Genotype of the donor plant plays a significant role in androgenesis (Guha-Mukherjee, 1973; Jones and Petolino, 1987; Zhou and Konzak, 1989; Lu et al, 1991; Prakash and Giles, 1992; Reddy et al, 1985; Sadasiviaah et al, 1999). One of the major problems encountered with cereal anther culture is that some genotypes are poor responders to the techniques used. Even in a major cereal crop like wheat, use of anther culture is limited (in breeding programs) by strong genotype specificity in androgenic response and a related problem of production of albinos.

Genotype that shows maximum number of androgenic structures might not show good rate of green plant regeneration and vice versa (Dunwell et al, 1992). Genotypic differences on in *vitro* pollen embryoid formation were studied in rice (Guha-Mukherjee, 1973). For pollen callus induction rate in wheat (Lu et al, 1991) genotypes showed 0 to 35% response.

The choice of a suitable genotype for a study depends on the objective of the experiment. When attempting DNA transfer into haploid genome, it is essential to select genotypes that are best able to regenerate plants. However, even genotypes which produce a greater number of initial androgenic structures (irrespective of their final regeneration capacity) are useful for physiological and biochemical analysis.

2.3.2 Physiological status

The frequency of haploid production can be enhanced by modifying the environmental factors under which the donor plants are

grown. The physiological conditions of the donor plant determine final success in tens of number of haploid embryos in cultured anthers. A correlation between plant age and anther response has been demonstrated (Maheswari et al, 1980; 1982). Factors like temperature variation and light intensity during growth of the donor plant also affect androgenic response (Sopory and Munshi, 1996).

2.3.3 Anther and pollen developmental stage

Nitsch and Nitsch (1969) were the tint to show that high embryo yields could be obtained by restricting the culture of only those anthers that contain microspores at optimal developmental stages. While working with 20 cultivars of rice, Guha-Mukherjee (1973) recommended that anthers containing uninucleate microspores are optimal for androgenesis. Generally, this is the case with most of the monocotyledons, especially, the cereal crops.

2.3.4 Preculture treatments

Various preculture treatments that were shown to enhance androgenesis include cold treatment, chemical treatment, atmospheric conditions, etc. Out of these, cold treatment has been most widely used. Effect of cold treatment was first reported by Nitsch and Norreel (1973) and subsequently confirmed by several reports using various crops (Sopory and Munshi, 1996). Besides cold treatment, other successful pretreatments include centrifugation, high temperature treatments, and maintenance of detached inflorescence in water for several days (Sangwan-Norreel, 1977). Carbon diozide pretreatment increased the callusing efficiency of the rice panicles by more than 28 times (Raina, 1997a; 1997b)

2.4 Culture conditions

2.4.1 Physical factors

Physical factors such as pre- and post-culture temperature treatments. Different light regimes for incubation of cultures, pH, atmospheric conditions and addition of various chemicals to the media to suit the requirements of plant species or even specific cultivars (Maheswari et al, 1980; 1982). Sometimes even factors such as orientation of anthers under culture also contributed significantly in altering the induction rate (Powell et al, 1988).

2.4.2 Temperature

Temperature influences induction of pollen embryo or callus development. High temperature treatments benefited embryo induction frequencies in dicotyledonous (reviewed by Sopory and Munshi, 1996). However, in cereals, usually low, rather than high temperature treatments increase embryo induction frequency.

2.4.3 Light

Effect of light on androgenic induction was extensively studied in Datura and Nicotiana. Despite many reports, the precise role of light in anther culture remains obscure because of the contradictory results. Nitsch (1977) reported beneficial effect of red light in Nicotiana while Sopory and Maheswari (1976) reported inhibitory effect of the red light on androgenesis in the same crop.

2.4.4 Anther density and orientation

The number of anthers or microspores per volume of culture vessel affects the percentage success. Gaillard et al, (1991) working with maize reported that 6-8 x lo4 microspores per ml was optimum density, while in wheat, Hoekstra et al (1993) found the optimal density to be **2** x 10~microsporesp per ml. Positioning of the anther onto the medium has pronounced effect on embryo formation. Orientation matters more in cereals where anther size is usually small; the surface tension induced film of liquid around the anther is large compared to that on the large anthers (Shannon et al, 1985). This aspect was reported to effect embryo production at least in two important cereals, rice (Mercy and Zapata, 1987) and barley (Shannon et al, 1985; Powell et al, 1988; Lu et al, 1991). In barley, embryo production has increased when a single lobe was in contact with the medium. However, anthers, when cultured flat (both the lobes in contact with the culture medium) did not show any response (Shannon et al, 1985; Powell, 1988).

2.4.5 Chemical factors

Anther and pollen in vivo are known to contain usual range of hormones including ABA. Despite this fact, they require chemical or other stimulus to release the pollen from a hormonally controlled dormancy and simultaneously to deviate from gametophytic to sporophytic pathway. Large-scale induction and derivation of a complete embryo from a single haploid cell depends upon the use of refined culture media.

In Solanaceous species, regeneration from embryos is so simple that germination of embryos can occur in induction media itself. In cereals, the induction medium contains complex combination of growth regulators and high sucrose levels (which are essential for embryo

induction). Usually, after initial induction phase, plantlet development does not occur unless the embryos are transferred to suitable regeneration media with fewer hormones containing lower sucrose levels. An exception to this is rice, where a single step culture method was developed for some cultivars (Karim and Zapata, 1990). Both the type and concentration of auxin influence somatic embryogenesis from in vitro from explants. Complete absence of auxin and increase in cytokinin content favors regeneration of embryos. Even if present, 2, 4-D at a minimal concentration or a weak auxin like IAA is recommended. Sustained activities of nucleic acid (DNA and RNA) and protein synthesis are very much essential for the microspore to follow the successful sequence for haploid plant regeneration, i.e. from multinucleate microspore -> multi-cellular microspore -> pro-embryo -> mature embryo -> differentiated plantlet. This is emphasized by studies that demonstrated the negative influence of inhibitors of nucleic acid and protein on the frequency of pollen embryogenesis. Availability of amino acid pool is required for all steps in this process. Pollen of certain species (e.g., *Petunia* ***hybrida:*** Sangwan and Norreel, 1977) may have a particular amino acid profile, which may allow embryogenic induction and growth without supply of exogenous supply of the same through culture media. However, cereal pollen embryogenesis depends on amino acids supplied exogenously (Nitsch, 1982) or available through conditioning from tapetal cells of anthers. Difficulties in plant regeneration have been encountered in several cereal species. Induction of large number of multicellular microspores is observed often, but majority of these fail to develop further. In cereals, shoot regeneration from macroscopic structures generally occurs at frequencies of less than 50%. Besides, albino plant regeneration (usually **>80** %) is a major problem with cereals. Elevated culture temperature, high concentration of 2,4-D in the induction media and multi-cellular pollen that contain micronuclei (Chu et al, 1978) increase the frequency of

