Tissue culture studies in pearl millet [*Pennisetum glaucum* (L) R. Br.] with special reference to *in vitro* doubled haploid production

by

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Certificate

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Declaration

I hereby declare that this thesis comprises of my own work except where specifically stated to the contrary, and that it is not substantially the same as any thesis that has been submitted for any degree at any other university.

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Dedicated to
My beloved Amma and
Nanna.....

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1. ABA	Abscisic acid.
2. 2,4-D	2,4-Dichlorophenoxy acetic acid.
3. BAP	6-Benzyl amino purine (N^6 Benzyl adenine).
4. BSA	Bovine serum albumen.
5. CTAB	Cetyl-trimethyl ammonium bromide.
6. dATP	deoxy adenosine triphosphate.
7. CH	Casein enzymatic hydrolysate (N-Z-Amine A).
8. DAPI	4',6-diamidino-2-phenylindole.
9. DH	Doubled haploid.
10. DMSO	Dimethyl sulfoxide.
11. dNTP	Deoxy nucleotide triphosphate.
12. DNA	Deoxyribonucleic acid.
13. EDTA	Ethylenediamine-tetraacetic acid.
14. FDA	Fluorescein diacetate.
15. GA	Gibberilic acid.
16. H_1	Hybrid 1 (ICMP451 x 843B).
17. H_2	Hybrid 2 (ICMH85410 x 81B).
18. HCl	Hydrochloric acid.
19. IAA	Indole acetic acid.
20. Kn.	Kinetin (6-Furfuryl aminopurine).
21. MS	Murashige and Skoog.
22. NAA	1-Naphthaline acetic acid.
23. NaOAc	Sodium acetate.
24. NaOH	Sodium hydroxide.
25. PCR	Polymerase-chain reaction.
26. PGM	Pollen grain mitosis.
27. PMC	Pollen mother cell.
28. PVP	Polyvinyl pyrolidine.
29. QTL	Quantitative Trait Loci.

30. RAPD	Randomly Amplified Polymorphic DNA.
31. RFLP	Restriction Fragment Length Polymorphism.
32. Rnase	Ribonuclease.
33. SDS	Sodium dodecyl sulphate.
34. SDW	Sterile distilled water.
35. TAE	Tris acetate-EDTA buffer.
36. TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thidiazol-5-yl) urea.
37. TIBA	2,3,5-Triiodobenzoic acid.
38. UV	Ultra Violet.
39. YP	Yu-Pei medium.
40. YPI	Yu-Pei induction medium.
41. YPR	Yu-Pei regeneration medium.
42. TPGI	Yu-Pei gynogenic induction medium.

Executive summary

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, cytogenetics 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. The present study aims at developing a protocol for doubled haploid production in pearl millet [*Pennisetum glaucum (L.) R. Br.*], using *in vitro* anther, microspore, ovary and spikelet cultures.

Brief resume of previous work done

In pearl millet [*Pennisetum glaucum (L.) R. Br.*], there are only three published reports on *in vitro* haploid production. Bui dang Ha and Pernes (1982) were the first to report regeneration through *in vitro* anther cultures for doubled haploid production in pearl millet [*Pennisetum glaucum (L.) R. Br.*]. In the same year, Nitsch et al (1982) obtained haploid plants through *in vitro* culture 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. Therefore, I explored different factors affecting *in vitro* development of doubled haploids from four different explants (anthers, isolated microspores, spikelets and isolated ovaries) of pearl millet to develop a suitable and repeatable protocol.

Collaboration with other departments and local institutions in Hyderabad

1. Training and Fellowship Programme (TAFP)
2. Information Management and Exchange program (IMEP)
3. Library and documentation unit
4. Electron microscopy, and Photography units
5. Statistics and computer unit
6. Pathology, and Radioisotope laboratories
7. Different institutions of the Indian Council of Agricultural research (ICAR) at Hyderabad: Directorate of Rice Research, Directorate of Oil seeds Research, and National Research Center for Sorghum
8. University of Hyderabad (mainly for use of library)

Technical program of work

In vitro studies	– Isolated microspore culture, and anther cultures,
	– Isolated ovary cultures and spikelet cultures.
Microscopy studies	– Anatomical studies using light and electron microscopy
	– Cytological studies using light and fluorescent microscopy
Molecular studies	– RFLPs and RAPDs
Agromorphological studies	– Growth, maintenance, and studies of the plants in greenhouse and field.

Results

Anther culture: Haploid and sterile androgenic plants were regenerated at a frequency of 14-18 % from the anther culture protocols developed by me. Anthers at uninucleate microspore stage of development were most appropriate for androgenic induction. Incubation of *in vitro* cultures under red light for initial 3 weeks followed by incubation under white light maximized androgenic embryo formation. Microspore origin of androgenic embryos (rather than somatic tissue of anthers as a source) was confirmed with extensive light, electron, and fluorescence microscopy studies. Both vegetative and generative nuclei underwent divisions and formed proembryos. Androgenic induction potential varied across the genotypes studied: genotype 7042 DMR showed highest response (48%) and genotype IP18298showed the least (2%). Androgenesis and subsequent regeneration of albino or green plantlets were genotype dependent.

However, the regenerated whole plants were albinos (In 7042 DMR only green stunted shoots with profound rooting were observed).

Isolated microspore cultures: Embryo formation occurred in isolated *in vitro* cultured microspores of the hybrid ICMP 451 x 843 B. Effects of addition of different hormone and organic compositions to Yu-Pei basal medium were tested for their effectiveness in inducing microspore embryos. L-proline (200 mg L^{-1}), glutamine (100 mg L^{-1}) and casein hydrolysate (500 mg L^{-1}), when added together to YP basal induction medium with 2,4-D (3 mg L^{-1}), thidiazuron (0.1 mg L^{-1}) and kinetin (0.5 mg L^{-1})], improved embryo induction. The induction rate was highest in microspores derived from pre-cultured anthers (12.3%) than from fresh anthers (1.7%). Filter-sterilized medium was five times more effective than autoclaved medium for inducing microspore embryogenesis. Three weeks after isolation, a high frequency (45%) of multicellular microspores turned to pro-embryos. By fifth week, they grew further, albeit slowly, into embryos showing initial polarity. By the end of six weeks, embryos turned brown and ceased to grow further.

Ovary and spikelet cultures: Isolated ovaries cultured *in vitro*, failed to produce callus or enlarged ovaries under *in vitro* conditions. However, success was obtained with the culture of spikelets. Gynogenic induction and subsequent pattern of regeneration (through callus phase or by direct plant production) depended on physical state of the medium. Cultures in liquid medium gave rise to regeneration through callus phase, and that on solid medium resulted into

direct plant regeneration. From spikelet cultures, doubled haploid and fertile plants were regenerated at a frequency of 6.7%. Out of a total of 21 regenerants obtained, 97% were green plants. Cytological analysis of gynogenic haploid (sterile) and doubled haploid (fertile) plants confirmed their ploidy levels. Further, RFLP and RAPD analyses of the doubled haploid progeny confirmed the homozygous status (absence of allelic segregation among progeny) of the fertile gynogenic regenerants. Uniformity and homozygosity within doubled haploid populations was confirmed by morphological evaluations under greenhouse and field conditions. This technique of producing doubled haploids *in vitro* from female gametophyte can contribute significantly to both pearl millet breeding and for studies such as QTL mapping and genetic transformation.

A total of 4 journal articles are being drafted. I am directly involved in transfer of this technology to both public and private sector professionals in India and Japan. Poster presentations were made during national meetings and to the visitors to ICRISAT at Patancheru, India.

1. Introduction

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, somaclonal 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 presence of a single dose of each allele in haploids, they can be employed in several areas of fundamental research in cytogenetics and genetics. 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. Recent developments in DNA-based genetic marker systems (such as RFLP, RAPD, and SSLP) improved possibilities for the detailed study of both qualitatively and quantitatively inherited traits. The protocol for mapping consists of generating a suitable population (F_2, back-cross, or DH populations) from a hybrid between parents showing different phenotypes The segregating offspring are carefully evaluated for their phenotype and subsequently for segregation of DNA markers. Finally, the genotype and phenotype data are used to identify markers linked to the trait of interest. 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. (More information on related to haploids in crop improvement is available in the list web sites described in *appendix 1*)

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^{-3}) 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 *Hordeum 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 dicotyledenous species like tobacco and sugarbeet (Horlow et al, 1996). Systems that induce parthenogenesis include alien cytoplasm substitution as in wheat, use of indeterminate gametophyte gene *ig* 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; Matzk 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 variation 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. Scope of the present thesis

1.3.1. Pearl millet as the candidate crop for study

Pearl millet is a diploid (2n = 2x = 14) C^4 species known by the scientific name *Pennisetum glaucum* (L) R. Br. Its common names are bulrush or cattail millet, or *bajra* (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 billions 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.

1.3.2. 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 (*T186*, *T23DA*, and *T23DB*) led to a sudden decrease in vigour 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. Heterozygotes produced by crossing inbred lines show significant increase in chiasma frequency. In addition, favourable 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.

1.3.3. 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/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 seggregating 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, I studied *in vitro* androgenesis using anther and isolated microspore cultures, and *in vitro* gynogenesis using spikelets or isolated ovary cultures.

1.4. Specific objectives of thesis research

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, microspores, spikelets and ovaries, and to characterize the doubled haploids produced to verify their haploid origin. The specific objectives are listed below:

Androgenesis

1. Study effects of physical, chemical, physiological and genetic factors affecting *in vitro* androgenesis.

2. Standardize the technique for *in vitro* anther culture and culture media for androgenic induction and regeneration.
3. Develop methods for isolating microspores and standardization of culture media for microspore embryogenesis and their subsequent regeneration.
4. Confirm microspore embryogenesis using light, electron, and fluorescence microscopy studies.

Gynogenesis

5. Compare the technique of spikelet cultures and isolated ovary cultures for *in vitro* gynogenic induction.
6. Establish optimal developmental stage of the explant for *in vitro* gynogenesis.
7. Compare the effect of liquid and solid media on gynogenic induction.
8. Standardize of the protocol and culture media for gynogenic plant regeneration.

Analysis of doubled haploids

9. Analyse *in vitro* regenerated haploid/doubled haploid plants by agromorphological and cytological studies.
10. Confirm the homozygosity and uniformity among the DH progenies using RFLP and RAPD techniques.

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 dicotyledenous species like onion and sugarbeet (Campion and Azzemonti, 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). Therefore, this review quotes mostly the work on from the related cereals

wherever the relevant reports are not available for pearl millet. For the sake of convenience, the literature will be reviewed under four sub-headings:

1. Anther culture
2. Isolated microspore culture
3. Spikelet and ovary culture
4. Analysis of the regenerants

2.1. Anther culture

Dang Ha and Pernes (1982) were the first to report successful androgenic haploids in pearl millet lines Tift 23 D_2B and a F_1 hybrid. In the same year, Nitsch (1982) reported haploid regeneration from anthers of *Pennisetum purpureum*. 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.

2.1.1. Technique for anther culture

Various workers modified the original technique of anther culture developed by Guha and Maheswari (1964; 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.

2.1.1.1. 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.

2.1.1.2. 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).

2.1.2. Factors 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.

2.1.2.1. Genotype 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.

2.1.2.2. 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 terms 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).

2.1.2.3. Anther and pollen developmental stage

Nitsch and Nitsch (1969) were the first 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.

2.1.2.4. 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).

2.1.2.5. Culture conditions

2.1.2.5.1. 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).

Temperature

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

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.

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 10^4 microspores per ml was optimum density, while in wheat, Hoekstra et al (1993) found the optimal density to be 2 x 10^4microspores 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).

2.1.2.5.2. 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 multi-nucleate 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 multi-cellular 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).

2.2. Isolated microspore culture

Microspore culture offers an efficient system to regenerate haploid plants as it provides access for direct cell manipulation (e.g., genetic transformation and *in vitro* selection). Microspore derived embryos are the potential targets for the delivery of foreign genes as the genes are integrated into a completely homozygous genome. Delivery of DNA into a homozygous genome would prevent the recovery of transformants with lethal mutants and avoids chimeric regenerates. At the same time, the isolated microspore cultures are complicated and less efficient when compared with anther cultures.

The first report on culture of isolated microspore appeared in *Brassica oleracea* (Kameya and Hinata, 1970). This has lead to the extension of the technique for both basic and applied studies. Subsequently, various efforts over the last several years lead to the improvement of the technique (reviewed by Dunwell, 1985; Dunwell, 1996). Regeneration was reported from isolated microspores of all major cereals like *Triticum aestivum* (Mejza et al, 1993; Hu et al, 1997). Apart from plant regeneration, genetic transformation studies using isolated microspores were reported in *Hordeum* (Jahne et al, 1994) and *Zea mays* (Gaillard et al, 1992; Jardinand et al, 1995). However, in pearl millet, until now, there is no report on the optimal hormonal combinations and techniques for initiating cultures of isolated microspores and their subsequent embryogenesis.

2.2.1. Genotype effects on embryogenic potential of isolated microspores

There is a good evidence of genotype influence on microspore cultures within the limited range of genotypes studied in tobacco, barley and oil seed rape. Recently, Orlov et al (1999) reported cytoplasmic effects on microspore embryogenesis in isolated microspore cultures of wheat. In barley, the cultivar *Igri* remains the most popular genotype for isolated microspore cultures (Ziauddin et al, 1990; Olsen, 1991;Hoekstra, 1993; Forster and Powell, 1997).

2.2.2. Influence of method of microspore isolation on induction potential of isolated microspores

There are various methods by which microspores can be separated from the surrounding anther tissue. Success rate of androgenic induction varied with different techniques of microspore isolation in different crops (Dunwell, 1996). These methods included grinding of anthers with a pestle and a mortar to release the microspores as in barley (Nitsch and Norreel, 1974), or a micro-blending method (Olsen, 1991). Micro-blending yielded three-fold increase in the induction when compared to the passive shedding method (where microspores are passively shed into the liquid medium from cultured anthers) followed by Ziauddin et al (1990). In maize, a blending method was successfully used by Pescitelli et al (1990; 1994), which resulted in three-fold increase in embryo yield when compared to the technique of pressing anthers through a stainless sieve.

2.2.3. Effects of culture medium on induction potential of isolated microspores

Various studies have examined the general nutritional and osmoticum effects of various carbohydrate sources (Hoekstra et al, 1993 in barley; Tuvveson and Ohlund, 1993 in wheat; Pescitelli et al, 1990 and Gaillard et al, 1991 for maize). The usual major salt concentrations varied from modified Gamborg's B5 medium for rice (Cho and Zapata, 1988; 1990) to modified MS medium for barley (Olsen, 1991). In cereals, less attention was paid to other medium constituents including the important plant growth regulators like auxins and cytokinins which were well studied in dicotyledenous crops.

2.3. Spikelet and ovary culture

Though anther culture is advantageous in several ways, its potential application to cereal crops is hampered by production of albinos. Ovary culture is an alternate technique to anther culture for obtaining haploid green plants. In ovary culture, though the available number of explants per flower bud is low, rate of induced embryosacs has generally been higher than for microspores. Another important factor in favor of gynogenesis is, once a somatic embryo is induced, its further development into plantlet occurs in near-natural environment inside the female gametophyte minimizing effect of somaclonal variation. Keller and Korzun, 1996, and references there in described the research efforts on ovary and ovule culture in higher plants.

After San Noeum (1979) obtained first haploid plant through gynogenesis in barley, gynogenic induction was attempted in other cereals such as rice, wheat, and maize during the past two decades (for review, Keller and Korzun, 1996). Gynogenic embryos are derived from egg cells (Andre and Demarly, 1984; Yang et al, 1986; Yang and Zhou, 1982) and synargids (Zhou et al, 1986) though occasionally they may arise from antipodals (Tian and Yang, 1989). In the light of the previous research done in cereals on this aspect, it is clear that conditions for successful gynogenic induction vary even within a species. Several factors contribute to these variations (for review, Keller and Korzun, 1996). Both the somatic and gametophytic tissue of ovary is sufficiently competent to develop into somatic embryos. Therefore, preferentially triggering of only gametophytic tissue helps successful regeneration of gynogenic haploid plants.

2.3.1. Factors affecting gynogenesis

Various factors such as genotype of the donor plant, developmental stage of the ovary, method of ovary culture, concentration of plant growth regulators etc., affect the induction frequencies of gynogenesis *in vitro* (reviewed by Keller and Korzun, 1996).

2.3.1.1. Genotype of the donor plant

Genotype of the donor plant is one of the important factors for induction of gynogenic plants. Genotypic differences in in vitro gynogenic induction response were demonstrated in *Hordeum vulgare* (San Noeum, 1979), *Oryza sativa* (Zhou, 1986), and *Triticum aestivum* (Zhu et al, 1981 and 1983a).

2.3.1.2. Choice between cultures of spikelet and isolated ovary for gynogenesis

When compared to the isolated ovary cultures, higher induction and regeneration frequencies have been obtained by culturing ovaries with the remaining peripheral tissues such as bracts, whole florets or additional parts of the inflorescence axis in several *Poaeace* species (Mathias and Boyd, 1988; Laurie and Bennet 1988; Higgins and Petolino, 1988). Therefore, in major cereals like wheat and barley, gynogenic haploids were obtained by culturing whole spikelets. The literature on ploidy distribution in ovary and ovule culture for haploid production in cereals is summarized in Table 1.

Table 1: Survey on ploidy distribution in ovary and ovule culture for haploid production in cereals.

(HP = haploid plant; DP = diploid plant; HC = haploid callus).

S.No	Species	Ploidy	Reference
1	*Hordeum vulgare*	- 100% HP, endopolyploidy in roots - DP, HP	Castillo & Cistue (1993, 2000)
2	*Oryza sativa*	- Directly from ovaries -100% HP; through callus – mixoploid	Liu & Zhou (1984)
		- More haploid (77.5%) than in anther culture (63.9%) and more green plants than in anther culture (89.3% vs. 36.4%)	Davoyan (1985)
		- Directly from ovaries - 100% HP; - Through callus – HP, DP, aneuploid	Lu & Wu (1986)
		- Haploid	Zhou & Yang (1981)
		- 100% haploid	Zhou et al (1986)
		- Mixolpoid from callus - 77.5% HP & 22.5% DP	Rongbai et al (1998)
3	*Triticum aestivum*	- HP	Lu & Wu (1986)
		- HC, HP	Zhu & Wu (1979)
		- HP	Zhu et al, (1983a)
4	*Zea mays*	- HP	Lu & Wu (1986)

2.3.1.3. Culture conditions

Solid media are frequently used for gynogenesis. In few cases, liquid medium was used for induction of gynogenic calli (Zhou et al, 1986; Gusakovskaya and Nadzhar, 1994) and later, these calli were transferred onto solid medium for further differentiation. Apart from other nutrient media as reviewed by Keller and Korzun (1996), modified MS medium (Murashige and Skoog, 1962) was the most commonly used medium for gynogenic induction. Regulation of growth hormones to enhance gynogenesis while inhibiting proliferation of somatic tissues is critical for ovary culture (Cagnet-Sitbon, 1980; Huang et al, 1982; Andre and Demarly, 1984).

2.3.1.4. Physical factors

Certain physical treatments (like cold pretreatment) given to explants prior to culture had strong influence on gynogenic embryo induction. These physical factors were studied in detail only in dicotyledonous crops.

2.3.1.5. Seasonal effects

Among the parameters influencing gynogenic haploid production is seasonal conditions. A significant seasonal effect was observed by Lux et al, (1990) for greenhouse grown plants. In their study conducted throughout the year, maximum gynogenic embryos were obtained during summer (May to September).

2.3.2. Study of gynogenesis

The developmental stage of the female gametophyte influences the gynogenic induction frequency of a given crop. Therefore, it is necessary to determine optimal stage of gametophytic development for *in vitro* gynogenesis. When compared to the microspores, megaspores are deeply embedded inside the embryosac. Therefore, study of megasporogenesis and *in vitro* development of gynogenic embryos or calli usually necessitated a range of light microscopic studies (of clear squashed ovules, isolated embryosacs or anatomical sections).