albinos. Chemical treatments applied to pollen or microspores with anti-mitotic herbicides (Bennett and Hughes, 1972), or antimitotic agents such as colchicin trifluralin, amiprophos-methyl-nitroside (APM) enhanced androgenic response (for review, Sopory and Munshi, 1996).

3.MATERIALS & METHODS

3.1Plant material

The Four genotypes of Pearl millet hybrids ICMB 89111, XL-51, 4201, 86M34 were used for anther culture. The inflorescence, which was still enclosed inside the flag leaf, was collected from the ICRISAT field.

3.2 Cold pretreatment

The spikes were placed in a polythene bag with 4-5 drops of water. They were then placed in refrigerator (dark) at **4^{o}c** for 2 or **7** days depending on the pretreatment.

3.3 Observation of microspore developmental stage

The pearl millet inflorescence is a contracted spike or a false spike, bearing densely packed clusters of spikelets borne in pairs. The anthers are extremely tiny and delicate, and therefore difficult to isolate. Hence, in most experiments we cultured spikelets. Anthers showing uninucleate microspore stage from the bisexual florets were used for initiating spikelet cultures. For confirming microspore developmental stage, one anther (out of three present in each spikelet) was removed from the spikelet, squeezed onto a microscopic slide and macerated in a drop of acetocarmine observed for microspore developmental stage. Only the portion of the spike containing uninucleate microspores was used for culture initiation and rest of the spike was discarded.

3.4 Media preparation

The culture medium with varied combinations of plant growth regulators were selected with Ch1 as basal media. It is used to study the effect of

intiation of androgenesis. For further induction the florets with emerged anthers containing multicellular microspores are transferred into Yu-Pei media.

Ch1 media composition:

Ch1 major stock

KH_2PO_4	170mg/lt
KNO_3	2500mg/lt
$MgsO_4.7H_2O$	370mg/lt
$CaCl_2.2H_2O$	600mg/lt

MS Iron stock: 10ml/lt

Ch1 minor stock

$CuSO_4.5H_2O$	0.05mg/lt
$CoCl_2.6H_2O$	0.025mg/lt
H_3BO_3	6.2mg/lt
$MnSO_4.4H_2O$	16.9mg/lt
$Na_2MoO_4.2H_2O$	0.25mg/lt
$ZnSO_4.7H_2O$	10mg/lt
KI	0.83mg/lt

Ch1 organic & vitamins

Casein hydrolysate	200mg/lt
myoinositol	100mg /lt
Nicotinic acid	5mg/lt
Pyridoxine HCl	0.5mg/lt
Thiamine HCl	0.5mg/lt

Sucrose :

Ch-1.7	17gm/lt
Ch-8	80gm/lt

Agar: 16gm/lt.

Yu-pei media composition:

Major stock

KNO_3	2500mg/lt
NH_4NO_3	165mg/lt
$CaCl_2.2H_2O$	176mg/lt
KH_2PO_4	510mg/lt
$MgSO_4.7H_2O$	370mg/lt

Minor stock (100x)

$MnSO_4.4H_2O$	4.4mg/lt
$ZnSO_4.7H_2O$	1.5mg/lt
H_3BO_3	1.6mg/lt
KI	0.8mg/lt

Chelated Iron stock(100x):

Na_2EDTA	37.3mg/lt
$FeSO_4.7H_2O$	27.8mg/lt

Vitamins stock(100x)

Glycine	7.70mg/lt
Thiamine HCl	0.25mg/lt
Pyridoxine HCl	0.25mg/lt
Nicotinic acid	1.30mg/lt
Casein hydrolysate	500mg/lt

Sucrose: 80gm/lit

Agar: 8gm/lit

Hormones :2mg/lt 2-4d+1mg/lt 2ip+1mg/lt Kn

	A	B	C	D	E	F	G	H	I	J	K	L
Basal media(Ch)+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose(1.7%)	1.7%	1.7%	1.7%	1.7%	1.7%	1.7%	1.7%	1.7%	1.7%	1.7%	8%	8%
Agar(1.6%)	+	+	+	+	+	+	+	+	+	-	+	+
P^H 5.8	+	+	+	+	+	+	+	+	+	+	+	+
2-4D(mg/lt)	-	-	--	-	-	2	2	2	2	2	2	-
NAA(mg/lt)	2	2	2	2	2	-	-	-	-	-	-	2
Kn(mg/lt)	1	-		-	-	1	-	-	-	-	-	--
Zn(mg/lt)	-	1	-	-	-	-	1	-	-	-	-	-
TDZ(mg/lt)	--	-	1	-	-	-	-	-	1	-	-	-
BAP(mg/lt)	--	-	-	1	-	-	-	-	-	-	-	-
2ip(mg/lt)	-	-	-	-	1	-	-	1	-	1	1	1
Light phase	+	+	+	+	+	+	+	+	-	+	-	-
Dark phase	-	-	+	+	+	+	+	+	+	+	+	+

Table 3.1: Composition of media used for Androgenesis

3.5Culture initiation

For initiation of floret cultures, individual florets were removed from spikes (control or pretreated), observed for the uninucleate stage of the microspore as described in **3.3,** and surface sterilized with mercuric chloride (0.1%) for 15 min. This was followed by 3 washes with sterile distilled water under Laminar air flow. Florets were then cultured under aseptic conditions on semi solid Ch-1.7 & Ch-8 media depending on the experimental requirements .These cultured plates were then incubated either under light (4000 lux white light) or in dark depending on the experimental requirements. Temperature of culture room was **24 ±2°C,** with a photoperiod of 8h in light phase. The florets with emerged anthers, few were transferred to semi solid YP media with 80g/l sucrose and few florets were retained on the same media.

For direct anther culture, controls, after cold pretreatment at 4^0C and after high speed and low speed centrifugation, the anthers containing uninucleate microspores were manually removed from individual florets and directly placed on JFK-1.7 medium

3.6 Observations and scoring of data

In all the experiments, anthers were collected at regular interval and they were squashed in acetocarmine to observe development of microspore into multinucleate, multicellular and proembryoid structures. Response is noted as number of responsive, non responsive, multinucleate or multicellular per anther.

Acetocarmine staining

Anthers for cytological observation were squashed in 4% acetocarmine and examined microscopically. Observations were made on fresh anthers, anthers collected from pretreated spikes, and anthers collected regularly during the period under In vitro culture.

4. RESULTS AND DISCUSSIONS

Anther culture is the most common technique for regeneration of haploid plants *in* vitro. Attempts were made to study all the three phases of androgenic doubled haploid production:

(a) Production of initial androgenic structures (microspore-derived calli or embryos) can be termed as androgenic induction.

(b) Differentiation of such androgenic calli or embryos into complete plants.

(c) Successful recovery of such haploid plants until greenhouse transfer and diploidization to obtain fertile seed set.

In androgenic induction microspores deviate from their gametophytic pathway (in vivo) to sporophytic pathway (In vitro). This androgenic induction in vitro is influenced by various factors like genotype of the donor plant, and the preculture pretreatments (cold and centrifugation) applied to the explants. Quality and intensity of the light used for incubation of cultures, physical state and hormone compositions of the culture media.

4.1 Effect of Explant

Direct culturing of control and pretreated anthers on semi solid Ch-1.7 medium and liquid Ch-1.7 medium with different combinations of plant growth regulators failed to respond. No divisions were observed and the microspores were pale in the 15 days squashes of the anther cultures. The anthers turned brown in colour after 7days of culture.

Florets cultured on both semi solid and liquid Ch-1.7 and Ch-8 medium showed response varying on different combinations of auxins and cytokinins.

4.2 Effect of Pre-treatment:

The panicles of pearl millet that were subjected to cold treatment for 2 and 7 days were non responsive without any divisions and they became pale within a week of culture. Florets with zero day cold treatment i.e controls responded when cultured on media with different combinations of auxins and cytokinins.

Table 4.1: Effect of cold treatment
(Ch+1.7% S+1.6% agar+hormones)

Number of days	**Response of anthers**
0	Anthers emerged out within 5-8 days
2	Florets turned pale and no anthers emerged out
7	Florets turned pale and no anthers emerged out

4.3 Effect of Medium

The culture medium composition varied with respect to growth regulators and JFK medium was used as basal medium.The anthers from the florets started emerging out from all hormones combinations within 6 days of culture except in NAA+Zn plate.

Different combinations of auxins and cytokinins were used for intiation of divisions in the microspores and observations have been recorded as follows:

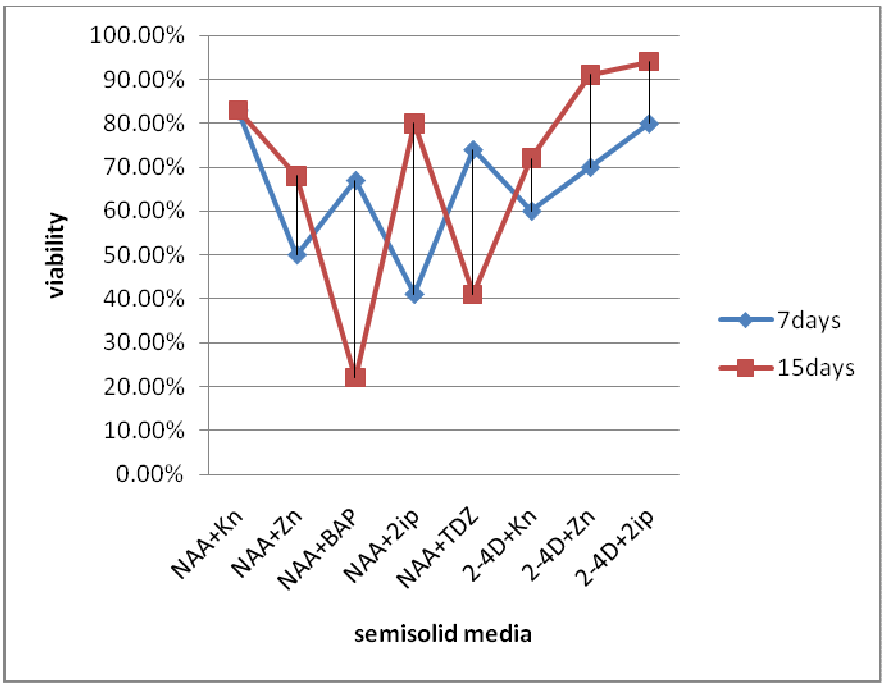


Figure 4.1: Effect of media (light phase)

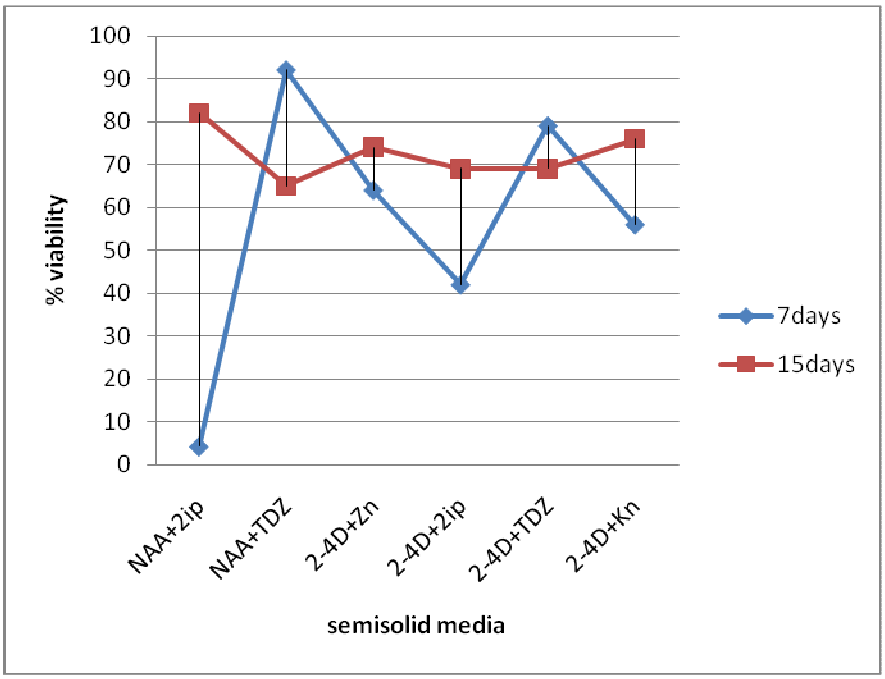


Figure 4.2: Effect of media (dark phase).