2.3.2.1. Optimal developmental stage of the female gametophyte for *in vitro* culture

Ideal method for optimization of female gametophyte developmental stage involves direct investigation of the embryosac during each time of culture initiation. However, such a direct investigation is difficult because of lengthy processes of thin sectioning, staining, and clear squashing (Herr, 1971) or embryosac isolation (Enaleeva and Dushaevaa, 1975; Huang and Russel, 1989; Wagner et al, 1989). Further, because of the tiny size of the flower buds in cereals and the difficulty in handling of dissection of the gynoecium, usually indirect judgment was made by observing developmental stage of microspore (Zhou et al, 1986). Studies in wheat (Castillo and Cistue, 1993) showed that trinucleate microspore stage of flower bud development is the optimal stage for gynogenesis. However, in rice most favorable developmental stage of explant was flower bud containing late uninucleate microspores (Zhou et al, 1986).

2.3.2.2. Study of the *in vitro* gynogenic embryos or calli using light microscopy

Any haploid cell present in the female gametophyte (embryosac) may show the totipotency and give rise to a haploid embryo and subsequently, a plantlet. Depending on the media used and the hormone combinations, sometimes even somatic tissues of the ovaries can give rise to plants. In order to know the exact origin of gynogenic embryos (even before confirming the ploidy levels of regenerants by cytology), their progressive developmental stages should be followed.

In dicotyledenous crops such as sugarbeet (van Geyt et al, 1987; Ferrant and Bouharmont, 1994), and sunflower (Yang et al, 1986), gynogenic regenerants were confirmed through anatomical and cytological studies. In cereals, these approaches have been used to confirm the origin of gynogenic embryos (San and Gelebart, 1986) and to preclude possible somatic origin of the regenerants.

In *rice* (Zhou et al, 1986), gynogenic proembryos were compared during the initial stages (below 3 weeks) in size and shape to zygotic proembryos *in vivo*. In addition to the normal gynogenic embryos, they also reported anomalous embryos - some with highly vacuolated cells, while others contained abnormally long suspensors.

2.3.3. Use of ovule, ovary and embryo culture in other methods of haploidization

Development of egg cell into a haploid embryo may also be induced by pollination with incompatible pollen. Haploids of *Triticum durum* have been obtained by crosses with maize. Embryo culture has been used to rescue haploid embryos resulting from chromosome elimination (as described by Keller and Korzun, 1996).

2.4. Analysis of the regenerants

Ploidy levels of the regenerants can be determined by various methods such as root tip chromosome counts, chloroplast counts in the stomatal cells or isozyme polymorphism (Bui dang Ha and Pernes, 1982). In dicotyledonous crops like sugarbeet (Geyt et al, 1987; Ferrant and Bouharmont, 1994) and sunflower (Yang et al, 1986), origin of gynogenic regenerants is confirmed by anatomically tracing origin of gynogenic embryos. In addition, ploidy of gynogenic plants was checked by counting the number of chromosomes and guard cell chloroplasts. Similarly in cereals, apart from the chromosome counts of the regenerants, most of the studies are confined to tracing the origin of gynogenic embryos by anatomical studies (San and Gelebart, 1986). Analysis of *in vitro* regenerated doubled haploid plants and their progenies in other cereals included molecular, cytological and agromorphological evaluations (Hyne et al, 1994; Martinez et al, 1994).

2.4.1. Use of cytological studies to determine the ploidy of the *in vitro* regenerants

In cereals, cytological studies are the most frequently employed tools to confirm the ploidy of haploid regenerants and their progenies. While analyzing intergeneric or interspecific progeny, chromosome-painting techniques such as *FISH* is fast becoming popular.

2.4.2. Use of agromorphological analysis of the regenerants and their progeny for studying the homogeneity of DH progenies

Anther culture derived DH lines were evaluated for their agronomic and morphological traits in sorghum (Kumaravadivel and Sree Rangaswamy, 1994). In barley, Powell et al (1986) compared the field performance of anther culture derived DH lines and *bulbosum* derived DH lines.

In pearl millet, all earlier reports of cyto-morphological analysis of haploids were restricted to naturally occurring haploids. Powell et al (1975) discussed the origin, cytology and reproductive characteristics of spontaneously occurring haploids of Tift 23A discovered in the field plantings.

2.4.3. Use of DNA markers for analysis of *in vitro* regenerants

The use of DNA markers for the genetic analysis of plants has become a major research tool for a broad spectrum of scientists from different research fields. Molecular technique at the DNA level can be a powerful tool to detect the homogeneity within DH population. Besides confirming the homozygosity, molecular analysis of the regenerants and their progenies are helpful to trace

back the gametophytic origin of gynogenic or androgenic regenerants. Maize doubled haploid lines were evaluated by using molecular and morphological markers (Murigneux et al, 1993). Androgenic DH lines were analyzed for segregation distortion in barley (Devaux et al, 1993). Using RFLP markers, Cowen et al (1992) identified four different locations on the maize chromosomes that affected number of embryos and plants obtained from anther culture. Devaux and Zivy (1994) used 28 protein markers to study anther culture response from 50 DH lines in barley. Degree of homogeneity, stability and conformity of DH plant progenies with respect to parental progenitors must be checked for successful application of DH approach for breeding purposes, QTL analysis or other genetic studies. Organelle DNA probes may also be used for evaluation of DH lines. In wheat, first and second cycle DH lines obtained from anther culture were analyzed for restriction patterns of mitochondria DNA and chloroplast DNA (Rode et al, 1985; Charmet et al, 1985).

3. Materials and methods

3. 1. Anther culture

3.1.1. Plant material

Two pearl millet hybrids, ICMP 451 x 843B (designated as H_1 hereafter) and ICMP 85410 x 81B (designated as H_2) were chosen for standardization of *in vitro* protocol. These hybrids were selected based on the recommendation of pearl millet breeder at ICRISAT, Patancheru. The parents of these hybrids have distinct morphological markers like presence or absence of ligule in the leaf nodes, or bristles in the inflorescence. Among these four lines, three are parental lines of released hybrid cultivars in India. A total of 17 genotypes (*see Appendix 2, for list of* genotypes) were used for comparing *in vitro* androgenic potential across different genotypes. Plants were grown in semi-controlled environment in a glasshouse where the maximum temperature did not exceed 30 °C. Plastic pots (30 cm diameter) were filled with potting mixture prepared by mixing soil and farmyard manure in the ratio of 3:1. A few (2-3) seeds were dibbled in each pot. The seedlings emerged within a week. Only one healthy seedling was retained in each pot. Urea was applied (@ 5 g per pot) twice, first when the plants attained 20 cm, and then at 40 cm. Spikes were harvested after the top 10% of the spike (approximately 2-3 cm) on the main shoot emerged from the flag leaf sheath. Anthers were removed from the spikelets, squeezed onto a microscope slide and released microspores were examined after staining with acetocarmine (*see Appendix 4, for protocol*) under a microscope. Unless otherwise mentioned, only spikelets with uninucleate microspores were selected for culture.

3.1.2. Cold pretreatment

The spikes were wrapped in an aluminum foil and placed in a polythene bag. They were then placed in incubators (dark) at 4 °C or 14 °C for 7 days depending on the pretreatment.

3.1.3. 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.

3.1.4. Culture initiation

For initiation of spikelet cultures, individual spikelets were removed from cold-treated spikes, observed for the uninucleate stage of the microspore as described in 3.1.3, and surface sterilized with mercuric chloride (0.1%) for 15 min. This was followed by 5 washes with sterile distilled water under Laminar airflow. Spikelets were then cultured under aseptic conditions on solid YP

medium (*Appendix 3, for composition of YP medium)* with 5 g L^{-1} charcoal and 60 gL^{-1} of sucrose. The spikelets were then planted on both solid as well as liquid YP media and incubated either under red light or white light (2000 Lux.) depending on the experimental requirements. Temperature of culture room was 24 ± 2°C, with a photoperiod of 16 h/8 h (light/dark).

Anther cultures were initiated using these precultured spikelets as their source of explants (**Figure 1**). Emerging anthers from the bisexual florets (about 3-7 days after initiation of spikelet cultures) were collected and recultured onto the YP medium as described above. Second flush of anthers from the male florets was discarded. For direct anther culture, after cold pretreatment at 14°C, the anthers containing uninucleate microspores were manually removed from individual spikelets and directly placed on YP medium.

3.1.5. Light and temperature conditions for culture

In all the above experiments, the cultures were subjected to a photoperiod of 16 h of light (2000 lux) and 8 h of darkness in a growth room maintained at 24 ± 2°C. After cold pretreatment, the spikelets were cultured and incubated under three conditions: dark, red light (fluorescent tubes wrapped with red polythene sheets), and white light. The light intensity was 2000 lux. After about 4 weeks in culture, the number of anthers responding (those with pro-embryoids) was recorded. Similarly, the number of anthers with embryoids was recorded after 8 weeks. While embryoids could be visualized directly, pro-embryoids were observed under microscope after staining with acetocarmine.

3.1.6. Heat shock treatments

To study the effect of temperature shock treatment, four sets of cultures were initiated (a) spikes with no cold treatment and no heat shock which served as a control, (b) regular cold pre-treated spikes, (c) heat shock employed and (d) cold pretreated and subjected to heat shock. For cold treatment, spikes were wrapped in aluminum foil placed in polythene bag, stored in an incubator at 14 °C for 6-8 days. For employing heat shock, the stalks of the spikes were placed in liquid YPCI medium in a conical flask, which in turn was placed in an incubator set at elevated temperatures (30, 35, or 40°C) for the required duration. After this temperature treatment, spikelets were cultured in liquid YPCI medium in petridishes (90 x 15 mm) and sealed with parafilm. Petridishes were then exposed to regular incubation conditions described above.

3.1.7. Sucrose starvation

In sucrose starvation experiment, spikelets were cultured initially in liquid YPCI media with 0 % sucrose for 72 h, subsequently transferred to three sucrose containing (3%, 6%, 9%) induction media and incubated under regular culture conditions.

3.1.8. Sterilization of TDZ and silver nitrate in induction medium

When used for experiments, TDZ (1-3 μM) and silver nitrate (0,10, 20, 30, 40 mg) (both autoclavable) were added to induction medium along with other components and autoclaved.

3.1.9. Observations and scoring of data

In all the experiments, anthers were collected at 4-day intervals during 6 weeks in culture. They were squashed in acetocarmine (as described earlier) to observe development of microspore into multinucleate, multicellular and proembryoid structures. Response is noted as number of proembryoid or embryos per 100 of the cultured anthers. Each experiment was performed on three different dates. While harvesting the spikes each day, three spikes were collected from three different pots for each genotype. Just before culturing, the spikelets were pooled to randomly to deal with the variation in them. A minimum of three replications was used for each treatment.

3.1.10. Test for viability of microspores

The number of viable microspores was determined at spike harvest after cold treatment, and after 15, 30, 45, 60 and 90 days of *in vitro* culture. Anthers were crushed and stained with FDA (*Appendix 4, for protocol*). The percentage of viability was calculated by counting the number of microspores fluorescing per every 100 microspores counted under a fluorescence microscope.

3.1.11. Tracking pollen embryogenesis

Anthers were sampled from cultures at weekly intervals. Anthers were placed on a slide, crushed to release the microspores, and stained with different stains. Stages of microspore development were examined either under ordinary

light using a binocular microscope or under fluorescence microscope as appropriate for the stain used.

3.1.12.1. Light microscopy

3.1.12.1.1. Acetocarmine staining

Anthers for cytological observation were squashed in 0.1% acetocarmine and examined microscopically. Observations were made on fresh anthers, anthers collected from cold pretreated spikes, and anthers collected daily during the first 10-day period under *in vitro* culture.

3.1.12.1.2. Histological staining

Anthers were fixed in ice-cold gluteraldehyde with 2% DMSO (buffered to pH 7 by 0.1% phosphate buffer) aspirated for up to 5 days, rinsed twice in buffer (12 h each time), dehydrated in a 30-100% alcohol series, and embedded in *spur* resin (*Appendix 4, for protocol*). Transverse sections (5 to 8μ thick) were obtained with the help of an ultra-microtome. Sections were stained with a combination of Toulidine blue and Azure-II.

3.1.12.2. Fluorescence microscopy

3.1.12.2.1. FDA staining

Microspores were stained with Fluorescein diacetate (*Sigma* F7378, *please see appendix 4 for protocol*), Pollen was sprinkled on a microscope slide containing a drop of above stain, a cover slip was placed and the slide was

sterilized and cultured for one week and the responding anthers from these were used to isolate dividing microspores according to Gaillard et al (1991). Microspores were suspended in 2 ml of Yu-Pei based induction media containing 3 mg L^{-1} 2,4-D alone or in combination with different cytokinins and amino acids at a plating density of 1.7-2.0 $x10^4$ microspores ml^{-1} as in barley (Hoekstra et al, 1993). The petridishes were sealed with *parafilm* and cultures were incubated in dark at 24 ± 2°C, and at relative humidity of 45%. In the second method (for *ab initio* cultures), anthers from cold-pretreated spikelets were sterilized, teased apart gently and microspores were released into series of the induction media (as mentioned above) under the same culture conditions. Microspores at different developmental stages were observed after staining them with acetocarmine at three-day intervals until eight weeks. After two weeks in dark, the developing microspores (pro-embryos) were transferred to the regeneration medium [2,4-D (0.5 mg L^{-1})· Kinetin (0.25 mg L^{-1}) and BAP (1 mg L^{-1})] at a photoperiod of 16 /8 h (light/dark). Microspore embryogenesis across different developmental stages was tracked as described in the section 3.1.11.

3.3. Spikelet and ovary cultures

3.2.1. Establishment of microspore and ovary developmental stages

Ideal method for optimization of embryosac developmental stage involves direct investigation of the embryosac at each time of inoculation. But such a direct investigation is difficult because of lengthy processes of thin sectioning, staining, and clear squashing (Herr, 1971) or embryosac isolation (Enaleeva and Dushaevaa, 1975; Huang and Russel, 1989; Wagner et al, 1989). Further,

because of the tiny size of the flower buds in cereals and the difficulty in handling of dissection of the gynoecium, usually indirect judgment was made by observing stage of microsporogenesis (Zhou et al, 1986).

In the present study, anthers were removed from the spikelets, squeezed gently onto a microscope slide to release microspores, stained in a drop of acetocarmine and observed under a microscope. Three developmental stages of microspores (uninucleate, binucleate, or late binucleate) were identified and the corresponding embryosac developmental stages were studied to establish a correlation. For observing embryosac developmental stage, spikelet samples at different developmental stages were fixed in 3% gluteraldehyde, processed and sectioned, stained with toulidine blue and observed under binocular microscope.

3.3.2. Isolated ovary culture, and sequential ovary culture

For culture of isolated ovaries, spikelets were sterilized (as described above), dissected under aseptic conditions and cultured under same incubation conditions as for the culture of whole spikelets. In the case of sequential ovary culture experiments, initially spikelets were cultured as described above. One week after *in vitro* culture, glumes were removed and rest of the spikelet was sub-cultured. After two weeks, bracts were removed before sub culturing. From the third week onwards, only the ovary (with stigma and style) was retained and gynogenic induction (initiation of a macroscopic callus, pro-embryo or direct plantlet from any region of cultured explant was defined as a unit of gynogenic induction) was recorded at weekly intervals.

3.3.3. Sampling and replications

For each experiment, explants collected from four spikes on the same day from different plants were pooled to minimize *in vivo* variation due to donor plant in the replicates. Each experiment was repeated thrice. Gynogenic induction was recorded as percent of the responding explants out of the total number of explants cultured. Statistical analyses were performed using *SIGMASTAT 3* software.

3.4. Analysis of the regenerants and their progeny

3.4.1. Molecular marker analysis

3.4.1.1. RFLP analysis

In total, 150 gynogenic progeny lines, along with *in vivo* derived hybrid and parental lines (served as controls) were studied for their RFLP genotype. The seed lots used were the original seed (R_0) from each of the *in vitro* regenerated gynogenic plant. Original seeds from each of the *in vitro* regenerated plants were sown in 20 pots and plants were raised @ 5 plants per pot. Approximately 15 days after sowing, five grams of fresh leaf material (from bulk of five plants per pot) was collected and fixed in liquid nitrogen until their further use for DNA extraction.

3.4.1.1.1. DNA extraction and blotting

After the fresh material was ground in liquid nitrogen, DNA extraction was performed as previously described by (Sambrook et al, 1989) except that additional Proteinase K treatment was done before the chloroform-isoamyl alcohol extraction (*Appendix 6*, for *protocol*). DNA was restricted with four

restriction enzymes, *EcoR* I, *EcoR* V, *Dra* I, *Hind III* according to the manufacturer's instructions (*Amersham*) with 6 units of enzyme per microgram of DNA over 15 h. at 37°C. Aliquots of 15 μg of digested and purified DNA were loaded onto a 0.8% agarose gel in TAE buffer (Sambrook et al, 1989). After electrophoresis, the gel was denatured in 0.25 N HCl for 15 min. and subsequently in 0.4 N NaOH for 1 h. The DNA was blotted onto a *Hybond N*$^{+}$ membrane (*Amersham*).

3.4.1.1.2. Preparation of the probe and hybridization

The pearl millet RFLP probes used for the present study were kindly provided by John Inns Centre, Norwich, United Kingdom. They were labeled with the random primer DNA labeling kit (*NEBlot* Kit, *Biolabs*, New England) for 2h. with [^{32} dATP] to a specific activity of $1x10^{9}$ cpm per microgram (*Appendix 7, for protocol*). Prehybridization and hybridization were carried out in a buffer containing 6X HSB, 0.5% SDS, 5X Denhart's (Sanbrook et al, 1989) and 25 % 5 μgml^{-1} of sonicated and denatured salmon sperm DNA. The blots were pre-hybridized for four h at 65°C in 30 ml buffer. The prehybridization solution was then removed and replaced with 10 ml of buffer containing denatured radioactive probe at $1x10^{6}$ cpm per milliliter; hybridization was then performed overnight at 65 °C. The blots were then washed at 65 °C for 30 min. each in 2X SSC, 0.5% SDS and once in 2X SSC, 0.1 % SDS and finally once for 30 min. in 0.1X SSC, 0.1 % SDS. The blots were then wrapped in *Saranwrap* and exposed for 4 days to Hyperfilm (*Kodak*) with two intensifying screens at - 70 °C.

3.4.1.2. RAPD analysis of the progeny

3.4.1.2.1. DNA isolation and purification

The stock of DNA (from 450 progeny lines derived from plants A, B, D, E, F, and G and the corresponding controls) that was used for RFLP analysis was diluted according to the requirement. RAPD-PCR was performed (*Appendix 8, for protocol*). Random 10-mers (random primers) employed in this study were purchased from *Operon Technologies*, USA.

3.4.1.2.2. PCR amplification

PCR reaction was performed (under a Laminar air flow chamber) with 25 µl of a total reaction mixture containing 25 ng of genomic DNA, 2.5 µl 10x PCR buffer, 1.5 µl 25 mM $MgCl_2$, 1µl 2.5 mM dNTP, 1 µl 10 µM RAPD primer and 0.4 µl *Taq polymerase* (*Gibco BRL* 5 units/µl). The volume was made up to 25 µl with sterile distilled water. A control without template DNA was included in each set of reactions with each primer. The amplification reaction was performed in a *Perkin Elmer Gene Amp 9600* thermal cycler programmed for 40 cycles.

3.4.1.2.3. Electrophoresis

The amplified DNA fragments were mixed with 2 µl of 6x loading dye [25 mg xylene cyanol and 1.5 g ficoll (type 400) for 10 ml]. PCR products were electrophoresed overnight on 1.5% agarose (Sigma) gels at of 25 V. The gels were stained with ethidium bromide (5 µg ml^{-1}) and photographed under UV illumination.

3.4.2. Agromorphological analysis

Morphological and agronomic evaluations were done both under field and greenhouse conditions. For this, 560 lines from 7 DH populations (referred to as A-G) were used. Six agronomical characters - plant height, spike length, width and length of the first three leaves, percent seed set and 100-grain mass were measured. When planted in the field, seed from each regenerant (R_0) was sown in 20 nursery rows of 4 meters length containing 20 plants per row. For morphological marker analysis, a total of 5 plants (in the middle section of the row) were studied per row. When the progeny was raised in greenhouse, all plants in the progeny of each regenerant were scored.

3.4.3. Cytological studies

Root tips for cytological studies were collected from *in vitro* regenerated gynogenetic plant (R_0) and from the seedlings derived from R_0 plants. To trap haploid plants and to avoid diploidization, 6 of the 27 regenerants were not treated with colchicin. In case of the other plants, completely regenerated haploid plants at 2-3 leaf stage were washed with sterile distilled water, treated with 0.1% colchicin + 2% DMSO + 10 mg l^{-1} GA for 5 h at 22°C under light. These treated plants were later washed thoroughly with water before transferring them to soil and establishing them in pots. Pollen mother cells (PMCs) for studying meiosis were obtained from the spikes of *in vitro* generated gynogenic plants.

Actively growing root tips were collected from young *in vitro* regenerated gynogenic plants kept for at least 12 h in dark. They were pretreated with 1%

saturated α-bromonaphthalene for 3 h at room temperature to condense mitotic metaphases. The root tips were then fixed in acetic ethanol (1:3) for 24 h at 4°C. After three successive rinses with distilled water for 5 min. each, they were enzyme digested in 1% each of *Pectinase* and *Macerozyme* for I h at room temperature; hydrolyzed in 1 N HCl for 10 min. at 60°C. They were stained with Schiff's reagent for 20 min. before dilacerating the meristematic zone in a drop of 45% acetic acid. Same procedure was followed for meiotic studies with a single alteration that the false condensation of chromosomes (α-bromonapththaline treatment) was avoided, as already there was problem of severe clumping with the haploid meiotic chromosomes.

4. Results

From different explants used, doubled haploids could be successfully produced only from spikelet cultures. Protocols were standardized for production of microspore-derived embryos or calli using *in vitro* anther culture technique, and subsequent differentiation of such androgenic structures into plantlets. Haploid plants of two F_1 hybrids were obtained (at a frequency of >14% from H_1, and >8% from H_2) from microspore-derived embryos. Microspore origin of these embryos was confirmed by light and fluorescence microscopy. However, all the regenerated plants were albinos, except in a single genotype (7042 DMR) where green shoots were produced which failed to regenerate further. Embryogenesis was induced successfully (12.3%) using isolated microspore cultures. However, again the differentiating embryos gave rise to only albino shoots. Fertile, gynogenic doubled haploids were regenerated from *in vitro* spikelet cultures, and successfully transferred to green house and field. Ploidy levels of these regenerants were confirmed using cytological studies. Doubled haploid (homozygous) nature of these regenerants was confirmed through morphological and molecular studies (RFLP and RAPD) of their progenies.

4.1. Anther culture

Anther culture is the most common technique for regeneration of a 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 the present study various factors were successfully optimized for the first two phases and microspore-derived embryos or calli were obtained. Subsequently, combinations of various plant growth regulators were tested and concentrations were optimized for regenerating such androgenic embryos or calli. However, all the regenerants thus produced were albinos. It was observed that androgenic induction depended on successful deviation of microspores from their gametophytic pathway to sporophytic pathway. On the other hand, genotypic factors and the pathway of microspore embryogenesis are crucial factors for albino or green plant production. Therefore, in addition to genotypic, physical, physiological and chemical factors influencing androgenic induction, I studied different pathways of microspore embryogenesis in pearl millet.

4.1.1. Inflorescence structure and pollen development *in vivo*

The pearl millet inflorescence is a false spike ranging from 5 to >150 cm in length and from 1 to 5 cm in diameter. An important feature of the floral biology of pearl millet is its conspicuous protogynous nature that facilitates cross-pollination. Stigmas generally emerged on flowers just below the tip of the partially exerted spikes, and dehiscence proceeded downward. The first stamens appeared usually

2-3 days after the appearance of stigmatic branches. Complete emergence of stigmas on a spike generally occurred within 2 days.

Each spikelet bears one bisexual fertile floret and one staminate floret. The fertile floret **(Figure 2)** develops considerably earlier than that of the staminate one. Along the length of each panicle, spikelets at the panicle apex are oldest and those at the base are youngest, the difference being 3-6 days depending on the temperature. Normal sequence of development *in vivo* (**Figure 3**) consists of first pollen grain mitosis (PGM), which results into vegetative and generative cell and subsequent spermatogenesis from generative nucleus resulting in two sperm nuclei. **Figure 4** shows first pollen grain mitosis where uninucleate microspore passes through prophase (4A and B), early metaphase (4C), and alignment of chromosomes on to metaphase plate (4C), anaphase (4D), and finally telophase to produce two nuclei (4E). After mitosis, the generative cell divides to form two sperm cells (4F). At later stages of spermatogenesis, intine deposition and starch accumulation occurs.

The first anthers generally emerged from apical florets on the panicle approximately one day after most styles have emerged. Most panicles shed pollen for 4-6 days. When the temperature exceeded 25°C, anthesis occurs anytime during the day with the greatest flush of anthers appearing soon after sunrise. However, when cultured *in vitro*, instead of producing normal pollen grains, a section of microspores repeatedly divided to produce androgenic embryos.

4.1.2. Factors effecting androgenic induction *in vitro*

Various factors like genotype of the donor plant, and the preculture temperature treatments (cold and heat) applied to the explants affected diversion of microspores from gametophytic to sporophytic pathway of development. Quality and intensity of the light used for incubation of cultures, physical state and hormone compositions of the culture media influenced the frequency of subsequent androgenic structures. Many of these treatments were statistically significant (**Table 2**).

Table 2: Statistical analysis of different factors effecting androgenic induction.

Treatment	***TMS***	***F***
Genotype	54.0	54.10**
Light regime	38.3	19.50***
Medium state	67.2	25.35**
Cold pretreatment	137	137.0***
TDZ	81	51.74**
TDZ + Cold pretreatment	122	37.14**
Sucrose conc.	59	33.00**
Silver nitrate	19	NS
Heat shock	23NS	NS
Sucrose starvation	67	56.00**

TMS = treatment mean square; **F** = F value, **NS** = Not significant

*Significant at 5%; **significant at 1%; ***significant 0.1%

4.1.2.1. Effect of genotype and viability of microspores

Androgenic ability greatly depended on genotype of the explant (average response of various genotypes, 20.12%). Androgenic induction varied among the 17 genotypes compared (**Figure 5**). Maximum response was observed in 7042 DMR (48.0%) and the response was least (2.0%) in the genotypes IP18298. However, between the two F_1 hybrids (which were studied in detail to standardize protocol), androgenic response in H_1 was better than in H_2 for all the parameters studied.

One of the reasons for the low androgenic response in crop genotypes is the high mortality of microspores in *in vitro* cultures. However, it was shown that pearl millet pollen could be stored at -73°C for 6 months without losing its viability (Hanna et al, 1983). Observations across different genotypes suggested that the average viability was 80% at the time of panicle harvest, more than 60% after cold pretreatment, and more than 46% after 3 weeks in culture (**Figure 6**). Thus, the high percentage of viable microspores in culture may suggest that factors other than microspore viability may be responsible for the low yield of embryoids from anther culture in some genotypes of pearl millet.

4.1.2.2. Effect of microspore developmental stage

Androgenic induction levels were compared at three developmental stages of microspores (late tetrad stages, uninucleate microspores, and binucleate microspores). Anthers containing uninucleate microspores gave best response ranging from 21-39% across various genotypes. Anthers at other

stages showed less response: those at late tetrad stages showed 0-3% and those at binucleate stages showed a response of 2-5%.

4.1.2.3. Effect of explant source

Direct culturing of anthers (without preculturing of spikelets) in YP callus induction (YPCI) medium resulted in very poor androgenic response (3%) with slow and low frequency of microspore divisions. This may suggest that anthers and microspores depend on surrounding tissues for their nutritional requirements during initial stages of androgenic induction. Besides, direct culture also carried risk of occasional anther injury during isolation, and triggering of cell divisions in diploid tissue. On the other hand, anthers that were released (after 7-9 days; **Figure 7A**) from *in vitro* cultured spikelets showed rapid divisions of the microspores. When collected and cultured again on YPCI medium (**7B**), these released anthers showed multi-nucleate and multi-cellular microspores, which subsequently formed proembryos. Browning of the anther wall was noticed two weeks after their first subculture; still the healthy proembryos continued to develop and gave rise to mature embryos.

4.1.2.4. Effect of cold treatment on frequency of embryoids

The treatment at 4°C was not effective for increasing androgenic response (12.0% in control vs. 10.5% in treated). The effect of cold temperature treatment at 4°C and 14°C was studied with two hybrids (H_1 and H_2) and their respective parents. Cold treatment of 14°C for 7 days increased androgenic response in all

the genotypes studied (**Figure 8**). The average response of seven genotypes was 4.1% in control, while the same was 12.02% in cold treated panicles.

4.1.2.5. Effect of heat-shock

Effect of cold treatment was compared with that of heat shock (total 6 treatments containing 2 temperatures, 30°C and 35°C and three durations each, 12h, 24 h, and 36 h). Cold pretreatment has increased the androgenic response in all the genotypes, but enhanced androgenic response after heat shock was observed only in some genotypes (**Figure 9**).

Heat shock treatment at 30°C for 36 h increased androgenic response in two genotypes (ICMH85410 and H_2). In rest of the genotypes i.e. H_1 and 81B, heat shock effect was marginal. However, in ICMP451, 843B and 7042DMR, heat pretreatment actually decreased the frequency of androgenic response.

Heat shock at 30°C for 12 h or 24 h did not show increase in androgenic response in any of the genotypes studied. Heat shock at 35°C for 12 h was inhibitory for microspore development in all the genotypes and a shock at 35 ° C for either 24 or 36 h drastically effected microspore viability.

4.1.2.6. Effect of different light regimes

Initial incubation of anthers under red light for 3 weeks was beneficial to increase androgenic response when compared to the anthers incubated under dark or under white light. Proembryo formation was highest in cultures incubated under red light (17.9% was the average response across genotypes) followed by

those cultured in dark (10.8% was the average response). Minimum response (6.1%) was observed in cultures incubated under white light (**Figure 10**). However, white light encouraged further conversion of such proembryos into mature embryos **(Figure 11)**. Thus, red light seems to enhance the formation of proembryos while white light enhanced their further maturation.

Providing donor plant with additional light did not increase androgenic response.

4.1.2.7. Effect of sucrose concentration in culture media

To study effect of concentration of sugar used in induction medium, cultures were initiated with an induction medium (YPCI) supplemented with 3, 6 and 12% sucrose. Maximum induction rate (28.5%) was observed in medium provided with 6% sucrose in both the genotypes (**Figure 12**).

4.1.2.8. Effect of sucrose starvation

To study effect of sucrose starvation on androgenic induction two genotypes (H_1 and H_2) were used. Cultures were initially subjected to sucrose starvation for three days using YP liquid media with no sucrose. Explants were later transferred to media containing 3, 6 or 12% sucrose. It was observed that, sucrose starvation and subsequent transfer of these cultures to 6% sucrose marginally increased androgenic induction (**Figure 13**) in both the hybrids tested when compared to respective controls, which were not starved.

4.1.2.9. Effect of TDZ

Thidiazuron (TDZ); a phenyl urea compound was used at 3.0 μM concentrations in induction medium to observe its effect on microspore embryogenesis. TDZ increased the frequency of microspores shifting from normal pollen grain formation to follow sporophytic pathway. Starch formation in developing microspore is a characteristic feature of the gametophytic microspore development. TDZ could successfully arrest starch formation (**Figure 14**) during microspore development and increased frequency of microspore embryogenesis.

4.1.2.10. Effect of different culture media on formation of embryoids

Cereal anther cultures require the application of exogenous plant growth regulators (Ferrie et al, 1995). Both YP and N6 media are widely used with or without different growth regulator combinations in cereal anther cultures. Therefore, based on previous report (Suresh, 1995) a single combination of growth regulators [2,4-D (3.0 μM) + KN (3.0 μM) +BAP (2.0 μM)] was used with YP, N6 and MS basal media and tested for their effectiveness. Out of three basal media used, YP media was found to be the best (29%) for androgenic induction followed by N6 (11%) and MS (5%).

4.1.2.10. Effect of different plant growth regulators

Effects of different plant growth regulators used in YP basal medium were summarized in **Table 3**. When used singly, either 2,4-D (YPI 1 and YPI 2) or NAA (YPI 5 and YPI 6) did not produce either calli or embryos from microspores. When a high concentration of strong auxin like 2,4-D is used in combination with

lower concentration of cytokinin in YPI 4 medium, callus production from microspores was observed (**Table** 3). On the other hand, when a high concentration of weak auxin like NAA was used with low concentration of cytokinin (YPI 7.8), both pollen calli and embryos were produced (**Figure 15 D-G)**.

When a cytokinin like BAP was used alone (YPI 9), there was no formation of calli or embryos. However, a combination of NAA, BAP and TDZ in the presence of an antiauxin, TIBA (YPCI 13-YPCI 15), resulted in only pollen embryogenesis (**Figure 15H**) without any callus formation.

Table 3: Effect of different growth regulator combinations on androgenic induction frequency.

*D denotes 2,4-D; N - NAA; K - kinetin; B - BAP; T- TDZ; TB – TIBA.

S.No.	Modified YP Induction medium	Growth regulator* composition (mgl^{-1})	No. of anthers cultured **	Mean induction frequency (%)	
				Calli	Embryos
1	YPI 1	1.0 (D)	98 (3)	0	0
2	YPI 2	2.0 (D)	103 (3)	0	0
3	YPI 3	2.0 (D) + 0.5 (K)	186 (3)	0	0
4	YPI 4	3.0 (D) + 0.5 (K)	110 (4)	29 ± 3	0
5	YPI 5	1.0 (N)	136 (4)	0	0
6	YPI 6	2.0 (N)	104 (4)	0	0
7	YPI 7	2.0 (N) + 0.5 (K)	130 (4)	15± 4	5±2
8	YPI 8	2.0 (N) + 0.5 (K)+ 0.1 (TB)	126 (4)	11±3	9±1
9	YPI 9	1.0 (B)	96 (3)	0	0
10	YPI 10	2.0 (B) + 1.0 (N)	112 (3)	0	0
11	YPI 11	2.0 (B) + 2.0 (N)	108 (3)	0	4±1
12	YPI 12	2.0 (B) + 2.0 (N)+ 0.1 (TB)	140 (4)	5±2	25±4
13	YPI 13	0.25 (T) + 2.0 (N) + 0.1(TB)	265 (5)	0	29±1
14	YPI 14	0.5 (T) + 2.0 (N)	180 (5)	0	11±3
15	YPI 15	0.5 (T) + 2.0 (N) + 0.1 (TB)	146 (4)	0	32±4

' Value in bracket indicates number of individual experiments performed.

4.1.3. Study of different stages of microspore embryogenesis in vitro

Different stages of microspore embryogenesis were studied in detail using anthers collected from spikelet cultures. The first microspore division was observed 1-3 days after initiation of spikelet culture while the anthers are still located inside the spikelets. At this stage, microspores varied in their size and staining capacities. For example, when stained with acetocarmine, or DNA specific stains like DAPI, intensity of generative nucleus was significantly more than generative nucleus. After 4 weeks under culture, microspores had many nuclei (multinucleate microspores), and some microspores showed initial stages of cell divisions (initiation of multicellular microspores).

Different types of multicellular microspores were observed as judged by the intensity of their nuclear staining (vegetative cell derived, **Figure 16A**; undifferentiated cell derived; **Figure 16B** and generative cell derived **Figure 16C**) were observed after 6-8 weeks in culture (**Figure 16D**). These multicellular structures increased in size, and ultimately 3-5% of such structures emerged from the anther by bursting through anther wall. Such proembryoids did not develop further. Only those proembryoids residing within the anther wall developed into globular embryoids

4.1.3.1. Factors effecting initial stages of proembryo development *in vitro*

I studied the patterns of microspore divisions starting from cold pretreatment of panicles until 3 weeks under *in vitro* culture. In addition to microspores that underwent mitosis normally and gave rise to trinucleate pollen or microspores that followed sporophytic pathway to produce multi-nucleate

microspores, there were other types of abnormal microspores as described in the following sections.

4.1.3.1.1 Anomalous pollen development *in vivo* and *in vitro*

Apart from the normal pollen grains (that undergo mitosis, give rise to vegetative and generative cells), three types of anomalous pollen grains are of relevance in anther culture. The first type, **unreduced spores** are a potential source of diploid heterozygous regenerants. Such grains were not observed in the present study. The second type pollen are the grains in which the primary pollen grain mitosis is symmetrical and gives rise to **two identical nuclei** (possible source of diploid homozygous regenerants) rather than dividing into a generative and vegetative cell. Such pollen was observed in 14±3% of the *in vitro* cultured pollen. The third type pollen (***p* grains**) are the **small, faintly cytoplasmic** grains, often found at low frequency among mature pollen at anthesis. In the present study with pearl millet, *p* grains were observed both *in vivo* and *in vitro* (**Figure 17**) but their frequency increased *in vitro* because of cold pretreatment.

4.1.3.1.2. Effect of cold pretreatment on *p* grain frequency

There was significant variation in *in vivo p* grain frequencies across different genotypes. The frequencies of *P* grains were compared both during *in vivo* phase (during panicle harvest) and *in vitro* (after cold pretreatment). Genotype 843B showed maximum number of *p* grains both *in vivo* and *in vitro*, followed by 7042 DMR and H_1 (**Figure 18**). Frequency of this *p* pollen has

increased up to five-folds under cold pre-treatment. Thus, though cold treatment generally increased *p* grain frequencies in all the genotypes, their level of enhancement is genotype dependent.

4.1.3.1.3. Pattern of microspore divisions during cold pretreatment

Cytological examination was conducted on anthers from panicles pre-treated for 8 days at 14°C. After pretreatment, overall pollen viability as estimated by staining capacity with FDA varied between 75-90% depending on the genotype. By the end of the cold pretreatment, first pollen grain mitosis (PGM) produced a generative nucleus and a vegetative nucleus in 6% of the microspores. These microspores followed further route of normal microspore gametogenesis as in *in vivo* conditions. For example, **Figure 19 A&B** show vegetative nucleus and generative nuclei adjacent to each other in early stages after PGM, in normal *in vivo* microspores. During later stages **(Figure 19C-F)** vegetative and generative nuclei migrated to the opposite ends. However, in up to 14±3% of the microspores, PGM produced two similar nuclei **(Figure 20)**.

4.1.3.1.4. Pattern of divisions in *in vitro* cultured anthers

In embryogenic anthers, 3% of pollen grains are still uninucleate even after 48-72 hours in culture. This indicates the delay or absence of PGM. Among the remaining 97% microspores, two types were noted: *Class I*- where the first PGM resulted in formation of two types of nuclei with different staining capacities (which resembled vegetative and generative nuclei of *in vivo*, **Figure 21**); and

class II-, where first PGM produced two similar nuclei. In *class II* microspores, both the nuclei showed equal intensity when stained with DNA specific stain like DAPI (**Figure 20 A**). By the end of fourth day, both types of microspores (class *I* and *II*) underwent divisions and formed microspores with three or more nuclei. But the division products of *class I* microspores showed less intensity of staining indicating their predominant vegetative nucleus origin (**Figure 20 B;** A pathway, Sunderland 1974).

After six days in culture, *class II* microspores contained six or more similar nuclei (B pathway). In *class I* microspores, vegetative cell derivative was either in the process of division or had completed the next division so that there were three derivatives of vegetative cell (**Figure 22A**). In some microspores, division of the vegetative nucleus is not followed by cell wall formation and the resulting nuclei are of the same size. Sometimes, both derivatives of the first division simultaneously underwent divisions and formed four or six nucleate structures. However, there was no evidence of division of the generative cell (C pathway) until the end of sixth day.

The generative cell of a potentially embryogenic microspore divided only after 6 days (**Figure 22 F**). This suggests that, microspores following A and B pathways are capable of responding more rapidly to induction signals, than microspores that follow C pathway. After the sixth day, in some microspores both vegetative and generative nuclei participated in divisions simultaneously **(Figure 22 C, D and E**). At the end of day 9, embryogenic pollen contained both multinucleate and multicellular pollen derived from all three pathways, (**22 G-I**). Usually the cell wall formation was complete in microspores with rich cytoplasm

than in vacuolated microspores. In latter case, microspores showed division started sometimes with formation of additional germ pore (**Figure 23 A**), and sometimes even in the absence of pore formation, centripetal ingrowth of cell plate was observed {both incomplete (**Figure 23 B & C**) and complete (**Figure 23 D & E**)} was observed. Insufficient synthesis of wall precursors may be the reason for incomplete cytokinesis in these microspores. Most of the nuclear divisions in *p* grains were not followed by cell wall synthesis, thus resulting in multi-nucleate structures. After six days of culture, the exine of some multicellular units had ruptured releasing a proembryo.