From the above observations it can be understood that NAA+Zn in light, NAA+2ip, 2-4D+ZN, 2-4D+KN and 2-4D+2ip in both light and dark phase are responsive for controls with respect to viability of microspores. But when anthers from these media were collected and squashed after 15days i found multinucleated microspores in only 2-4D+2ip and NAA+2ip dark phase cultures which were considered to be the best media among the above listed responsive media.

Table 4.2: Effect of media and light

Data recorded after 15days per 3 anthers

Media used: Ch1+ 1.7% sucrose+1.6% agar+hormones

	light	Dark
2-4D+Kn	Binucleates (8%)	Binucleates (23%)
2-4D+Zn	Binucleates (18%)	Binucleates (15%)
2-4D+2ip	Binucleates (22%) Trinucleates (6%)	Multinucleated microspores(24)
NAA+2ip	Binucleates (10%)	Multinucleated microspores(27)
NAA+Zn	Binucleates (5%)	-

In 2-4D+2ip anther squashes it was observed that in few multinucleated microspores generative nucleus was dormant and vegetative nucleus divided and in one or two microspores cellularisation was observed.

The microspores in which four or multi nuclei was observed, those anthers with florets were placed on Yu-Pei media to promote callus

induction with a few florets retained on the original media. Those florets retained on the original media were found be better than those transferred on to Yu-Pei media. The florets on Yu-Pei media turned brown and the media turned yellow in colour within 15 days of transfer and the microspores were pale.

4.4 Effect of Genotype of the plant:

Androgenic ability greatly depended on genotype of the explant Androgenic induction varied among the 4 genotypes compared (fig4.3)**.** Maximum average response with respect to anthers with viable microspores was observed in XL-51(85%) and the response was least in the genotype 4201(45%). One of the reasons for the low androgenic response in crop genotypes is the high mortality of microspores in *In vitro* cultures.

Observations across different genotypes suggested that the average viability was 71% in Ch-1.7 semi solid media with 2mg/lt of 2-4d and 1mg/lt of 2ip, and 64% in same media with 2mg/lt NAA and 1mg/lt 2ip

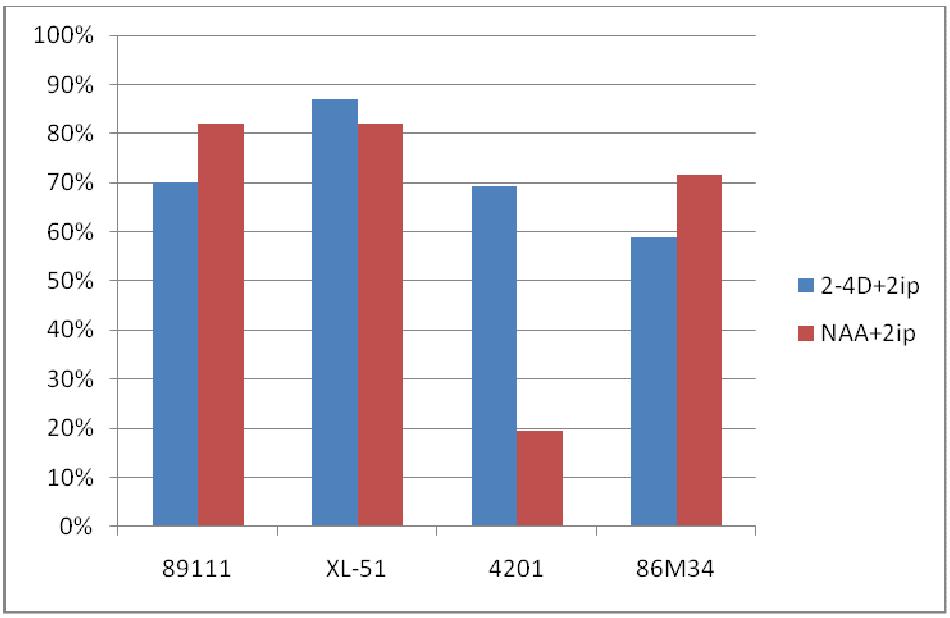


Figure 4.3: Genotype Vs % microspore viability.

Data recorded after 15 days(dark phase) for an average of 3 anthers

Media used: Ch+1.7%S+1.6%agar +hormones.

Maximum response of genotype with respect to average number of multinucleate per 3 anthers was 89111 and least was 4201.

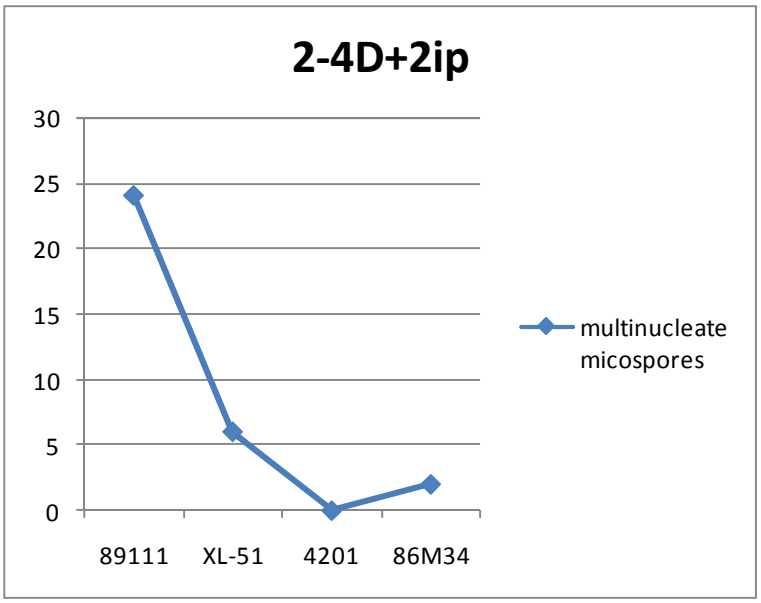


Figure4.4: genotype Vs No. of multinucleate microspores

Data recorded after 15 days for 3 anthers.