4.1.4. Factors effecting regeneration of microspore derived embryos.

It was observed that in pearl millet, as in the case of other cereals, the process of androgenic induction is independent of the process of regeneration. Increased production of androgenic embryos or calli obtained by optimized combinations of growth regulators in the induction media, or the various pretreatments applied to microspores were not always resulted in increase in frequency of regenerants. For example, in certain genotypes, though the pre-culture heat shock or pre-culture sucrose starvation was helpful for enhancing initial androgenic induction (**Figure 24**); such androgenic structures were aborted after transferring them onto the regeneration media. Additionally, physical state of the media influenced the induction frequency. Liquid medium (**Figure 25 A, B, F & D**) is favored during induction phase while solid medium (**Figure 25 D & E**) was best for further regeneration and germination of androgenic embryos or calli.

Of the ten regeneration media tried (**Table 4**), two media combinations (YPR 3 and YPR 4) were favorable for regeneration from both pollen calli and

embryos (frequencies summarized in **Table 4**). Another growth regulator combination (YPR 7) was useful to produce maximum regenerating structures from pollen embryos. When rests of the media combinations were used, both pollen calli and embryos failed to regenerate.

Using the above-described favorable regeneration media, regeneration was achieved from both embryogenic embryos (**Figure 26)** as well as microspore derived calli (**Figure 27**). A total of 91 albino regenerants (**Figure 28**) were obtained from H_1 and 43 regenerants (again albinos) from H_2 **(Figure 29**). The somatic embryos and the embryogenic callus from the genotype 7042 DMR developed green patches within two weeks after transferring to regeneration medium. However, instead of a balanced germination giving rise to shoot and root induction, there was an abnormal rooting, which stunted the shoot production. As a result, the regenerating structures became brown and eventually disintegrated.

Table 4: Effect of different growth regulators on regeneration of androgenic calli or embryos.

S.No.	Regeneration medium	Growth regulator composition (mg L^{-1})	Mean regeneration frequency (%)	
			From Calli	From Embryos
1	YPR 1	2,4–D, 2.0 + IAA, 0.5+ GA, 0.5	0	0
2	YPR 2	2,4-D, 2.0 + IAA, 0.5	0	0
3	YPR 3	2,4-D, 1.0 + BAP, 1.0+ TIBA, 0.2	5±2.1	4±1
4	YPR 4	2,4-D, 0.5 + BAP, 1.0 + TIBA, 0.2	3±1.2	9±1.5
5	YPR 5	2,4-D, 1.0 + BAP, 0.5 + GA, 0.5	0	0
6	YPR 6	2,4 - D, 0.5 + BAP, 0.5 + GA, 0.5	0	0
7	YPR 7	2,4-D, 0.5 +BAP, 1.0 + TIBA, 0.2	0	15±2
8	YPR 8	2,4-D, 0.0 +BAP, 1.0 + TIBA, 0.2	0	0
9	YPR 9	NAA, 1.0 +BAP, 1.0+ TIBA, 0.2	0	0
10	YPR 10	2,4 -D, 0.0 +NAA, 1.0+ BAP,1.0	0	0

4.1.5. Analysis of the anther culture derived regenerants

Cytological studies of root tips conducted on a total of 30 anther derived albino plants (from H_1) showed that 28 of them were haploid and 2 were mixoploids. Root tip squashes of the 10 plants from H_1 revealed that all of them were haploids only.

4.2. Isolated microspore cultures

4.2.1. Effect of different hormone compositions on microspore embryogenesis

Out of different combinations of hormones tested, maximum frequency of multi-cellular microspores was obtained from YPI 5 medium (**Table 5**). When compared to the YP medium having only 2,4-D, most other combinations increased the induction frequency. Presence of amino acids increased the embryo induction significantly (YPI 1,3,5 *vs.* YPI 2,4,6).

Though TDZ alone could increase induction response (YPI 6 *vs.* YPI 3), it's positive effect was synergistic when amino acids are also present in the medium (YPI 5 *vs.* YPI 6).

4.2.2. Effect of source of microspores on microspore embryogenesis

Just as for anther cultures (described above), induction potential of microspores depended greatly on their source (of anthers) whether they are from pre-cultured anthers or fresh anthers (**Figure 30**). While the maximum induction frequency with the best medium (YPI 5) was only 1.3 % in fresh anther derived

microspore embryos, the response was as high as 12.3% in microspores isolated from pre-cultured anthers. Microspores isolated from pre-cultured anthers showed maximum frequency of active divisions and pro-embryo formation by the end of two weeks (**Figure 31**). Three weeks after transferring onto regeneration medium (1 mg L^{-1} IAA + 1 mg L^{-1} BAP + 0.5 mg L^{-1} GA), such pro-embryos matured and formed embryos with root and shoot initials.

Table 5: Embryo induction percentage in different media combinations.

Medium	Hormones (mg L^{-1})	Amino acids*	Induction rate (%) Mean ± SE
YPI 1	2,4-D (3.0)	Yes	2.9 ± 0.85
YPI 2	2,4-D (3.0)	No	0.8 ± 0.85
YPI 3	2,4-D (3.0) + Kn 0.5	Yes	5.3 ± 0.17
YPI 4	2,4-D (3.0) + Kn 0.5	No	1.8 ± 0.12
YPI 5	2,4-D (3.0)+ Kn (0.5) +TDZ (0.1)	Yes	12.3 ± 0.31
YPI 6	2,4-D (3)+ (Kn 0.5) +TDZ (0.1)	No	7.95 ± 0. 21

* L-Proline (200 mg L^{-1}), Glutamine (100 mg L^{-1}) & Casein hydrolysate (500 mg L^{-1})

4.2.3. Effect of method of media sterilization on microspore embryogenesis

Method used for sterilizing culture media for microspore cultures influenced frequency of microspore embryogenesis. In each of the media combinations tried, filter sterilized medium was nearly four times more effective than autoclaved medium for inducing androgenic response in microspores.

Table 6: Effect of method used to sterilize the culture media on microspore embryogenesis.

Medium	Method of sterilization	Induction (%)
YPI 3*	Autoclaved	1.2 ± 0.2
	Filter-sterilized	4.7 ± 2.4
YPI 4	Autoclaved	0.5 ± 0.2
	Filter-sterilized	1.8 ± 0.9
YPI 5*	Autoclaved	2.8 ± 1.2
	Filter-sterilized	10.8 ± 5.3
YPI 6	Autoclaved	1.6 ± 0.7
	Filter-sterilized	6.2 ± 2.8

4.3. Spikelet and ovary cultures

Gynogenesis may be an alternate tool to produce doubled haploids in cereals, where anther cultures usually produced albinos. In pearl millet, *in vitro* gynogenic induction was affected by a number of factors. They included factors like embryosac developmental stage, cold pretreatment, technique of ovary culture, different growth regulator combinations, and the physical state of the medium, etc. I studied effect of most of these factors in order to develop a suitable protocol for gynogenic induction. For standardization of the explant and to study the effect of cold treatment, YP basal medium containing 2,4-D (2 mg L^{-1}) and BAP (1 mg L^{-1}), which was effective in inducing androgenesis (Shyamala et al, 1997) was used.

4.3.1.Effect of embryosac developmental stage

Study of sectioned embryosacs and anther squashes from the same spikelets were used to establish a correlation between embryosacs and pollen developmental stages. Of the three stages of spikelets tested for *in vitro* culture, gynogenic induction rate was highest (27 ± 4%) in stage 2 spikelets, which showed late uninucleate microspores. This stage of microspore corresponds with eight nucleate stage embryosac. Induction was much less with spikelets at other stages (4 ± 1 % with stage 1 spikelets containing tetra-nucleate embryosac, and 7±3 % with stage 3 spikelets with advanced embryosacs).

4.3.2. Effect of cold pre-treatment on gynogenic induction

Cold pre-treatment of the panicles helped to increase the frequency of enlarged ovaries (**Table 7**), but there was no significant increase in embryogenic callus formation. However, the frequency of non-embryogenic callus decreased because of cold pre-treatment (**Figure 32**). Overall, cold pretreatment was favorable because it reduced unwanted (non-embryogenic) callus formation and increased ovary enlargement (**Table 7**). Subsequently it was observed that such enlarged ovaries are capable of regenerating plants directly without any callus formation.

Table 7: Effect of pre-culture cold treatment of panicles on gynogenic response.

		Induction frequency (mean ± S.E)		
No. of spikelets cultured		% of spikelets showing non-embryogenic callus	% of spikelets showing embryogenic callus	% of spikelets showing enlarged ovaries
Control	130	36.3±8.1	5.0±1.7	12.0±2.3
Cold treated	156	9.7±2.5	5.3±0.9	43.8±7.4

4.3.3. Isolated, and sequential ovary culture

Cultures of spikelets showed callus formation in liquid medium (**Figure 33 B & C**) and ovary enlargement on solid medium (**Figure 33 A, D and E**). Experiments conducted using both solid and liquid media showed (**Table 8**) that sequential and ovary cultures were not effective in inducing gynogenesis. Sequentially cultured ovaries were healthy (**Figure 34 A & B**), when they were in association with surrounding bracts and glumes for the first three weeks started turning brown or gave rise to brown non-embryogenic calli (**Figure C & D**) only after surrounding tissues were completely removed during the last stage of reculture. Cultured ovaries turned brown by the end of the sixth week indicating their dependence on surrounding tissues for their survival. On the other hand, spikelet cultures produced Healthy, embryogenic callus (**Figure 34 E**) or enlarged ovaries (**Figure 34 F**).

Table 8: Responses of sequential and isolated ovary cultures and spikelet cultures.

Type of Explant	Physical state of the Medium	Induction frequency (mean % ± S.E)			Regeneration frequency		Ploidy of the regenerants
		No. of explants cultured	Explants showing non embryogenic callus	Explants showing embryogenic callus	Explants showing enlarged ovaries	No. of regenerated plants through embryogenic callus	No. of regenerated plants directly from enlarged ovaries	
Isolated ovary cultures	Liquid	170	8.1±1.5	0	0	0	0	-
	Solid	172	0.0	0	0	0	0	-
Sequential ovary culture	Liquid	168	6.2±1.7	0	0	0	0	-
	Solid	186	3.4±0.5	0	5.0±2.0	0	0	-
Spikelet culture	Liquid	185	42.9±8.2	15.5±4.3	3.2±0.8	0	0	-
	Solid	179	11.0±2.1	7.1±1.8	48.4±6.5	2*	0	one diploid, one aneuploid

4.3.4. Effect of different hormonal combinations on gynogenesis

Various combinations of plant growth regulators were tested with *Yu Pei* basal medium (numbered as YPGI 1 - YPGI 7, **Table 9**). Effects of different hormone combinations are summarized in **Table 9.** When used alone, 2,4-D (YPGI 1) produced loose, non-embryogenic callus. Combination of 2,4-D + Kn (YPGI 2-YPGI 3) also lead to production of loose, non-embryogenic callus. 2,4-D with BAP (YPGI 4-YPGI 5) induced enlargement of ovaries but no plantlet regeneration. However, this combination also induced callus formation. The calli, upon transfer to regeneration medium gave rise to plants with different ploidy levels (**Table 9**). A Combination of 2,4-D, BAP and TIBA used in the induction media (YPGI 6 – YPGI 7) not only resulted in embryogenic callus but also direct plant regeneration from enlarged ovaries in 60-70 days after culture without further transfer into regeneration medium (**Figure 35 A & B**). Majorities of such regenerated plants were green (**Figure 36 A),** with single exception of an albino (**Figure 36 B**). They were successfully transferred from petridishes (**Figure 37 A**) to autoclaved vermiculate in Jiffy cups (**Figure 37 B**); the plants were subsequently established in pots in greenhouse (**Figure 37 C-E**). Root tips from the *in vitro* regenerated plants, showed diploid (**Figure 38 A**).and haploid (**Figure 38 B**) chromosome counts

4.3.5. Comparison of solid medium with liquid medium for gynogenic induction

Spikelets cultured in liquid induction medium (YPGI 7) produced loose non-embryogenic callus. Rate of callus induction was high (57%) and ovary enlargement was low (3%) in liquid medium (**Table 10**). Histological sections of the cultured ovaries showed profound callusing from ovary walls in addition to the occasional development of somatic embryos inside the layers of ovary wall. However, such structures subsequently turned brown. Spikelets cultured on solid induction medium (YPGI 7) showed low (2%) callus production and high frequency (43.0%) of enlarged ovaries after 2 weeks. Light microscopy confirmed multinucleate stage of embryosac (**Figure 39 A & B**) during initiation of spikelet cultureand when dissected and observed after 4 weeks of culture, some of such ovaries showed somatic embryogenesis (**Figure 39 C**) and callus (**Figure 39 D**) formation from all over the ovary. Light microscopy studies also revealed that most of the swollen ovaries were empty (**Figure 40 A and C**). However, 28% of the swollen ovaries contained developing embryos mostly in the embryosac of responding spikelets (**Figure 40 B, D, E and F**). Subsequently, direct plantlet regeneration from the embryos was observed from such swollen ovaries (**Table 10**).

Table 9: Effect of different hormone combinations on gynogenic induction.

			Induction frequency (mean ± S.E)			Regeneration frequency		
	YPGI Media	No. of spikelets cultured	% of spikelets showing non embryogenic callus	% of spikelets showing embryogenic callus	% of spikelets showing enlarged ovaries	No. of regenerated plants through embryogenic callus	No. of regenerated plants directly from enlarged ovaries	Ploidy of the regenerants
YPGI 1	2,4-D, 2.0	102	16.5±3.4	0.0	0.0	0	0	-
YPGI 2	2,4-D, 2.0 + Kn,0.5	115	8.9±0.5	3.0±0.4	0.0	0	0	-
YPGI 3	2,4-D, 2.0 + Kn,1	98	12.6±1.8	7.5±0.9	0.0	0	0	-
YPGI 4	2,4-D, 2.0 + BAP,0.5	106	19.8±2.1	12.3±1.3	0.0	0	0	-
YPGI 5	2,4-D, 2.0 + BAP,1	119	17.4±1.9	17.2±3.5	6.5±1.0	1*	1**	*diploid ** haploid
YPGI 6	2,4-D, 2.0 + BAP,0.5 + TIBA, 0.1	125	5.0±0.4	4.8±0.8	39.0±4.2	0	0	
YPGI 7	2,4-D, 2.0 +BAP,1 + TIBA, 0.1	108	3.1±0.9	7.4±1.1	49.2±8.6	3$	8$$	$ two diploids, one hypo-haploid $$ 7 haploids, one diploid

4.3.6. Effect of activated on gynogenic induction

Activated charcoal is considered beneficial for cereal androgenesis (Sopory and Munshi, 1996). However, in the present study activated charcoal did not influence or enhance gynogenic response. Frequency of direct plantlet regeneration was the same on the medium without charcoal (**41 A & B**) as that on the medium containing activated charcoal (**41 C & D**).

Table 10: Effect of solid vs. liquid medium on gynogenic induction.

(Cold pre-treated spikelets are cultured on YPGI 7 medium)

		Induction frequency (mean ± S.E)			Regeneration frequency		
Medium	No. of spikelets cultured	% of spikelets showing non - embryogenic callus	% of spikelets showing embryogenic callus	% of spikelets showing enlarged ovaries	No. of plants through embryogenic callus	No. of plants from enlarged ovaries	Ploidy of the regenerants
Liquid	217	57.0±9.0	21.0±4.8	3.0±0.5	2	0	-
Solid	230	2.0±0.2	4.9±1.0	43.0±6.9	0	13	11 haploids, (1albino) 2diploids

4.4. Analysis of the regenerants

4.4.1. Morphological and agronomic analysis

A total of nine (6 dihaploid plants and 3 haploid) gynogenic plants (R_0) were evaluated for their agronomic characteristics along with normal hybrid plants (as controls). There was no significant difference between diploid controls and gynogenic doubled haploids for any of the six traits studied (**Table 11**). However, the difference between haploid plants and the controls was significant for all the traits. Of the three haploid plants studied, one plant had set small seeds (**Figure 42 B**). The 100 seed weight of this plant (0.17 g) was 1/18 of the control (**Figure 42A**). On the other hand, all the six of the doubled haploid plants had set healthy seeds (100 seed weight = 1.5± 0.35 g). A preliminary attempt was made to screen two of the DH progenies for their tolerance to downy mildew infection under glasshouse conditions ((**Figure 43**). However, the resistance level among the progeny lines was not significantly uniform and the work was not continued further considering the complexity involved in the mechanism of DM resistance.

The female parent (ICMP 451) of the hybrid used for induction of gynogenesis is characterized by the presence of conspicuous ligule in the leaf internode and bristles in the mature panicle (**Table 12**). Both these markers are absent in the male parent (843 B). Presence of ligule and absence of bristles characterize the hybrid. To monitor the inheritance of these two morphological markers, 7 R_1 progenies (100 plants per each progeny) of gynogenic plants (6 dihaploids, referred to as plants A, B, D, E, F, G and one aneuploid referred to as

C) were studied in the greenhouse and field (**Table 12**). Among these, three progenies resembled the male parent (843 B) in morphological features and

Table 11: Agronomic characteristics of gynogenic regenerants (R_0)

Plant	No. of plants	Plant height (cm)	Panicle length (cm)	Main stem girth (cm)	AVERAGE LEAF LENGTH	No. of internodes visible at maturity	100 seed wt. (g.)
Diploid (Control)	5	95.0±11.2	31.5±4.7	3.3±1.1	48.9±9.0	7±1	1.85 ± 0.4
Haploid	3	32.0±7.5	9.2±2.2	0.9±0.3	21.5±4.1	2±1	0.17* (for main panicle) 0.25 * (for tillers)
Doubled haploid	6	89.0±6.0	28.0±5.3	2.9±1.5	51.0±9.4	6±3	1.15±0.35

*Seed setting was observed in only 1 haploid plant out of 3 studied.

Table 12: Analysis of morphological markers in the progeny (R_1) of gynogenic regenerants

S. No.	% of plants with ligule	% of plants with bristle	Remarks on ploidy and homogeneity within DH lines
ICMP 451	100	100	Parent 1 (P1)
843 B	0	0	Parent 2 (P2)
ICMP 451 x 843 B	98	0	Hybrid
Gynogenic progenies	94	9	Heterozygous /diploid
Progeny of Plant A	2	0	Homozygous /dihaploid (P_2)
Progeny of Plant B	53	17	Variants/aneuploid
Progeny of Plant C	0	0	Homozygous /dihaploid (P_2)
Progeny of Plant D	97	95	Homozygous /dihaploid (P_1)
Progeny of Plant E	92	96	Homozygous /dihaploid (P_1)
Progeny of Plant F	3	0	Homozygous /dihaploid (P_2)
Progeny of Plant G			

agronomic characters. Two progenies resembled female parent bearing both the ligule and bristles. In all the five of these progenies, there was no segregation for these morphological markers. In each progeny, all the plants were uniform in their morphological and agronomic characters. The other diploid plant showed the presence of ligule and absence of bristles, as is the case of hybrid plants. However, the progeny of the aneuploid regenerant showed segregation for both the markers. Variance levels for agronomic traits among each R_1 progeny (A-G) indicated uniformity of DH lines except in the case of plant C (**Table 13**).

Table 13: Agronomic characteristics of progeny (R_1) of gynogenic regenerants

Plant designation	Plant A	Plant B	Plant C	Plant E	Plant F	Plant D	Plant G	Controls		
								ICMP 451 (P1)	843B (P2)	Hybrid
Plant height (cm)	27.5	24.0	10.0	34.0	38.7	24.8	22.6	45.0	23.7	41.4
Minimum	44.2	26.5	58.6	49.0	41.4	27.2	25.8	52.3	28.5	47.3
Maximum	37.00	0.87	271.30	14.63	0.95	0.55	0.46	4.27	3.82	6.19
Variance										
Panicle height (cm)										
Minimum	16.2	11.3	4.8	10.9	13.7	15.7	12.1	17.6	13.2	14.5
Maximum	19.5	16.5	17.4	14.5	18.9	18.4	13.6	21	16.5	19.0
Variance	7.63	9.45	13.83	8.35	9.21	5.32	3.74	6.83	5.27	4.65
Seed set (%)										
Minimum	83.2	45.4	58.1	51.0	62.3	75.0	83.7	65.5	87.0	78.2
Maximum	92.5	49.0	73.8	53.6	65.1	78.4	89.0	71.3	91.0	85.9
Variance	9.60	0.86	20.76	2.90	4.70	3.72	5.80	6.05	4.70	7.92
100 seed mass (g)	0.9	1.1	0.8	1.4	0.95	1.0	0.93	1.4	1.23	1.0
Minimum	1.2	1.5	1.3	1.6	1.4	1.3	1.24	1.6	1.4	1.3
Maximum	17.53	5.95	52.89	3.92	7.40	4.75	4.82	2.97	1.78	2.15
Variance										

4.4.2. Cytological analysis of the regenerants

Mitotic studies of root tips showed that 6 regenerants were haploids, 20 regenerants were diploids, and one was aneuploid. The meiosis was normal in diploids. Pollen mother cells (PMCs) of the haploids showed numerous cells with extreme chromosome clumping (**Figure 44 A, C & D**) even until the stage of metaphase I. A few cells contained a single bivalent and five univalents (**Figure 44 B**) indicating autosyndesis. During anaphase, single chromosomes migrated to poles instead of chromosome pairs (**Figure 44 E**). At anaphase I, a bridge configuration was clearly observed (**Figure 44 F**). The PMCs at metaphase I revealed unpaired univalents showing unusual alignments (**Figure 44 G & H**) because of non-availability of homologous chromosomes. At the end of telophase, cells with three and four chromosomes were observed (**Figure 44 I**). However, the diploids and doubled haploids showed normal meiosis.

4.4.3. RFLP studies

For RFLP analysis, 13 radiolabeled probes were Southern-hybridized to different combinations of the restricted fragments of doubled haploid genomic DNA. With some of the enzyme-probe combinations, there was clear polymorphism between parents while the hybrid showed both the bands. However, there was no polymorphism (any segregation) in the banding pattern among the progeny lines, indicating uniformity of DH plants.

For example, the progeny of gynogenic plant B, when digested with *EcoR* I and probed with a single copy insert *psm* 410 (**Figure 45**) showed clear polymorphism between parents. A single band of 9.41 Kb was generated in P_1

that is absent in P_2 and a band of 4.36 Kb was generated in P_2, that is absent in P_1. In the case of hybrid, both of these fragments were seen. In all progeny lines, only the band corresponding to 4.36 Kb (as that of P_2) was present and 9.41 Kb band was absent. Similarly, the DNA from progeny of same gynogenic plant, when digested with *EcoR* V and probed with a single copy insert *psm* 412 (**Figure 46**) showed clear polymorphism between parents. A polymorphic band of 8.7 Kb was present in P_1 and the same was absent in P_2. A band of 5.1 Kb was generated in P_2, which is absent in P_1. In addition to these, there were two common bands (6.2 Kb and 2.8 Kb each), which were seen in both the parents. In the case of hybrid, all four of these bands were seen. However, in all progeny lines, only the unique band corresponding to 5.1 Kb (as that of P_2) was present in addition to the two common bands, and 9.41 Kb band was absent. However, in the progeny of gynogenic plant A, both parents were polymorphic and hybrid contained the bands corresponding to both the parents. The profile of all the progeny lines resembled that of the hybrid (**Figure 47**).

4.4.4. RAPD studies

Out of 126 primers, only 12 primers revealed clear and repeatable polymorphism between both the parents and the hybrid **(Figure 48)**. Genomic DNA from progenies of plant B, D, E, F when amplified with 7 primers (OPD-2, OPE-2, OPE-16, OPG-8, OPJ-4, OPD-11, OPJ-1) did not show even a single polymorphic band among progenies, and all of them resembled either P_1 or P_2 with the bands generated using any single primer. For example, in the case of gynogenic plant B, when amplified with the primer OPE-16, a band of 1.1 Kb was

generated in P_2, which was absent in P_1. Hybrid contained both the bands. In all the progeny lines, this band was absent indicating that the progeny resembled P_1.

In the progeny of another gynogenic regenerant E, when amplified with the primer OPG-8 (**Figure 49**), a band of 900 bp was generated in P_1 and hybrid that was absent in P_2. In all the progeny lines, the band was absent as is the case with P_2. In the same progeny, when a different primer (OPD-2) was used (**Figure 50**), a band of 1.6 Kb was seen in P_1 and hybrid that was absent in P_2. All the progeny lines also lacked this band. However, in the same gynogenic regenerant E, all the progeny lines resembled P_1 (**Figure 51**) when amplified with yet another primer, OPD-11. In this profile, a band of 560 bp was amplified in P_1, which is also present in hybrid, but absent in P_2. All the progeny lines also showed presence of this band, resembling P_1. This confirmed the homozygous nature of the gynogenic regenerants B, D, E and F and consequently homogeneity in their progenies.

In the case of progeny G, when all the above 7 primers were used for amplification, the progeny showed no polymorphic bands when primed and amplified with 5 of the above 7 primers. However, when amplified with OPD-11 and OPE-16, this progeny showed one polymorphic band with each of the primer. Thus, in this progeny, of the 510 bands generated (using 7 primers), only two were polymorphic, making the residual heterozygosity of this progeny as 0.4 %. This is less than the heterozygosity observed in maize (1%; Murigneux et al, 1993). In the progeny of plant A, primer OPD-2, amplified four polymorphic bands. Band-1 is segregated among the progeny in 12:3 (presence: absence),

band-2 segregated in the ratio of 5:10, band-3 in a ratio of 13:2, and finally band-4 again segregated in the ratio of 5:10. This confirmed the heterozygous nature of the gynogenic plant A and consequently heterogeneity among its progeny.

5. Discussion

Haploids can be produced *in vitro* by triggering embryogenesis in either male or female gametes. Subsequent differentiation of such embryos and diploidization produces complete plants capable of sexual reproduction. In cereals crops, there are different methods to induce haploids *in vitro*. Androgenic haploids can be induced using anther or microspore cultures, and gynogenic haploids can be induced through ovary or spikelet cultures. Frequency and quality of the regenerants may vary with each of the above-mentioned techniques used for production of haploids.

5.1. Androgenesis

There are a number of factors that trigger androgenesis such as genetic, developmental, physiological, physical, and chemical factors [Maheswari et al, 1980; 1982; Bajaj, 1983], which affect the pollen grains to switch to a new developmental pathway. Present study with pearl millet confirmed the influence of various factors such as genotype of the donor plant, microspore developmental stage, various pre-culture treatments, physical state of the *in vitro* culture medium, light and temperature conditions during culture incubation on triggering androgenesis.

5.1.1. Effect of genotype

Androgeneic response of a genotype is controlled by genetic constitution of the donor plants. Studies in other cereals like wheat indicated existence of an

independent multi-gene control for androgeneic induction and green plant regeneration (Agche et al 1989). Many researchers have shown that the genotype of the donor plant plays an important role in the induction and development of the haploid plants (Prakash and Giles, 1992). Lu et al (1991) showed in wheat genotypes that the ability to induce pollen calli could vary from 0% to 35%. Present study with pearl millet genotypes, is the first to report relative androgenic induction potential under similar culture conditions. Out of 17 genotypes, only 6 showed >20% response.

5.1.2. Effect developmental stage of the explant

The pollen developmental stage at the time of anther excision for *in vitro* culture is a critical factor for successful induction of pollen embryos and calli. Besides affecting the overall response, developmental stage of the microspore had an important bearing on the nature of the plants produced in anther culture (Sunderland and Dunwell, 1977). Nitsch and Nitsch (1969) were the first to show that high regeneration frequency could be assured by restricting the culture of pollen only at uninucleate stage. In general, plantlets obtained from pollen at uninucleate stage were mostly haploids while at later stages, plants with higher chromosome numbers were found. It was reported that when microspores at e advanced developmental stages were used, for *in vitro* culture, ploidy level of the resulting embryos proportionately increased (Sopory and Munshi, 1996). Present study in pearl millet showed that anthers containing microspores at tetrad stage failed to respond. Anthers showing binucleate microspores showed minimum response (2.4%), while the response was maximum (42.5%) when uninucleate

microspores were used. This confirms the previous reports on other cereals where uninucleate microspores were found to be more optimal for successful androgenic induction (Guha-Mukherjee, 1973; Maheswari et al, 1980; 1982).

5.1.3. Effect of pretreatments

Even after selecting the most suitable genotype and optimal developmental stage, a preculture treatment (physical or chemical) to the explant further enhances frequency of doubled haploids. Various preculture treatments (cold treatment, chemical treatment, atmospheric conditions, etc.) have been shown to influence induction of androgenesis (Sopory and Munshi, 1996). Of all these factors, cold treatment is the most critical one playing significant role in increasing androgenesis, especially in the case of monocotyledons.

During the last two decades, a number of papers have appeared on the technique of preculture cold treatment (Maheswari et al, 1980; 1982). Cold pretreatment of panicles is believed to switch the genetic program from a gametophytic mode of development to a sporophytic mode (Nitsch 1982). Therefore, it has been successfully used to increase the proportion of dividing microspores in a number of cereals. For optimal anther culture response, the cold pretreatment requirement varied depending on the species and even on cultivars (Yamaguchi et al, 1990). In maize, typical cold pretreatment applied is for 7-14 days at temperatures ranging from 4 -14 °C (Nitsch et al, 1982; Genovesi and Collins 1982; Tsay et al, 1986). Several workers had reported the beneficial effects of cold treatment in rice (Tsay et al, 1988; Ogawa et al, 1992).

In the present study, of the different temperature pretreatments (4 °C, 14 °C and 30 °C) studied for their effect on androgenesis, a pretreatment of 14 °C for 6-8 days increased the androgenic response remarkably in all the genotype used (as is the case with most of other cereals). In fact, it was found that pretreatment was an essential and important step to obtain pollen embryos of pearl millet. However, in spite of many evidences testifying the requirement of cold pretreatment for successful androgenesis, the mechanism of cold-induced beneficial effect is still obscure. Several hypotheses have been put forth. It was suggested that cold treatment might cause a re-orientation of the spindle axis, resulting in two similar nuclei at the first pollen grain mitosis stage (Nitsch and Noreel, 1973). Alternatively, it may cause the accumulation of inducible microspores. Cold pretreatment can also influence indirectly by delaying the anther senescence, thus ensuring survival of induced microspores for longer duration (Sunderland, 1978). Cold pretreatment may also cause repression of gametophytic differentiation which is a branch-out phase for sporophytic pathway induction.

In the present study, while a pretreatment of 4°C was found to be detrimental, results from an elevated temperature treatment indicated genotype dependent alterations in androgenic response. This may suggest possible role of a complex, genotype dependent ethylene inhibition or reversion pertinent to each genotype because of high temperature treatment. In general, elevated temperatures were found to stimulate pollen embryogenesis in much researched dicotyledonous crops like *Brassica* (Keller et al, 1990).

5.1.4. Effect of *in vitro* culture conditions

Despite having selected the right genotype, optimal developmental stage and proper pretreatment, *in vitro* culture conditions play crucial role in increasing the frequency of haploid production. The factors that need to be standardized are both physical and chemical. Of two pearl millet hybrids used in my study to assess androgenic response under different physical and chemical factors, response in hybrid H_1 was better than H_2. Androgenic response depended significantly on physical state of culture medium. Response in liquid medium was more than in solid medium probably due to better aeration to the developing microspores in liquid medium.

There are many reports on the effects of light during incubation on anther cultures. However, the precise role of light regimes used for anther culture incubation is still not clear. Not much work was done on cereal crops on this aspect. In model dicotyledonous crops, the results on light effects are contradictory. An alternate light and dark period increased pollen embryo formation (Sopory and Munshi, 1996). In the present study with pearl millet, initial incubation of anther cultures under red light for three weeks followed by their exposure to the 16h /8h (light/dark) photoperiod was found to be optimal for pollen embryogenesis. The effectiveness of red light for pollen embryoid induction may suggest a possible regulatory role of phytochrome if there is any photoreceptor involved. In case of cereals, there is a need for further work to understand the precise role of light on anther culture.

Placement of the anther onto the culture medium has pronounced effect on microspore embryo formation. Orientation matters more in cereals where anther size is small and where the surface tension induced film of liquid around the anther is large in relation to the anther size (MarkShannon et al, 1985). This aspect was reported to affect 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 was more when a single lobe was in contact with the medium. However, anthers, when cultured flat (i.e., both the lobes in contact with the culture medium) did not show any response (Shannon et al, 1985; Powell, 1988). Similarly, in pearl millet, anthers when cultured flat did not show any response whereas anthers with single lobe in contact with the medium showed maximum induction response.

5.1.5. Effect of media components and phytohormones

Apart from the above-discussed physical factors, various organic and inorganic substances have been shown to affect the ability of microspores to produce calli or embryos and subsequently haploid plantlets. The constituents of the basal medium serve as important factors in eliciting successful androgenesis. Though the requirements varied from crop to crop (Sopory and Munshi, 1996), in general, source and amount of the total nitrogen, type and concentration of the sugars used, the kind of growth regulators, and exogenously added amino acids are some of the crucial factors considered for optimizing the media combinations. Monocotyledons show a high degree of phytohormone autotrophy. However, cereal anther cultures require the application of exogenous plant growth regulators

(Ferrie et al, 1995). Both YP and N6 are widely used with or without different growth regulators. MS medium is effective and popular with explants other than anther (for latest review, see Sopory and Munshi1996). To select the best basal media for pearl millet androgenesis, a single combination of growth regulators [2,4-D (3.0 μM) + KN (3.0 μM) +BAP (2.0 μM)] was used with YP, N6 and MS basal media. Out of these basal media used, YP media was found to be the best (29%) for androgenic induction followed by N6 (11%) and MS (5%).

Carbon supply is an obligatory requirement in culture medium for microspore embryogenesis (Nitsch and Nitsch, 1969). Sucrose at a concentration of 2-3% has generally been used as the major carbohydrate source in the culture medium. However, increase in sucrose concentration might lead to beneficial morphogenetic potential, which can be exploited in the case of gametophytic explants (microspores and ovaries), owing to their shift from gametophytic to sporophytic mode and consequent recalcitrance to morphogenesis. Higher levels of sucrose have also been used with improved response in maize microspore cultures (Pescitelli et al, 1990).

In addition to its role as a carbon source, sugar content in the culture medium may also play additional role as an osmoticum in nutrient medium. As a result of these dual roles played by the sugars, their response can vary with the crops. The type of sugar used in induction media plays important role in increasing the number of calli or embryos originating from microspores (Peter and Lyne 1994; Otani and Shimada 1993). However, the beneficial effect of maltose over sucrose in cereal anther cultures is not universally acknowledged and mostly confined to rice. In the case of bread wheat, maltose partially inhibited the androgenesis of

three responsive genotypes (Trottier et al, 1993). In contrast, maltose as a sole source of carbon was proved critical for the development of microspore-derived embryos in wheat by Mejza et al, 1993. Maltose served as a better osmoticum than sucrose in cereals (Ziauddin et al, 1990). In barley and wheat, maltose and occasionally glucose or cellobiose was found to be superior to sucrose in terms of embryo and plant production. Therefore, the effect of four sugars (maltose, sorbitol, mannitol and sucrose) in YP media was examined in pearl millet. Proembryoids formed in YP medium with 6% maltose, but they aborted later. There was no androgeneic response in medium supplemented with 6% sorbitol or 6% mannitol. Embryoids were most frequently observed in YP medium supplemented with 6% sucrose. Our present results are in agreement with previous findings of Buter et al (1995) in maize, which found that sucrose was the best source among eight sugars tested. Genovesi et al (1994) and Pescitelli et al (1994) also reported superiority of sucrose for anther culture of barley over other sugars.

Within the same crop, genotypes differ in their *in vivo* phytohormone composition. Proper balancing of growth regulators in the medium plays a critical role in pollen calli for embryo formation. The reports available so far on anther culture suggest that in the majority of cases, an auxin or a cytokinin is required as a component of the induction medium (Maheswhwari et al, 1980; 1982). According to the earlier reports, it was also found that a combination of 2,4-D and kinetin was effective for androgenic induction. However, some specific combinations worked better for callus induction in different crops, and for different genotypes within the same crop (Sopory and Munshi, 1996). In our

present study with pearl millet, it was observed that, when a high concentration of strong auxin (2,4-D) is used in combination with lower concentration of cytokinin, there was callus production, which in many cases did not translate into successful plant regeneration. On the other hand, when a high concentration of weak auxin (NAA or IAA) was used with low concentration of cytokinin, both pollen calli as well as embryos were produced. However, when a cytokinin like BAP or TDZ was used in combination with a weak auxin like NAA, only pollen embryogenesis (without any callus formation) was observed. It has been suggested that specific pathways (either callus based embryogenesis or direct embryogenesis) of microspore development in anther culture are regulated by the type and concentration of auxins in the induction medium. In barley (Liang et al, 1987), callus induction was observed when 2,4-D was used in the induction medium but when they replaced 2,4-D with NAA, haploid plants were produced directly. In the present study, TIBA (an anti-auxin) increased the androgenic response in all the genotypes studied. Ethylene production during initial stages of androgenic induction is known to interact and effect induction either positively or negatively (Tiainen, 1996). Therefore, in the present study, 6 genotypes (2 hybrids and their 4 parents) were compared for their androgenic response under treatments such as temperature shock or addition of the silver nitrate in the induction medium. Both of these treatments indirectly act on ethylene inhibition or alteration of ethylene atmosphere surrounding the developing tissue. But there was no significant increase in the androgenesis of otherwise recalcitrant genotypes as a result of these treatments.

Apart from sugars and plant growth regulators, another crucial factor for pearl millet anther culture was presence of activated charcoal in induction medium. It is widely known that accumulation of inhibitory substances during culture could repress the development of pollen plants. In view of this, Anagnostakis (1974) observed the beneficial effect of activated charcoal in tobacco anther cultures. This was later found to be true for other crops too including cereals (Maheswari et al, 1980). In the present study, when activated charcoal (0.5%) was added to the induction medium, it enhanced microspore embryogenesis from a mere 11% to 39%.

5.1.6. Effect of source of explant

Anther and microspore cultures were compared for embryo and calli production and subsequent plant regeneration. Plant regeneration was successful through anther culture, but the regenerants were all albinos. In microspore cultures, well-developed embryos were observed but their further development was arrested. It is believed that the anther wall provides a physical and chemical environment supporting pollen embryogenesis in many species (Heberle-Bors 1985). This may explain the difference in the response between anther and microspore culture. Androgeneic embryoids were also obtained from spikelets cultured on solid and liquid medium. In both solid and liquid media, anthers emerged from the spikelets and they dropped on the surface of the medium. Such released anthers were collected and subcultured onto the same medium. Subsequently, embryoids were observed in such anthers after 6-8 weeks of re-culture. No androgeneic response was observed when anthers were picked up

manually from spikelets and cultured directly (no preculturing of spikelets to release anthers), which suggests the role of some nourishing factors in spikelet in addition to those in anther wall for microspore embryogenesis.

5.1.7. Possible influence of *P* grains on androgenic ability

Production of androgenic embryos requires microspores to deviate from their normal gametogenesis. All microspores are not uniformly programmed for this switchover. While some do not respond at all, others might respond, but reach developmental dead-ends after few initial atypical divisions. Microspore-derived haploid plants were regenerated in a number graminaceous species (Dunwell, 1996). In addition to the normal green and haploid plants, cereal anther cultures often result in albinos, and heterozygous and abnormal regenerants. Variations in the pathway of microspore embryogenesis, and pattern of subsequent cell wall formations may be the reason for varied ploidies and abnormalities of the regenerants. Zhou and Yang (1981) showed that in barley both multi-nucleate and multi-cellular microspores are formed *via* different pathways of embryogenesis. This variation in androgenic induction could be explained by predisposing factors either like genetic and cytoplasmic effects or by pre-determined pollen grains '*p*' grains (Heberle-Bors, 1985).