4.5 Effect of sucrose concentration

The anthers from the florets cultured on the 8% sucrose media emerged out early when compared to 1.7% sucrose media . But when the anthers were squashed after 15days, the microspores were round , big and took up stain well in 8% sucrose media but multinucleate and multicellular microspores were found in 1.7% sucrose media. Considering the viability of microspores , it was found to be high in 8% sucrose media.

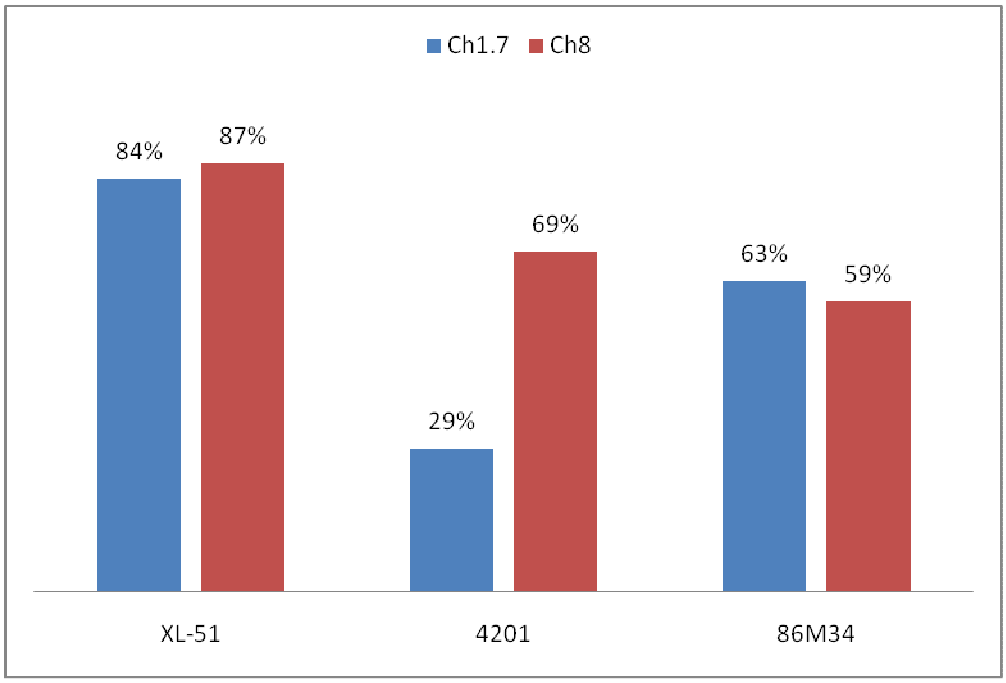


Figure4.5: Effect of sucrose concentration.

Data recorded after 15days per 3anthers

5.Conclusion:

By the end of the term project, I have been able to reach the multicellular microspore stage of androgenesis but could not optimize factors for callus induction. However I was able to answer some questions discussed in introduction , regarding the factors affecting anther culture in pearl millet like Explant, cold treatment , media, genotype and sucros concentration.

Direct culture of anthers was found to be non- responsive. Florets that were cold treated for 2 and 7 days were also non responsive but florets with out any treatment showed varied response on different media combinations.

The florets cultured on 2-4D+2ip and NAA+2ip showed multinucleated microspores. Multicelular microspores were constantly observed in 2-4D+2ip media even for different genotypes hence it was found to be the best media fo culture intiation.

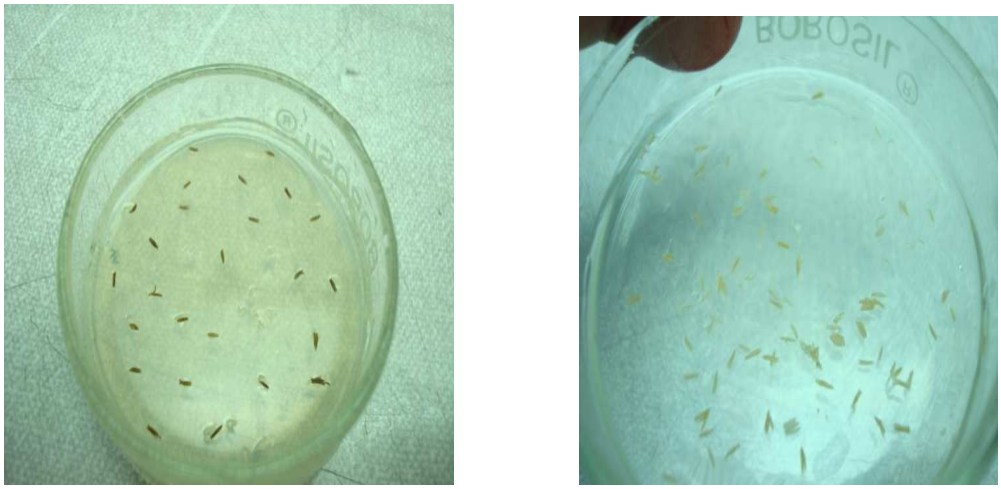


Figure 5: Direct Anther culture plates.