Early indicators of androgenic potential may assist to select suitable genotypes for production of doubled haploids. Though hormonal level or amino acid profile (Dunwell, 1978) may distinguish low and high embryogenic lines, still simpler criteria exist. For example in wheat, frequency of '*p* grains served as an indicator for androgenic ability of a genotype. Occurrence of *p* grains is also

reported in barley in which genotypes producing large number of *p* grains are easy to culture (Sunderland and Dunwell, 1974). Therefore, frequency of *p* grains may be an easier and cost-effective tool to screen several genotypes for their androgenic ability. In pearl millet, it was shown that frequency of *p* grain could be used as an indicator for the inherent androgenic potential of each genotype. It was possible to select the genotypes with high frequency of androgenic ability by selecting genotypes that showed high *p* grain frequency and *vice versa*.

5.1.7. Pathways of microspore embryogenesis and their influence on androgenic ability

Though the atypical microspores serve as the precursors for microspore-derived embryos, their subsequent development towards embryogenesis varies among cereals. In the process of microspore embryogenesis, if nuclear divisions are uniformly followed by cell wall synthesis, multi-cellular microspores are produced. Such multi-cellular microspores play important role in increasing androgenic response because they are the precursors of microspore derived embryos. On the other hand, multi-nucleate microspores, in the absence of cell wall synthesis abort and thus do not have any role in increasing androgenic response. Formation of either type of these structures may depend on the specific pathway followed by dividing microspores. Therefore, any pathway that produces multi-cellular microspores is considered to be advantageous than the pathway leading to multi-nucleate microspores. The different pathways followed by the cultured pollen have been studied in species like *Hordeum vulgare* (Sunderland et al, 1979), *Triticum aestivum* (Henry et al, 1984) and *Sorghum*

bicolor (Rose et al, 1986). Two pathways for androgenic embryo development exist in rice (Yang and Zhou, 1979). There are at least eight reports on similar lines in maize (Buter, 1997).

Microspore-derived plants have been regenerated pearl millet (Bui dang Ha and Pernes, 1982; Nitsch 1982; Shyamala et al, 1997). However, in pearl millet, microspore embryogenesis *per se* is not yet studied in detail using histological and cytological approaches. My study established: (a) different pathways of microspore embryogenesis and their role in androgenic response, and (b) the occurrence of *p* grains and their possible contribution for androgenic ability of each genotype. My study indicated that A and B pathways are predominant in pearl millet microspore embryogenesis as in the case of rice. In all the genotypes studied, microspores following A pathway are rich in cytoplasmic contents, and larger in size. Cell wall synthesis is complete in these microspores (following A pathway) leading to multi-cellular microspores. On the other hand, microspore embryogenesis via B pathway is predominant in *p* grain derived microspores, which have faint cytoplasmic contents and are small in size. Cell wall synthesis in these microspores is partial or fragmented leading to multi-nucleate microspores.

Enhancement of *p* grain frequency after cold pretreatment is genotype dependent which suggests that cold shock has only maximized the expression of pre-existing *p* grains inherent to each genotype, rather than converting normal pollen to *p* pollen. Most of the nuclear divisions in *p* grains were not followed by cell wall synthesis, so resulted in multi-nucleate structures. A portion of

microspores showing B pathway, contained poorly stained, vacuolated cytoplasm indicating *p* grains as their possible precursors. This assumption can be further justified by the finding that, in genotypes with higher frequency of *p* grains, after 10 days in culture, it is the frequency of multinucleate pollen grains that increased in exact proportion with *p* grains but with no increase in multicellular microspores. So, further manipulations of *in vitro* culture media (such as addition of inositol which plays role in cell wall synthesis) might compensate for the incompetence of *p grains* for cell wall formation. This may increase the frequency of androgenesis in recalcitrant genotypes.

The importance of *p* pollen grains in pollen embryogenesis is still a controversial issue (Dale, 1975; Dunwell, 1985; Hebrle-Bors, 1985). Some authors suggest that they represent the main if not the whole source of prospective proembryos while others feel the yield of total proembryos is greater than the number of *p* pollen in the same material (Zhou, 1980). In *H. vulgare* the origin of *p* pollen is well studied histologically. It was shown that normal size pollen grains develop in contact with the tapetum, while the *p* pollen grains are in the centre of the locule and so are not in contact with the tapetum. Rose et al (1986) reconfirmed this for *Sorghum bicolor*. Sections of the anthers both *in vivo* and in *vitro* suggested that this was also the case in pearl millet.

5.2. Gynogenesis

Uchimiya et al (1971) first reported induction of haploids from megaspores by culturing unpollinated ovaries of *Zea mays* and *Solanum melongena*. Later,

different researchers reported haploid production through megaspores (Keller and Korzun, 1996). The success of gynogenesis through ovary culture depends on the developmental stage of the ovary, culture conditions, combinations of growth regulators, and seasonal effects. Present study clearly established that developmental stage of embryosac, cold pretreatment of spikelets, culture media constituents and technique of the ovary culture influenced the frequency of gynogenic response.

5.2.1. Optimum developmental stage of embryosac for gynogenic induction

Ideal method for optimization of embryosac developmental stage for gynogenic induction involves direct study of embryosac in cultured ovules. However, such a study is not possible particularly in cereals in which only one ovule is present in each spikelet. Direct observation of the stages in ovules is also cumbersome as it involves thin sectioning and staining, or clearing and squashing (Herr 1971), or embryosac isolation (Enaleeva and Dushaevaa 1975; Huang and Russel 1989). In the present study, microspore developmental stages were used as a guiding factor to determine the corresponding embryosac developmental stage to optimize the induction frequency as has been done in rice by (Zhou et al, 1986). My results showed that optimal developmental stage of the explant for *in vitro* culture is the spikelet containing late uninucleate microspores. For induction of gynogenesis in *Hordeum*, spikelets with trinucleate microspore have been shown to be optimal (Castillo and Cistue 1993; 2000). However, the reports on rice are contradictory: Zhou et al (1986) reported that late uninucleate microspore stage was optimal, whereas Rongbai et al 1998; Raina 1997; Raina

and Zapata (1997) suggested spikelets with trinucleate microspores were the most suitable explants for gynogenesis.

5.2.2. Ideal explant for gynogenic induction

Comparison of different explants showed that only spikelets are most suited for induction of gynogenesis. Culture of isolated ovaries or sequential cultures of ovaries after removal of other parts of the spikelets were not successful. This observation may suggest a high degree of dependence of ovaries (as is the case in anther culture) on the surrounding tissues during gynogenic induction and differentiation. In rice also, culture of isolated pistils (detached from receptacle) failed to show any gynogenic response to (Zhou and Yang 1981). In barley, whole spikelet inserted vertically on solid medium proved to be a better explant than isolated pistils (Huang et al, 1982). In several members of *Poaceae*, higher induction and regeneration frequencies have been obtained by culturing ovaries with the remaining peripheral tissues such as bracts, whole florets or additional parts of the inflorescence axis (Mathias and Boyd, 1988; Laurie and Bennet, 1988; Higgins and Petolino, 1988). The simulative effect of other floral parts has been attributed to the supply of nutritive and other active substances for gynogenesis. A better understanding of the requirements of embryo development *in vivo* may help in formulating *in vitro* conditions to achieve plant regeneration from isolated ovule cultures, as in the case of barley and wheat .

5.2.3. Influence of cold pretreatment on gynogenic induction

Cold pretreatment of the ovaries increased gynogenic response in rice (Zhou and Yang 1981), while it had no effect on gynogenic induction in barley (Castillo and Cistue, 1993). However, in pearl millet cold pretreatment was favorable for gynogenic induction. It increased the frequency of enlarged ovaries, which gave rise to gynogenic plantlets and reduced non-embryogenic callus formation.

5.2.4. Comparison of solid and liquid media for gynogenic induction

Zhou et al (1986) and Gusakouskayan and Nadzhen (1994) used liquid medium for gynogenic induction, and subsequently dissected and transferred the embryogenic structures to solid medium for differentiation. My effort to repeat the same procedure for pearl millet was not successful as somatic tissue was stimulated in liquid culture to produce callus. In liquid culture, larger part of the spikelet comes in contact with the medium. Therefore, the availability of nutrients is more than in solid medium. As a result, glumes and other inner somatic tissues gain access to nutrients faster than the female gametophyte. Only the pedicel is in contact with the solid medium and the nutrients reach the vasculature of the embryosacs through pedicel (Huang et al, 1982). This may result in differential accessibility of nutrients to peripheral tissues in competition with embryosac. Above results suggest that the supply route and accessibility of nutrition between the peripheral and gametic tissues may vary depending on the physical state of the medium.

5.2.5. Role of activated charcoal in enhancement of gynogenic induction

Many researchers reported beneficial effect of the activated charcoal for cereal androgenesis. However, there was no report about its effect on gynogenesis. In the present study, it was observed that activated charcoal did not influence or enhance gynogenic response. Direct plantlet regeneration was achieved at the same frequency on the medium containing activated charcoal as that of the medium without charcoal.

5.2.6. Role of phytohormones in gynogenic induction

Regulation of growth hormones to enhance gynogenesis while simultaneously inhibiting the proliferation of somatic tissues has been critical for ovary culture (Cagnet-Sitbon 1980; Huang et al, 1982; Andre and Demarly 1984). Auxin has been reported to stimulate embryo development in members of *Poaceae* both *in vivo* (Matzk 1988; 1991) and *in vitro* (Holm et al, 1999). However, in the present study, 2,4-D when used alone produced loose, non-regenerable callus. In many species, exogenous cytokinins improved the frequency of somatic embryogenesis (Norstog 1970; Dale 1980). Our results indicate that, BAP was more effective for gynogenic induction than Kn in pearl millet as in barley (Castillo and Cistue 1993). Use of TIBA, along with an auxin and a cytokinin greatly improved gynogenic response. TIBA may reduce the extent of non-embryogenic callus formation and promote gynogenic pathway. The hormone combination in the medium, YPGI 7 may probably have complementary effect with *in vivo* concentrations of growth regulators in pearl millet ovaries. Auxins like MCPA and

picloram (Yang and Zhou, 1982) were found to be effective for rice ovary culture. However, present study suggests the favorable effect of an anti-auxin (TIBA) for direct regeneration of plantlets from *in vitro* cultured, swollen ovaries of pearl millet. Thus for pearl millet, the optimal protocol for gynogenic induction involves the culture of cold-pretreated spikelets (showing microspores at late uninucleate stage) on solid Yu Pei media containing 2 mg L^{-1} 2,4-D, 1 mg L^{-1} BAP and 0.1 mg L^{-1} TIBA. This protocol maximized the frequency of the enlarged ovaries and the subsequent regeneration of green, doubled haploid plants that can be grown to mature plants capable of setting seeds. Having successfully established a protocol for production of gynogenic doubled haploids; present study also confirmed the ploidy of the regenerants. Further, the level of uniformity in DH progenies was studied using morphological and molecular markers (RFLPs and RAPDs).

5.2.7. Origin and nature of gynogenic embryos

Any haploid cell present in the female gametophyte (embryosac) may show the totipotency and give rise to a haploid embryo and subsequently to a plantlet. Depending on the media and the hormone combinations, even somatic tissues of the ovaries can sometimes give rise to plants. Study of progressive developmental stages of gynogenic embryos is important to know the exact origin of gynogenic embryos. In dicotyledonous crops such as sugarbeet (van Geyt et al, 1987; Ferrant and Bouharmont 1994), and sunflower (Yang et al, 1986), gynogenic regenerants were confirmed through anatomical and cytological studies. In cereals, these approaches have been used to confirm the

origin of gynogenic embryos (San and Gelebart, 1986) and to preclude possible somatic origin of the regenerants. In *Oryza sativa* (Zhou et al, 1986), in the initial stages (below 3 weeks) gynogenic proembryos were similar in size and shape to zygotic proembryos *in vivo*. However, only 2% of the gynogenic proembryos developed further and produced normal plants. Rest of the gynogenic proembryos developed into anomalous embryos, some with highly vacuolated cells, others contained abnormally long suspensors. Here, many proembryos degenerated in culture by vacuolation of peripheral cells followed by their disintegration. In pearl millet, present study confirmed (through anatomical studies) different developmental stages of gynogenic embryos in 8% of the induced ovaries. In addition to the embryo formation, histological sections of the cultured ovaries also showed profound callusing and embryo development in the outer layer of ovary walls.

5.2.8. Detection of haploidy and study of homogeneity in doubled haploid populations

In pearl millet, all earlier reports of cyto-morphological analysis of haploids were restricted to naturally occurring haploids. Gill et al, (1973) observed pearl millet haploids among the progeny of trisomics and analyzed their meiotic behavior. Powell et al, (1975) discussed the origin, cytology and reproductive characteristics of spontaneously originated haploids of 'Tift 23A' isolated from field plantings. Present results show that majority of the gynogenic regenerants had originated from reduced cells of female gametophyte and that the progressive doubling of chromosomes occurred during development. The

general observations of the DH lines under field and greenhouse conditions revealed a very high homogeneity among the doubled haploids generated *in vitro*. Absence of segregation in five of the progenies for morphological markers suggests that all these plants are homozygous and identical. Out of the two heterozygous populations (in one case derived from Plant A, which showed segregation for the agro-morphological characters), the regenerant might have been the product of unreduced female gametophytic cell. However, the hypothesis of a total non-reduction is to be excluded in the progeny of other plant (Plant C), as the variation observed in this progeny is abnormal and might suggest possible *in vitro* selection pressure on developing gynogenic embryo. In the present study, the cytological observations of haploid plants are comparable to the results of *in vivo* haploids (Powell et al, 1975). Occasional pairing in the haploid chromosomes of pearl millet may be due to duplicated loci or a marked tendency for non-homologous pairing. This tendency may indicate segmental partial homology. Different kinds of abnormal alignments between chromosomes may indicate intra-haploid affinity.

Biochemical and molecular techniques at the DNA level can be used as powerful tools to detect the homogeneity of DH populations. Molecular analysis of the regenerants and their progenies are helpful not only to confirm the gametophytic origin of gynogenic regenerants but also their homozygosity. Doubled haploid lines of maize were evaluated by using molecular and morphological markers (Murigneux et al, 1993). Similarly, wheat-triticale doubled haploids were characterized by cytological and biochemical markers. While

analyzing intergeneric or interspecific progeny, chromosome-painting techniques such as *FISH* are fast becoming popular. Compared to the pedigree estimates, DNA markers provide better description of genetic relatedness among the clones.

The homogeneity observed in RFLP and RAPD observations may suggest that, at the time of reduction division in megagametophyte, the precursor cell of gynogenic embryo inherited genetic material from either P_1 or P_2. The resultant haploid plant was subsequently diploidized to get the homozygous regenerant (for the single parent it inherited). For example, two parents were clearly polymorphic with a given RFLP probe and the hybrid contained both of these bands. Both types of DNA markers (RFLP and RAPD) proved the absence of segregation among progeny lines as no single polymorphic band was observed in the progeny lines. The absence of segregation among the progenies of gynogenic lines confirm earlier reports in other cereals with little or no segregation distortion in the DH lines derived from gynogenic plants in comparison to the unusual distortion in anther culture derived DH lines. In barley, segregation distortion is compared between androgenic DH lines and DH lines derived from *bulbosum* technique. In all these studies, microspore derived lines exhibited significant departure from the expected 1:1 ratio whereas the *bulbosum* technique derived DH lines showed very less distortion for the number of markers studied (Kjaer et al, 1991). Present results with pearl millet gynogenic DH lines are comparable to that of *bubosum* derived DH (essentially gynogenic) lines of barley with least segregation distortion.

Very low frequency of the heterogeneity observed in the DH progeny might suggest the possible role of the direct regeneration system without any callus phase. RFLP and RAPD analysis of *in vitro* derived DH lines permitted unambiguous identification of their origin. Results from the present study throw light on the process of *in vitro* gynogenesis in pearl millet and helps to better understand gynogenic DH production in pearl millet.

6. Conclusions

Production of doubled haploids of pearl millet (*Pennisetum glaucum* (L.) R. Br.) was studied using anther and microspore cultures, and by spikelet and ovary cultures (for gynogenesis). Effects of *in vitro* culture conditions, chemical factors and genotype on androgenic induction were established in two pearl millet hybrids H_1 (ICMP 451 x 843 B) and H_2 (ICMH85410 x 81B). Androgenic response was better in liquid medium than solid medium. Subjecting donor plant to additional light intensity was not effective. The light quality used for incubation of anthers affected androgenic response. A cold pretreatment of 14 °C for 6-8 days increased the frequency of androgenic induction. Sucrose starvation pretreatment also was effective in increasing androgenic response. Out of eight different regeneration media evaluated for their ability to induce regeneration from androgenic embryos and calli, a combination of 1 mg L^{-1} NAA, 2 mg L^{-1} BAP and 0.1 mg L^{-1} ABA with 3% of sucrose was found to be the best. TDZ could arrest starch formation in the developing microspores *in vitro* and increased the frequency of microspores deviating from gametophytic to sporophytic pathway. This beneficial effect of TDZ was further improved when used in combination with cold pretreatment of panicle prior to culture.

Based on a study of 17 genotypes of pearl millet (13 inbred lines, 2 hybrids and their 2 reciprocals), it was revealed that androgenic induction rate significantly depended on the genotype. Androgenic response was not uniform across the genotypes, even when a common induction medium was used. Highest induction was obtained from an inbred line 7042 DMR, and the lowest

from IP16976. However, all the regenerants from anther and microspore cultures were albinos. A total of 91 haploid albinos were regenerated from H_1 and 45 albinos from H_2. When the above regeneration medium was used with the genotype 7042 DMR, differentiating embryos showed green patches. Subsequently they produced profound rooting but failed to produce shoots. Further manipulation of media specific for each genotype may help to regenerate whole plantlets.

Microscopy studies on microspore embryogenesis offer clues for manipulating culture conditions that might maximize number of responding microspores, thus increasing the final yield of haploids. In the present study, I compared microspore embryogenesis in *in vitro* cultured anthers with the pollen development occurring *in vivo* in freshly harvested anthers using both light and fluorescence microscopy. Three pathways of *in vitro* microspore embryogenesis were observed. These three types included vegetative cell derived (A), generative cell derived (C), and undifferentiated cell derived (B) microspore embryos. However, A and B types are predominant during microspore embryogenesis. Pollen dimorphism (*s* and *p* type pollen) was observed both *in vivo* and *in vitro,* and their frequency varied with the genotypes. After cold pretreatment of excised anthers, the frequency of *p*-grains increased in all the seven genotypes studied. Pollen from the genotype 843 B showed highest *p*-grain frequency after cold pretreatment and showed maximum microspore divisions under culture, suggesting a possible correlation between *p*-grain frequency and androgenic ability.

In addition to the anther cultures, isolated microspore cultures were established and factors affecting microspore embryogenesis were studied. The induction rate of microspore embryos was highest (12.3%) in microspores derived from precultured anthers than those from fresh anthers (1.7%). Filter-sterilized medium was five times more effective than autoclaved medium for microspore embryogenesis. Four *Yu-Pei* basal media with various additives were tested for embryo induction, and the one with 2,4-D (3 mg L^{-1}), TDZ (0.1 mg L^{-1}) and Kinetin (0.5 mg L^{-1}), L-proline (200 mg L^{-1}), glutamine (100 mg L^{-1}) and casein hydrolysate (500 mg L^{-1}) was found to be the best. Two weeks after their culture in above YP induction medium, microspores showed multicellular structures. These were sub-cultured onto regeneration medium with 2,4-D (0.5 mg L^{-1}), IAA (1.0 mg L^{-1}) and BAP (1 mg L^{-1}). After three weeks, a significantly high (45%) frequency of multicellular microspores turned to proembryos, and by the fifth week, they differentiated further into embryos showing initial polarity. By sixth week, embryos turned brown and ceased to grow further. Anther culture with the same genotypes in our laboratory produced only albino plantlets. I concluded that with appropriate genotype and the above-described microspore culture system, successful plant regeneration might be possible.

In model dicotyledonous species like tobacco and *Brassica*, isolated microspore embryos enabled the investigations into several molecular aspects like synthesis and regulation of mRNA and protein levels, *protein kinase* and phosphorylation activities, and heat-shock proteins during the induction process (Dunwell, 1996). The above microspore culture system described here also offers a possibility to study the bio-chemical and molecular events during pollen

embryogenesis. Such knowledge obtained would be very useful for optimizing further protocols for high frequency of doubled haploid regeneration. Once routine regeneration is achieved using these microspore-derived embryos, it may accelerate the process of successful genetic transformation and subsequent production of homozygous transgenics.

As mentioned earlier, though the anther and microspore cultures are the popular techniques for *in vitro* doubled haploid production in cereals, the high frequency of albinos among regenerants restricts its further application for crop improvement. Therefore, I standardized a protocol for *in vitro* gynogenesis in pearl millet, which generates green plants. Spikelets at late uninucleate microspore stage cultured on solid medium showed maximum frequency of androgenic embryos. Cold treatment of panicles at 14 ^{0}C for 6-8 days preceding spikelet culture improved gynogenic response. Of the different hormones tested, maximum number of gynogenic plantlets was obtained on modified Yu Pei medium containing 2,4-D (2 mg L^{-1}), BAP (1 mg L^{-1}) and TIBA (0.1 mg L^{-1}). Anatomical studies confirmed gynogenic origin of the plantlets. The regenerants were later established in soil. Study of various factors influencing gynogenesis helped us to improve our protocol for *in vitro* gynogenic induction and doubled haploid production in pearl millet.

Present study in pearl millet showed that spikelet containing late uninucleate microspores is the best explant for *in vitro* gynogenic induction when compared to the spikelets containing microspores either at late binucleate stage or late tetrad stage. Among the cytokinins, BAP is effective for induction of gynogenesis than Kn. For induction of embryogenesis, solid medium was better

than liquid as solid medium avoided stimulation of somatic tissues and favored direct plantlet regeneration when appropriate hormone composition (as in the medium YPGI-7) was used. Culture of isolated ovaries or sequential culture of ovaries after dissection were not successful, and may suggest the high dependence of responding gynoecium (as is the case in most anther culture experiments) on surrounding tissues during initial and late developmental phase. In addition to the ease in handling, present approach of culture of whole spikelets offers possibility of studying both androgeneic and gynogenic haploid induction at the same time. This was possible as spikelet cultures served as source material for harvesting responding anthers and microspores. Alongside, some of the spikelets when left undisturbed showed *in vitro* gynogenic induction after 3-6 weeks in culture Further efforts are needed to develop this system to regenerate a higher frequency of green haploid plants quickly so that the protocol can be used in applied plant breeding.

Confirmation of doubled haploids (DH) using cytological and molecular analysis is essential to ensure their value in breeding and for genetic studies. Gynogenic regenerants of H_1 were studied for their cytological status and their progeny was characterized using agro-morphological studies and molecular markers (RFLP and RAPD). Mitotic studies with root tips of 27 gynogenic regenerants, showed that 6 regenerants were haploid (n=7), 1 regenerant was aneuploid, and the rest 20 contained diploid chromosome number. Meiotic studies of pollen mother cells from gynogenic regenerants showed normal meiosis in diploids, but haploids showed abnormalities including non-homologous chromosome pairing. During metaphase, different kinds of abnormal alignments

involving haploid chromosomes were observed. Homozygosity of the gynogenic doubled haploid (DH) lines was evident by the absence of segregation for two morphological markers in the progeny. RFLP and RAPD studies showed that, a majority of the diploid or doubled haploid regenerants were homozygous haploids. No segregation in the progeny was detected with any of the RFLP probes or RAPD primers that could reveal polymorphism between parents.

Present study towards development of *in vitro* doubled haploid production in pearl millet could clearly establish the following protocols and basic information.

1. Optimized anther and microspore culture techniques could successfully induce androgenic embryos *in vitro,* and regenerate such embryos to finally produce haploid plants (albinos). However, genotype effect was prominent in deciding whether the regenerants were albinos or green plants. Therefore, further selection of favorable genotypes might increase chances of green plant regeneration.
2. By studying the mechanism of microspore embryogenesis, it was shown that frequency of *p* grain could be used as an indicator for the inherent androgenic potential of each genotype. It was possible to select the genotypes with high frequency of androgenic ability by selecting genotypes that showed high *p* grain frequency.
3. It was possible to establish a protocol for isolated microspore cultures that could induce sufficiently large number of androgenic embryos and the proper choice of genotypes might lead to green, haploid plant.

4. Induction responses in isolated ovary cultures or isolated microspore cultures are not as high as in spikelet cultures suggesting lack of nourishing factors, which are present in anther and spikelet cultures. Therefore, spikelet cultures are more suited for inducing *in vitro* haploids and can serve as a common source of explants for either androgenesis (anthers and microspores) or gynogenesis (ovaries).

Thus, my study has laid the foundation for further research on doubled haploids of pearl millet. Besides *in vitro* techniques described above, approaches based on intergeneric hybrids as profitably exploited in barley and wheat should be explored. The choice of genotypes and cultural conditions are to be carefully considered. For the immediate practical applications, spikelet culture as described in this thesis holds the promise.

7. References

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9. Appendices

Appendix I. List of some useful web sites related to tissue culture and haploid research

http://www.hos.ufl.edu/mooreweb/TissueCulture/tcclass.html
http://res2.agr.ca/research-recherche/agtran/agt-18e.html#intro
http://res2.agr.ca/research-recherche/agtran/agt-18e.html#benefits
http://res2.agr.ca/research-recherche/agtran/agt-18e.html#tech
http://res2.agr.ca/research-recherche/agtran/agt-18e.html#compet
http://www.agron.missouri.edu
http://www.nucleus.cshl.org/worldpac/eng/r000001/r000592.htm
http://www.rrz.uni-hamburg.de/biologie/b_online/e12/12a.htm
http://www.hos.ufl.edu/mooreweb/TissueCulture/anther.html
http://www.cclabs.missouri.edu/~c705340/news/news13.htm
http://www.nal.usda.gov/bic/Biotech_Patents/1994patents/05322789.html
http://www.pbi.nrc.ca/bulletin/jan99/intro.html
http://www.wheat.pw.usda.gov/ggpages/bgn/24/v24p60.html
http://www.genome.cornell.edu/rice/documents/newsletters/rgn6/v6p52.html
http://www.abs.sdstate.edu/plantsci/teaching/ps383/breeding/happroc.htm
http://www.cimmyt.mx/Research/ABC/Apomixis/ApomixisNews10-6.htm
http://www.css.orst.edu/classes/css530/Unit13.htm
http://www.biosci.ohio-state.edu/~plantbio/Faculty/scholl.html
http://www.agro.agri.umn.edu/courses/agro5021/lecture_19.htm
http://www.sbreb.org/86/production/86p185.htm
http://www.agctr.lsu.edu/wwwac/research/rice/98report.html
http://gnome.agrenv.mcgill.ca/breeding/happroc.htm

Appendix II. List of genotypes used in the present study

S. No.	Name of the genotype	
1	7042DMR	
2	ICMP451	(Parent 1 of H_1)
3	843B	(Parent 2 of H_1)
4	ICMP451 x 843B	(H_1)
5	ICMH85410	(parent 1 of H_2)
6	81B	(Parent 2 of H_2)
7	ICMH85410 x 81B	(H_2)
8	23 D_2B	
9	7042S	
10	81B x ICMH85410	
11	843B x ICMP451	
12	843A	
13	IP18986	
14	Sujatha line	
15	ECIL tall	
16	ECIL dwarf	
17	IP16976	

Appendix III. Composition of Yu-Pei basal medium

Components	Weight in mg L^{-1}
NH_4NO_3	165.00
KNO_3	2500.00
$MgSO_2 .7H_2O$	370.00
$MnSO_4 .4H_2O$	4.40
$ZnSO_4 7H_2O$	1.50
$CuSO_4 5H_2O$	0.00
KI	0.80
$CoCl_2 6H_2O$	0.00
KH_2PO4	510.00
H_3BO_3	1.60
$NaMoO_4 .2H_2O$	0.00
$FeSO_4 7H_2O$	27.80
Na_2 EDTA	37.50
Nicotinic acid	0.50
Pyridoxine- HCl	0.50
Thiamine –HCl	1.00
Glycine	2.00
Casein hydrolysate	500.00
TIBA	0.10
Sucrose	30000.00
Activated charcoal	2500.00
Agar	8000.00

pH *5.8 (with 1N NaOH).*

Appendix IV. Preparation of samples for LM, FM and EM studies and staining recipes

Recipe for Aceto-carmine staining

Anthers for cytological observation were squashed in 0.1% acetocarmine (Belling et al, 1974) and examined microscopically. Observations were made on fresh anthers, anthers collected from cold re-treated panicles, and anthers collected daily during the first 10 days period under *in vitro* culture.

Recipe for Histological staining

Anthers were fixed in ice-cold gluteraldehyde with 2% DMSO (buffered to pH 7 by 0.1% phosphate buffer) aspirated for up to 5 days, rinsed twice in buffer (12 h each), dehydrated in a 30-100% alcohol series, and embedded in *spur* resin. Transverse sections (5 to 8μ) were obtained with the help of an ultra-microtome. Sections were stained with a combination of Toulidine blue and Azure-II.

Recipe for FDA staining

Microspores were stained with Fluorescein diacetate (*Sigma* F7378) prepared according to Shivanna and Heslop-Harrison (1981). Pollen was sprinkled on a microscope slide containing a drop of above stain, a cover slip was placed and the slide was incubated at room temperature in a humid chamber for 8-10 min., before observing the slide under an *Olympus AX70* microscope using an excitation filter IF 490.

DAPI staining

The procedure of Jahier et al (1996) was followed for DAPI staining. Anthers were gently teased on a microscope slide in a drop of DAPI (*Sigma* D 1388) stain to liberate pollen grains. After removing anther debris, a cover slip was placed on pollen and incubated for 10-20 min, in a humid chamber at room temperature. Stained pollen was observed under an *Olympus AX70* microscope using UV light at a principal wavelength of 365 nm.

Appendix V. Preparation of buffers and other chemicals for RFLP and RAPD studies

0.5 M EDTA pH 8.0 (500 ml)

93.05 g EDTA + 300 ml sterile dH2O in 1000 ml beaker + little sodium hydroxide ⇒ stir while heating ⇒ add little amount of sodium hydroxide in between - it takes time to dissolve ⇒ continue till it gets dissolved and turns transparent ⇒ adjust the pH to 8.0 with sodium hydroxide ⇒ make up the volume to 500 ml with SDW.

RNase (2 mg/ml)
8 mg Rnase + 4 ml SDW ⇒ shake gently and divide into 4 aliquots of 1 ml each to get a conc. of 2 mg/ml ⇒ store at –20 oC

***Proteinase K* (2 mg/ml)**
20 mg *proteinase* K + 10 ml SDW ⇒ shake gently and divide into 10 aliquots of 1 ml each to get a conc. of 2 mg/ml ⇒ store at –20 oC

Saline A Extraction buffer pH 8.0

Constituent	l^{-1}	Final concentration.
NaCl	58.4	1M
0.5M Tris-Cl	100 ml	0.05M
0.5M EDTA (pH 8.0)	10 ml	0.005M
BSA (to be added fresh)	1 g	0.1mg/ml

Store in cool temperature (4°C)

2X Extraction buffer pH 8.0

Constituent	500 ml^{-1}	Final concentration.
0.5M Tris-Cl (pH 8.0)	150 ml	0.15M
0.5M EDTA (pH 8.0)	8 ml	0.008M
NaCl	2.92g	0.1M
SDS (kept at 65°C)	7.5g	1.5%

Store at room temperature

Saline wash buffer

Constituent	l^{-1}	Final concentration
NaCl	58.44g	1 M
0.5M Tris-Cl (pH 8.0)	100 ml	50 mM
0.5M EDTA (pH 8.0)	40 ml	20 mM

Store in cool temperature (4°C)

Shelf buffer

Constituent	500 ml^{-1}	Final concentration
Sucrose	102.69g	0.6M
0.5M Tris-Cl pH 7.5	10 ml	10 mM
0.5M EDTA (pH 8.0)	20 ml	20 mM

Store in cool temperature (4°C)

70% Ethanol

Constituent	l^{-1}	Final concentration
Absolute ethanol	700 ml	70%
SDW	300 ml	-

0.5M Tris-Cl pH 8.0

Constituent	gl^{-1}
Trizma base	60.507
Adjust pH with 6N HCl to 8.0	

0.5M Tris-Cl pH 7.5

Constituent	gl^{-1}
Trizma base	60.507
Adjust pH with 6N HCl to 7.5	

5M Potassium acetate

Constituent	100 ml^{-1}
Potassium acetate	49.07 g
pH not adjusted	

$T_{50}E_{10}$ Buffer

Constituent	100 ml^{-1}
0.5M Tris-Cl pH 8.0	10 ml
0.5M EDTA pH 8.0	2.0 ml

$T_{10}E_1$ Buffer

Constituent	l^{-1}
0.5M Tris-Cl pH 8.0	20 ml
0.5M EDTA pH 8.0	2.0 ml

Denaturing solution

Constituent	l^{-1}
1.5M NaCl	87.66g
0.5M NaOH	20.00g

Neutralizing buffer

Constituent	l^{-1}
1.5M NaCl	87.66g
1M Tris	121.1g
Adjust pH to 8.0	

Chloroform (24:1)

Constituent	100 ml^{-1}
Isoamyl alcohol	4.0 ml
Chloroform	96.0 ml

0.25N HCl

Constituent	l^{-1}
Conc. HCl (11.6N)	21.6 ml
SDW	78.4 ml

7.5M Ammonium acetate

Constituent	l^{-1}
Ammonium acetate	57.75g

10x TBE Buffer

Constituent	
Trizma base	108.0 g
Boric acid	55.0 g
0.5M EDTA	40 ml
(Adjust pH to 8.0 with 6N HCl)	

20x SSC

Constituent	$2\ l^{-1}$
3M NaCl	350.64g
0.3M Sodium acetate	176.46g

Prehybridization solution / 7% SDS Phosphate solution

Constituent	$500\ ml^{-1}$
Disodium hydrogen phosphate (Na_2HPO_4)	35.5g
BSA	5g
SDS Adjust pH with Phosphoric acid.	35g

20X SSPE (1LITRE)		
NaCl	3.6 M	210 g
Na_2HPO_47H2O	0.2 M	53.6 g
EDTA	0.02M	7.44 g

100X DENHART'S REAGENT (1LITRE)	
Ficoll 400	20 g
Polyvinylpyrolidone (PVP)	20 g
BSA fraction V	20 g

Constituent	l^{-1}
20x SSC	150 ml
10% SDS	10 ml

10x TBE

Constituent	l^{-1}
Tris base	108g
Boric acid	55g
0.5M EDTA (pH 8.0)	40 ml
pH automatically comes to 8 if weighed exactly. Otherwise, adjust the pH with 6N HCl.	

Probe stripping solution I

Constituent	$250\ ml^{-1}$

NaOH	4.0 g
10% SDS	10 ml

Probe stripping solution II

Constituent	250 ml^{-1}
20x SSC	2.5 ml
10% SDS	2.5 ml
Tris-HCl pH 7.5	100 ml

Developer

Constituent	l^{-1}
SDW	1000 ml
D-19 add slowly	157 g.

Stop Bath (3% of HAC)

Constituent	l^{-1}
HAC (Acetic acid)	30 ml

Rapid fixer

Constituent	l^{-1}
Solution A	250 ml
SDW	750 ml
Solution B (Add slowly at room temperature)	28 ml

Loading buffer

Constituent	10 ml^{-1}
Sucrose	4g
Bromophenol blue	25 mg
0.5M EDTA pH 8	400 µl

Appendix VI - DNA extraction and Southern blotting protocol.

Extraction of genomic DNA

1) Fresh young leaves (5.5 - 6.0 g) were harvested, lyophilized in liquid nitrogen and stored at -70^{0}C.
2) The leaves were pulverized to fine powder in a mortar and pestle, and 10 ml of freshly prepared CTAB buffer (1.0 M Tris, pH 8.0, 5.0 M EDTA, 2% β-mercaptoethanol, 2% CTAB) at 65^{0}C was added to freeze-dried, ground tissue in a 30 ml Falcon tube, mixed well on a rotating shaker and incubated for 2 h. at 65^0 C with occasional mixing.
3) The tubes were taken out from water bath, cooled to room temperature, and 10 ml chloroform-isoamyl alcohol (24:1) was added and mixed gently by inverting for 5-6 times and centrifuged using swing bucket rotors at 6000 rpm for 20 min at room temperature. The aqueous phase was transferred to a new 30 ml tube to which 10 ml chloroform-isoamyl alcohol was added and mixed gently 5-6 times.
4) The extract was centrifuged at 6000 rpm for 20 min at 2^{0}C and the aqueous phase was transferred to 30 ml corex tube. After chilling, 10 ml of isopropanol was added to the aqueous extract, and mixed gently by inversion for several times, and kept at -20^{0}C for 15-20 minutes. DNA was spooled with glass hook.
5) The spooled DNA was washed in a 5 ml corex tube containing 2 ml of 76% ethanol, 0.2 M NaOAc (Washing Buffer I) followed by a 100% ethanol wash. The tubes were inverted on a paper-towel and allowed for all ethanol to evaporate.
6) The DNA was treated with 2 ml of $T_{50}E_{10}$ containing RNase (0.2 μg/ml) and incubated at 37^{0}C for one h.

Purification of genomic DNA

1) After the *RNase* treatment, 200 μl of 5M NaCl was added, shaked gently and incubated at 4^{0}C for 15-20 minutes.
2) The tubes were next centrifuged at 6000 rpm at 2^{0}C for 20 minutes. The aqueous phase was transferred to a 5 ml corex tubes.
3) Next, 2 ml of phenol:chloroform (1:1) was added and centrifuged at 2500 rpm for 10 minutes at 2^{0}C.
4) Equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phase and centrifuged at 2500 rpm for 10 minutes at 2^{0}C.
5) For precipitation of DNA, 200 μl of 2.5 M sodium acetate pH 5.2 was added to the aqueous phase, mixed well and 2 ml of absolute alcohol was added and again mixed well, incubated at -20^{0}C for 15-20 minutes.

6) The precipitated DNA was spooled with a glass hook into a 1.5 ml eppendorf tube and washed with 76% and then 100% alcohol. $T_{10}E_1$buffer (10mMTris.Cl, 1mM EDTA pH 8.0; 300 µl) was added to dissolve the pellet and stored at 4^0C until further use.

Quantification of DNA

1) The quantity and purity of the DNA samples were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm with a *SHIMADZU* UV 160A spectrophotometer.
2) The ratio of OD_{260} to OD_{280} provides information about the purity of the DNA samples. DNA was quantified considering that 1.0 OD unit at 260 nm is equivalent to 50 µg of DNA per ml (Sambrook et al, 1989).

3) An aliquot of genomic DNA was run on 0.8% ethidium bromide stained agarose gel. The dye intercalates into the DNA double helix, and the intensity of florescence induced by UV light is proportional to the amount of DNA in the lane. Comparison with a standard λ Hind III digest of λ marker DNA, gives an estimate of the amount of DNA in the samples.

Restriction digestion of genomic DNA

Genomic DNA was digested with four restriction enzymes, *Eco R1*, *Eco RV*, *Dra I* and *Hind III*. 10-15 µg of genomic DNA was used for restriction digestion.

Reagents for 10 reactions

Restriction buffer	50 µl
Restriction enzyme (20 units/µl)	30 µl
SDW	20 µl
Total reaction volume	100 µl

1) Master-mix was made and 10 µl was dispensed into 9 reaction tubes 10µl each, and template DNA was added such that the final concentration of DNA was 10-15 µg.
2) The contents in the reaction tubes were briefly centrifuged and incubated at 37^0C for overnight for complete digestion of pearl millet genomic DNA.
3) The samples were run on the 0.8% agarose gel at 40 V.
4) The gel was stained with ethidium bromide and then de-stained in distilled water for about 30 min.
5) The gel was then transferred on to the vacuum blot apparatus to transfer the DNA fragments to the nylon membrane.

Southern Blotting

1) The nylon membrane was cut according to the gel size (Amersham **Hybond N$^+$**) and it was marked.
2) The gel was carefully transferred on to the membrane and the depurination was done using 0.25 M HCl, denaturation using 0.4 N NaOH and neutralization reactions were carried out for 20 min each.
3) The transfer was done in 20x SSC solution for one h.
4) After the transfer, the DNA was cross-linked to the nylon membrane, followed by baking at 80^{0}C for one h.
5) The blot was wrapped in *Saran wrap*, preserved at 4^{0}C.
6) The preserved blots were ready for the hybridization.