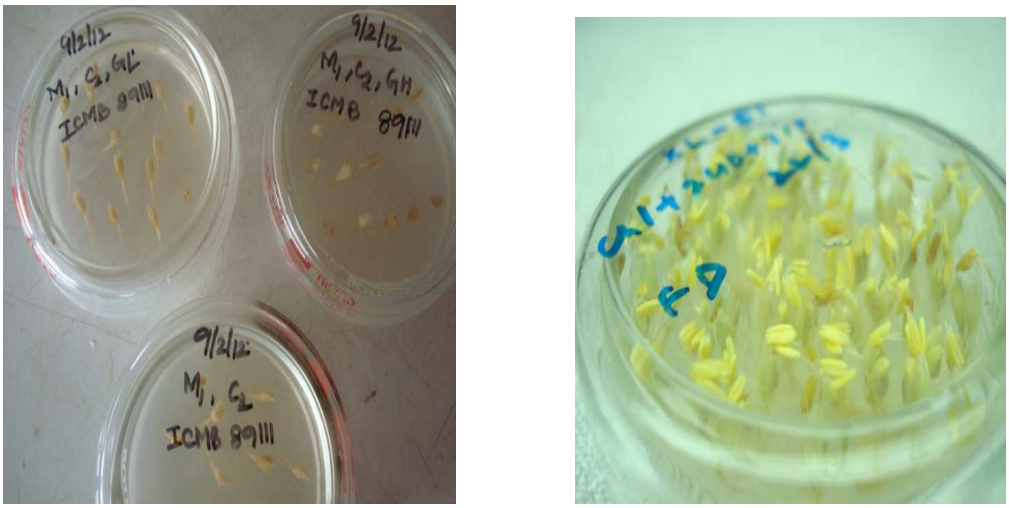


Figure 6: Cold treated plate(left), Control plate(right)

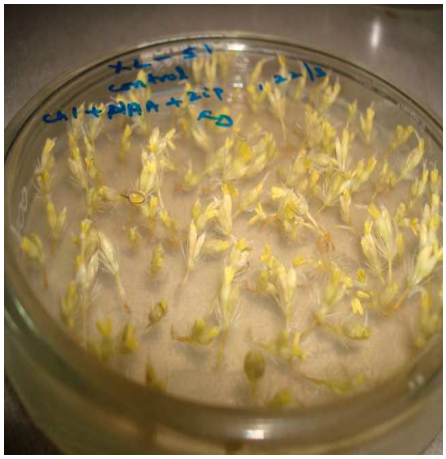
Figure7: ICMB 89111 plates

XL-51

4201

86M34

Figure8: 2-4D+2ip plates



XL-51

4201

86M34

Figure 9: NAA+2ip plates

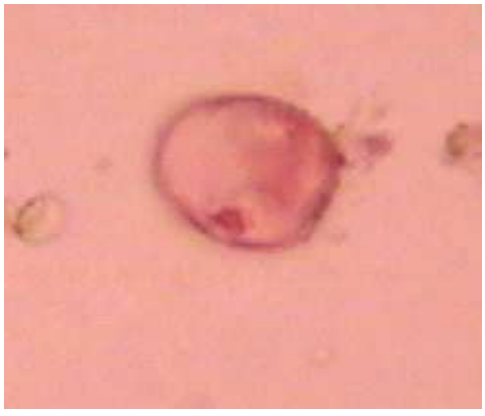


Figure 10: Before culture

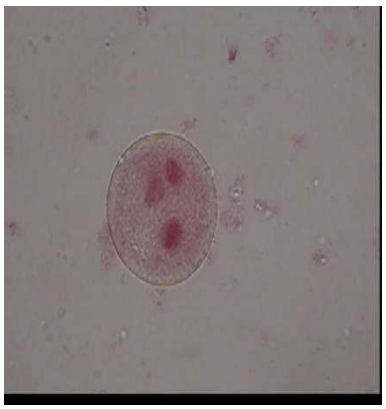


Fig 11: 3 nucleate on 2-4D+Zn plate

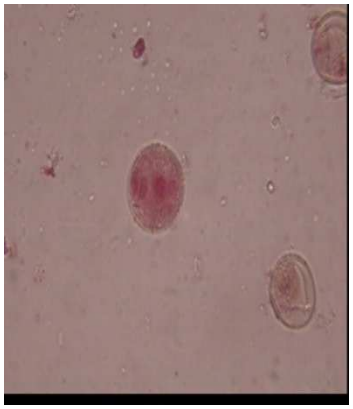


Fig12: 4 nucleate on 2-4D+Kn

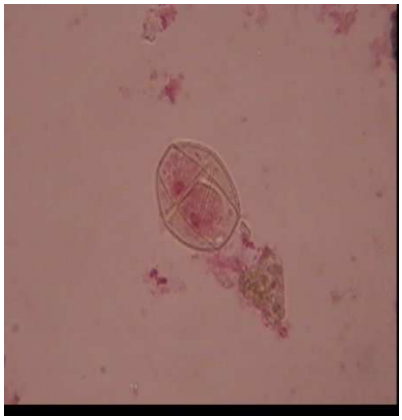


Fig 13: Binucleate on NAA+BAP

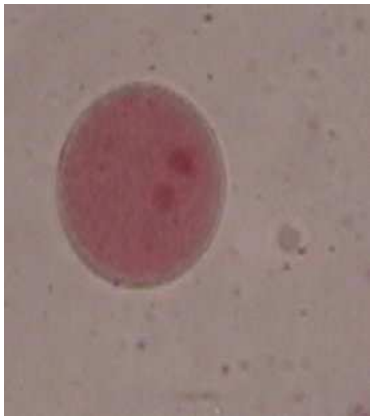


Fig 14:Binucleate on NAA+TDZ

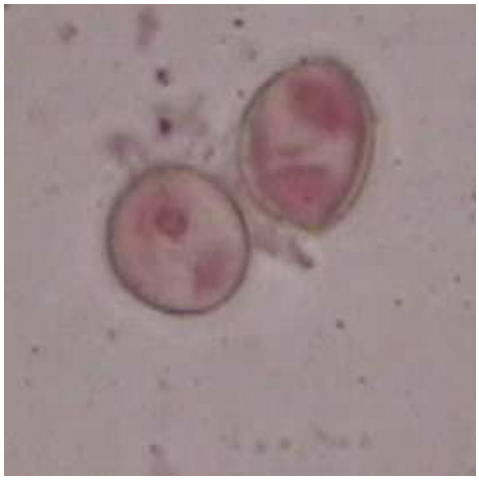
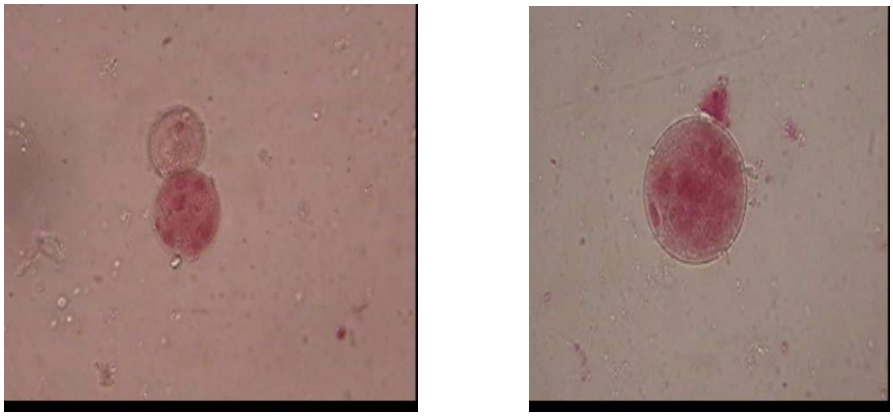
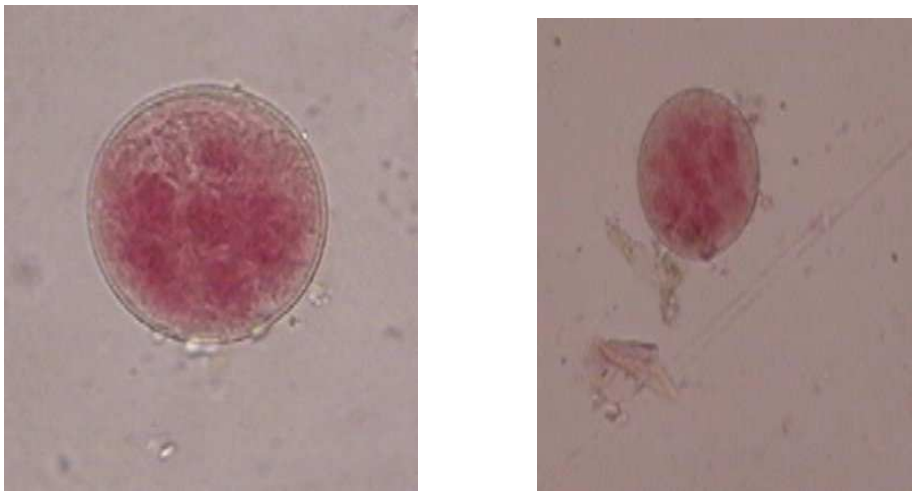


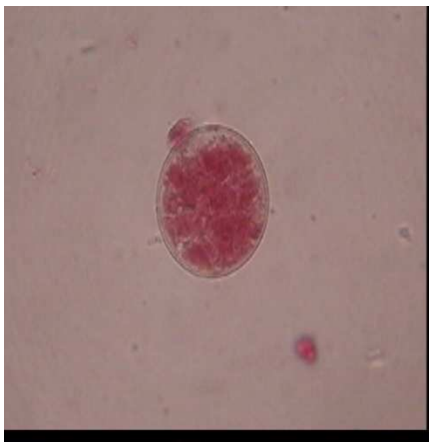
Fig 15: Binucleate & 2 celled on NAA+Kn



(a) Vegetative nucleus dividing

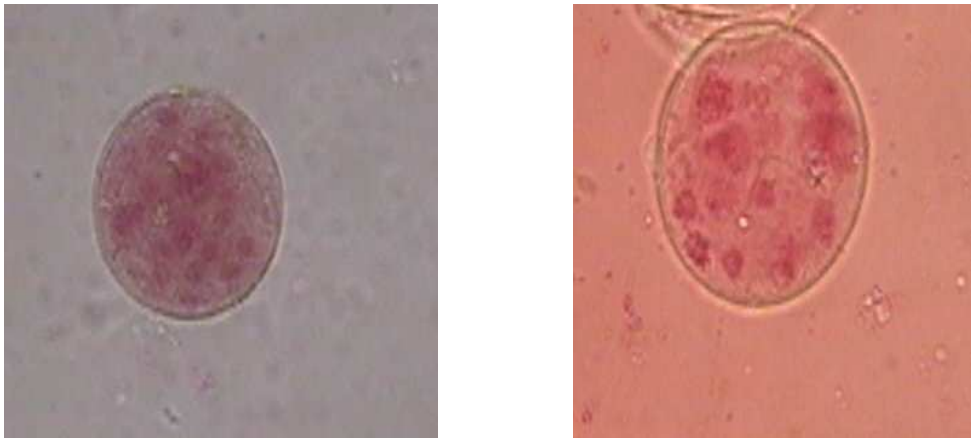


(b) Multinucleated microspores

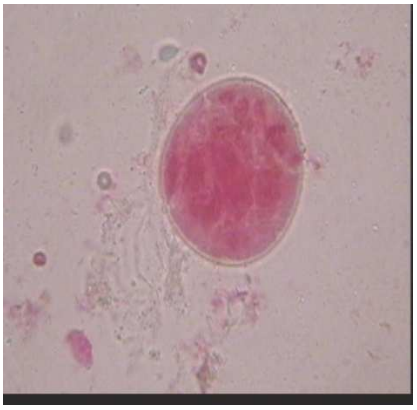


(c) multicellulat microspore

Fig 16: ICMB 89111(2-4D+2ip)



(a) multinucleate microspores



(b)Multinucleate microspore with veg and gen dividing

Fig 17: ICMB 89111 (NAA+2ip)

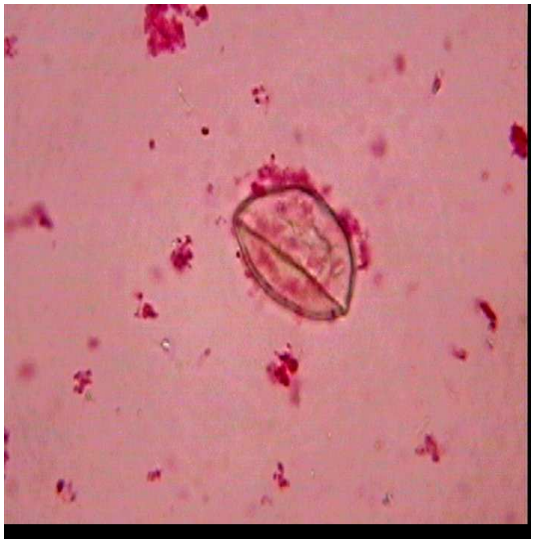


Fig 18 : Multinucleate microspore (86M34) on 2-4D+2ip

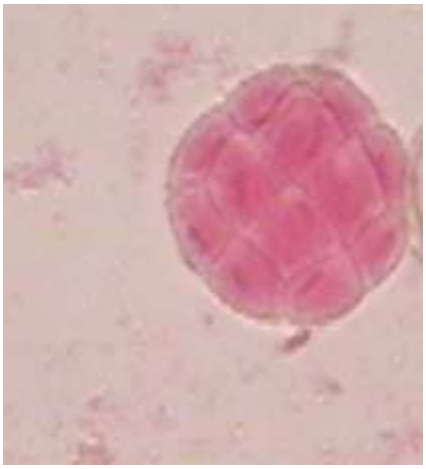
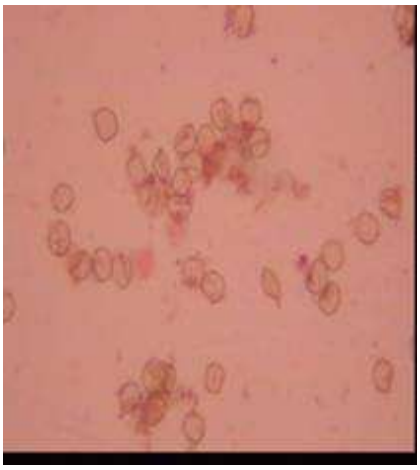
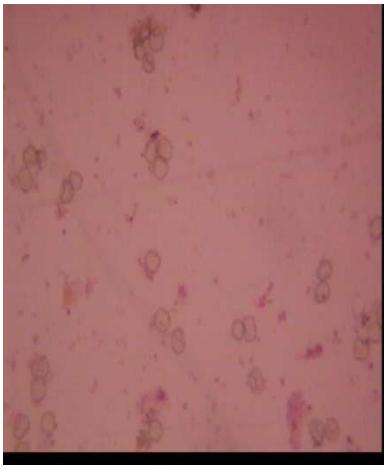


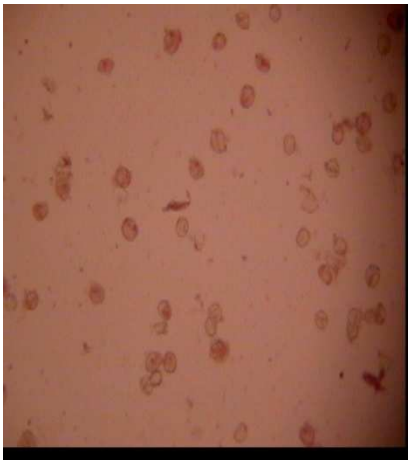
Fig 19: Multicellular microspore (XL-51) on NAA+2ip

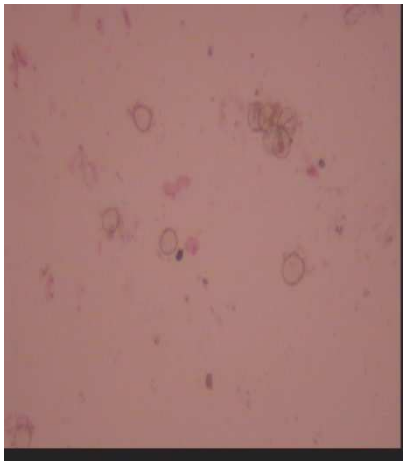




(a) Retained on original media (b) Transferred to Yu-Pei media

Fig 20: cultures on 2-4D+2ip after 31 days.





(a) Retained on original media (b) Transferred to Yu-Pei media

Fig 21: cultures on NAA+2ip after 31 days.

6.REFERENCES:

Buter B, Saisintong S, Schmid JE. Nageii M and Stamp P **(1995)** improved methods for *m*vrtm productlon of doubled haplo~ds In malze In U Wlenand (Eds), MEN 2-Malze Europ~anN etwork Meetlng, Hamburg 29-31 May 1995

.Buter B **(1997)** In vrtro haploid production In maize Vol **4** 37-71 in Jam SM Sopory SK, VelleuxR E (Eds) In vitro haploid productlon In hegherp lants Kluwer academlc publishers Netherlands

C.garcia-Llamas, M.C.Ramfrez, J.Balesteros (2004); Effect of pollinator on haploid production in drum wheat crossed with maize and pearl millet, Plant breeding123(2), Page no.201-203.

Chen C (1978) Effect of sucrose concentration on plant production in anther culture of rice, Crop science, Pagee no. 905-906.

D.T.Jones and G.B.Collins (1976); Factors effecting haploid production from in vitro anther culture of Nicotiana species, Crop science, Vol 16, page no.837-840.

Duc Bui Dang and Jean Pernes (1982); Androgenseis of Pearl millet, Analysis of plants obtained from microspore culture, Z.Pflanzenphysiol 108, page no. 317-327.

Dennis Genovesi and Clint W. Magil(1979); Improved rate of Callus and green plant production from rice anther culture following cold shock, crop science.

Haydu Z and Vasil I.K (1981); Somatic embryogenesisa and plant regeneration from leaf tissues and anthers of *Pennisetum purpureaum*, Theoritical and applied genetics 59, Page no. 269-273.

Judith B Rose, James M Dunwell, Norman sunderland (1986); Plant cell, tissue and organ culture, Page no. 15-22 Vol-6.

N.Mallikarjuna (1986); Investigations on haploid induction in sorghum, Pennisetum And Arachis.

S.C.Maheshwari, A.Rashid and A.K. Tyagi(1982); Haploids from pollen grains, American journal of Botany, Vol-69,No.5, Page no. 865-879.

Shigemune Akiko, Yoshida Tomoshiko(2000); methods of anther culture of pearl millet and ploidy level of regenerated plants, Japanese journal of crop science, Vol 69, Pag no. 224-228.

S.Mohan Jain, S.K. Sopory, R.E. Veilleux(1986); In vitro haploid production in higher plants, Current plant science and Biotechnology in Agriculture, Vol.23

Vasil I.K and Vasil V(1994);In vitro culture of cereals and grasses, Plant cel and tissue culture, Page no. 293-312.