Appendix VII - Protocol for preparation of RFLP probe, labeling and hybridization

Labeling of probe

Pearl millet genomic DNA was labeled with (α-^{32} P) dCTP in the following way:

DNA	5 µl
Buffer	5 µl
dNTP without dCTP	6 µl
dCTP (α-^{32}P)	5 µl
Klenow	2 µl
SDW	27µl
Total volume	50 µl

1) A total of 20 ng purified insert DNA was used as the probe.
2) DNA was denatured for 5 min in a boiling water bath, flash-cooled, and final volume was made up to 50 µl. and incubated at 37^{0}C for 3 hr.
3) The reaction was stopped using 0.5 M EDTA and the sample was diluted to 200 µl with distilled water.
4) The unincorporated radioactive material was removed by using spin columns packed with Sephadex G-50.
5) The radioactivity of 2 µl aliquot was monitored before and after purification to calculate the percent of incorporation of the label.
6) The samples were incubated at 37^0 C for 1 h. before stopping the reaction by adding 200mM EDTA (amount of EDTA to be added was adjusted according to the reaction volume).

7) The samples were denatured at 95^0 C for 5 min and immediately transferred to ice and kept for 5 min.
8) After 5 min, λ DNA marker cut with Hind III was added to the probe.

Prehybridization and hybridization

1) Prehybridization was carried out in boxes containing prehybridization solution (200 ml 20 x SSPE, 5 gm SDS, 50 ml 100x Denhart's reagent, 20 ml salmon sperm DNA (10 mg/ml), distilled water to make volume 1 liter at 65^0C for 4 hr.
2) The labeled probe was denatured for 5 min in boiling water bath, flash-cooled and added to the same prehybridization solution.
3) The hybridization was carried out in hybridization oven for 16 hr at 65^0C with constant agitation (rotation).

Posthybridization processing of Southern blots

1) After 16 hr hybridization the excess probe was removed by washing the blots in solution 1(2x SSC, 0.5% SDS) for 15 min and in solution II (0.1X SSC, 0.1% SDS) for 15 min at 65^0C with constant agitation.
2) The blots were wrapped in *Saran wrap* and exposed to X-ray film (*Kodak*) at -80^0C for 4-5 days, before developing the film.

Appendix VIII -Protocol for polymerase chain reaction (PCR) and RAPD

General procedures

1) RAPD-PCR was performed according to the protocols of Williams et al, (1990).
2) Random 10-mer primers employed in this study were purchased from *Operon Technologies*, USA.

Polymerase chain reaction

1) PCR reaction was performed under a lamina hood with 25 μl of a total reaction mixture containing 25 ng of genomic DNA, 2.5 μl 10x PCR Buffer, 1.5 μl 25 mM $MgCl_2$, 1μl 2.5 mM dNTP, 1 μl 10 μM RAPD primer and 0.4 μl *Taq* polymerase (*Gibco BRL* 5 U/μl).
2) Total reaction volume was made up to 25 μl with sterile distilled water.
3) A control without template DNA was included in each set of reactions with a single primer.
4) The amplification reaction was performed in a *Perkin Elmer GeneAmp 9600* thermal cycler programmed for 40 cycles with the following temperature profile - **First cycle:** Denaturation at 94^{0}C for 2 min.; Primer annealing at 40^{0}C for 1 min.; Primer extension at 72^{0}C for 2 min.; Next **38 cycles:** Denaturation at 94^{0}C for 1 min.; Primer annealing at 40^{0}C for 1min.; Primer extension at 72^{0}C for 2 min.; **Last cycle:** Denaturation at 94^{0}C for 1 min.; Primer annealing at 40^{0}C for 1 min.; Primer extension at 72^{0}C for 5 min.

Electrophoresis of amplified fragments

1) The amplified DNA fragments were mixed with 2 μl of 6x loading dye (25 mg Xylene cyanol and 1.5 g ficoll type 400 for 10 ml).
2) PCR products were electrophoresed on 1.5% Agarose (*Sigma*) gels at a voltage of 25 V overnight.
3) The gels were stained with ethidium bromide (5 mg/ml) and photographed under UV illumination.

Scoring of gels

1) The presence of a DNA band was scored as 1, and absence as 0.
2) Amplified bands from gynogenic regenerants (F_1 hybrid) were compared with the banding pattern in two of the parents.
3) The polymorphism in a gynogenic population was detected as presence of a band, which is not shared with another gynogenic line from the same population.

Figures 1- 51

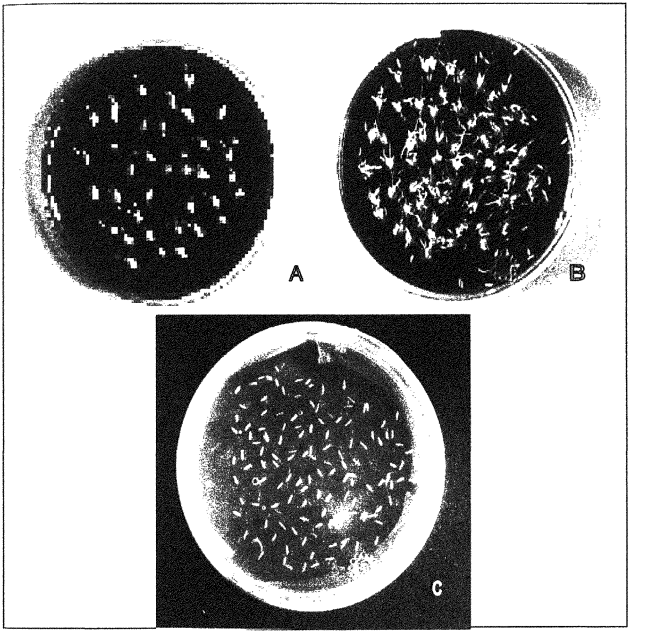


Figure 1. Initiation of in vitro anther cultures from precultured spikelet cultures. Spikelets cultured on solid YP medium (A). Anther release from spikelets six days after culture initiation (B). Initiation of anther cultures using such released anthers (C).

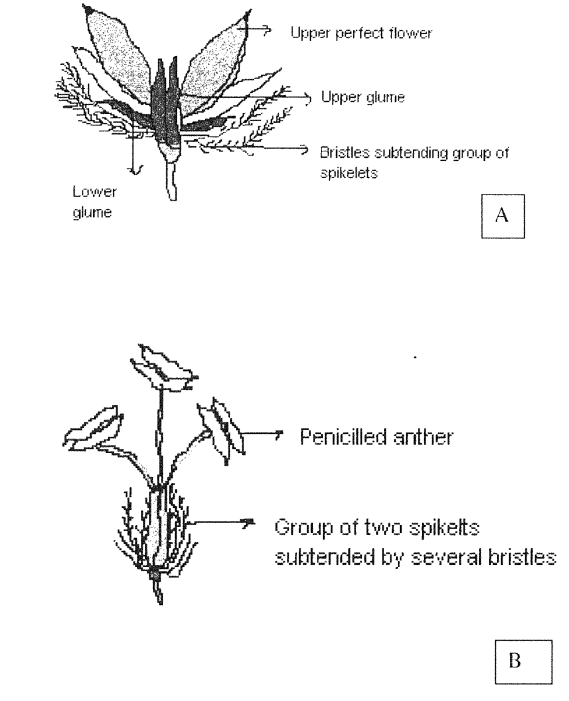


Figure 2: Line drawing of pearl millet floral structure. (A) Flower opened up to show different parts. (B) Flower seen during anther emergence.

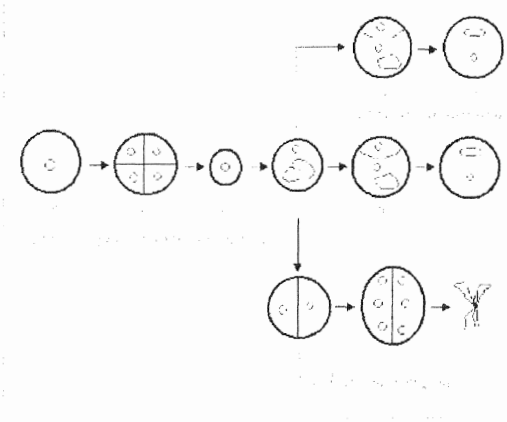


Figure 3. Schematic representation of development *in vivo* and *in vitro.*

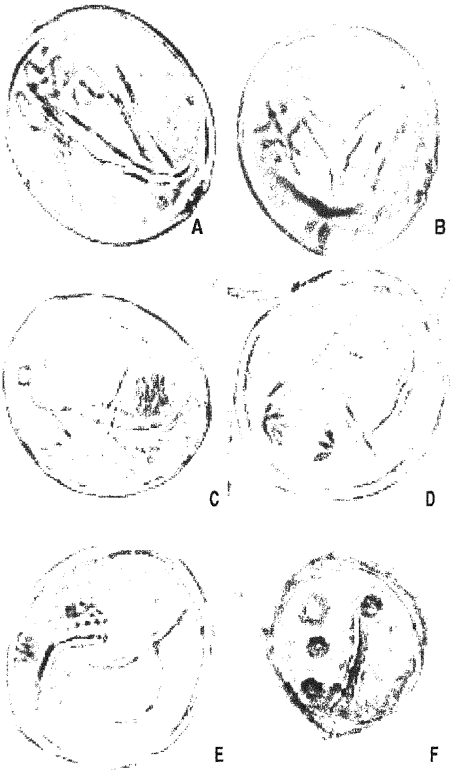


Figure 4. **Various stages of mitosis in dividing microspores**. Microspores showing long and thin chromosomes during early prophase **(A)**, showing short, thick chromosomes during late prophase **(B)**, alignment of pairs of chromosomes onto metaphase plate **(C)**, migration of chromatids to the opposite poles during anaphase **(D)**, microspore after telophase **(E)**, and a microspore with four nuclei after two rounds of mitosis (**F**).

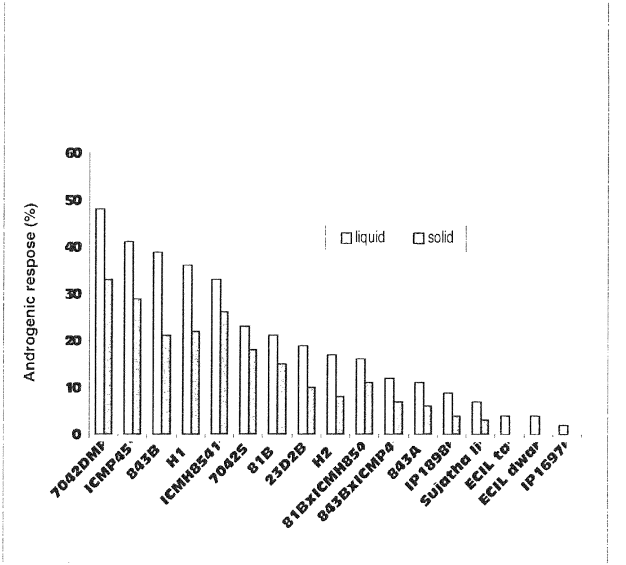


Figure 5. Comparison of androgenic response in 17 genotypes of pearl millet.

The genotype 7042 DMR showed maximum response in both liquid and solid media while the genotype IP18698 showed minimum response.

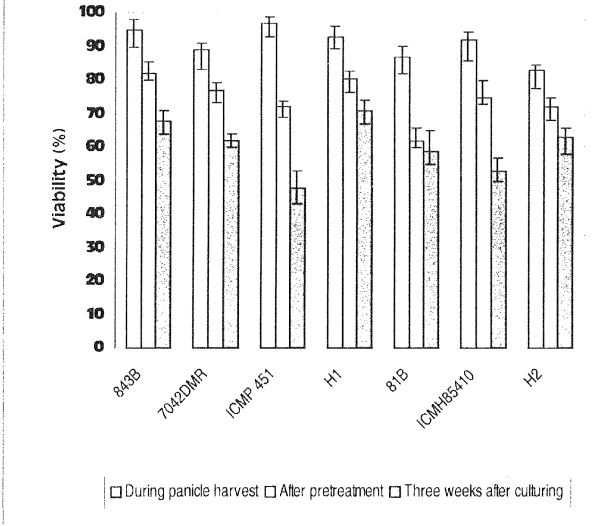


Figure 6. Viability of microspores before and after *in vitro* culture. Average viability across genotypes was 80% during panicle harvest, more than 60% after cold pretreatment (just during *in vitro* culture initiation) and around 50% after 3 weeks under culture.

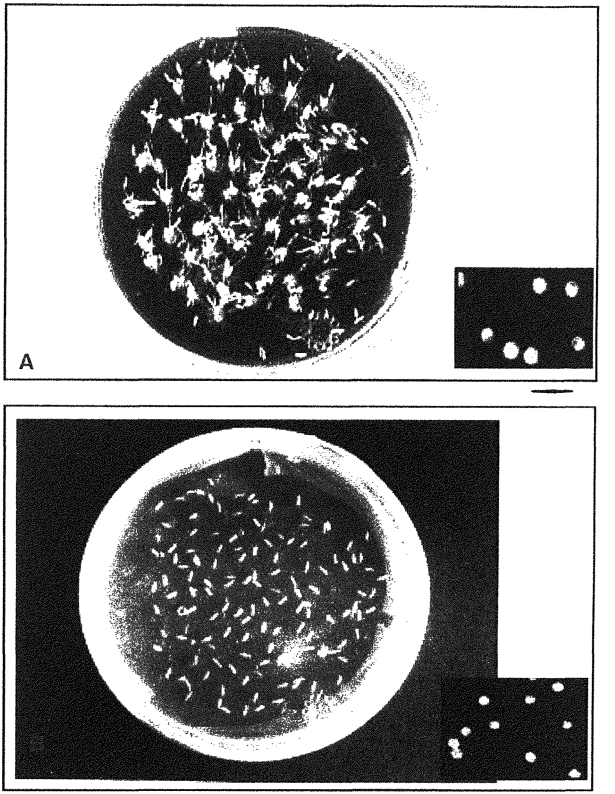


Figure 7. Released anthers from precultured spikelets (A) **Initiation of anther cultures using such released anthers (B)**

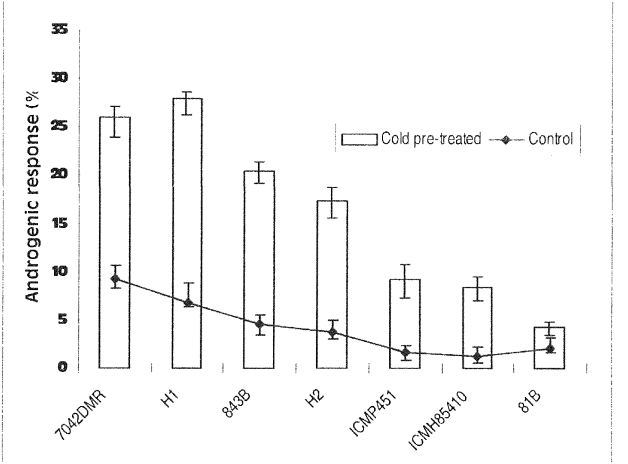


Figure 8. Effect of cold pretreatment on androgenic induction. In all the 7 genotypes studied, Androgenic induction frequencies were more in cold treated samples than in non-cold treated samples (controls).

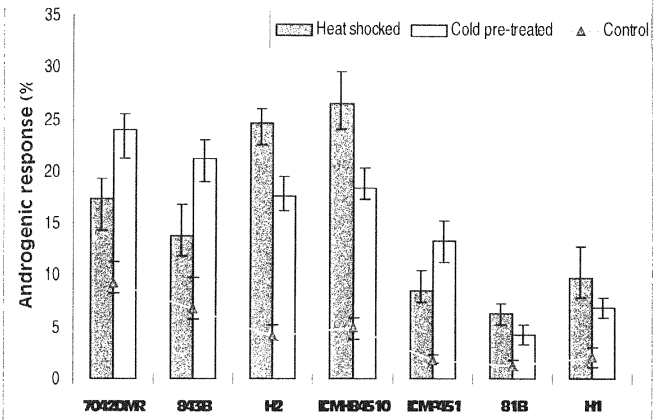


Figure 9. Effect of high temperature pretreatment on androgenic response. Heat-shock has increased the androgenic response in the genotypes H2 and ICMH85410 while it has inhibited the response in the genotypes 7042 DMR, ICMP451 and 843B. Effect of heat-shock was marginal in the genotypes 81B and H1.

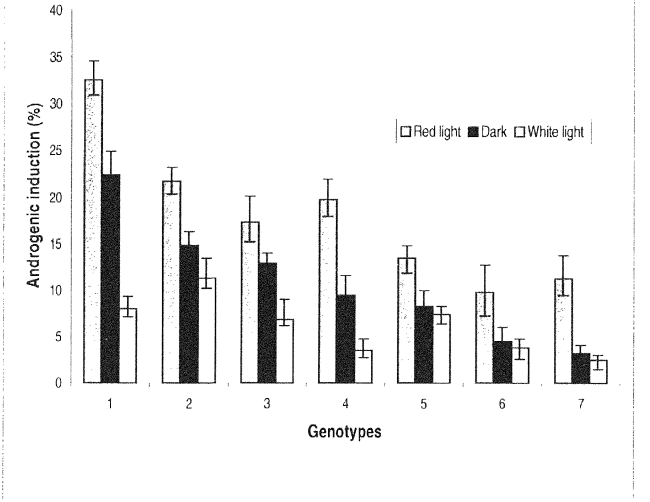


Figure 10. Effect of different light regimes on proembryoid formation. In all the genotypes studied, maxcimum frequency of proembryoids was obtained in cultures incubated under red light, followed by dark and white light.

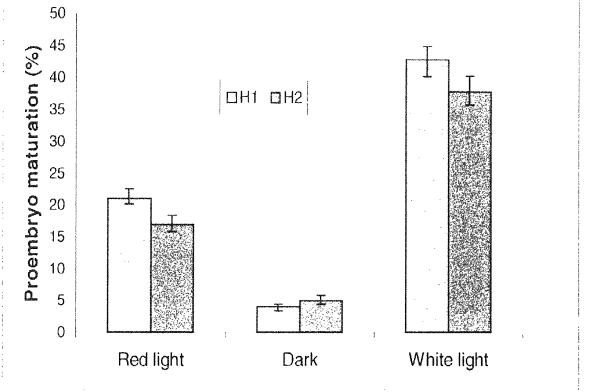


Figure 11. Effect of light quality on conversion of proembryos into embryos (embryo maturation). Maximum frequency of mature embryos was observed in cultures incubated under white light followed by red light. Embryo formation was minimum in cultures incub

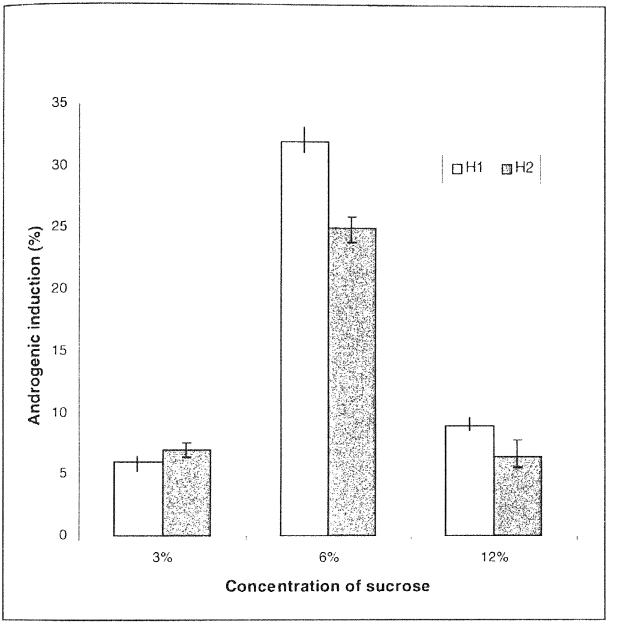


Figure 12. Effect of sucrose concentration on androgenic induction frequency.

In both the hybrids, maximum frequency of of proembryos was observed in 6% sucrose, followed by 12% and then 3%.

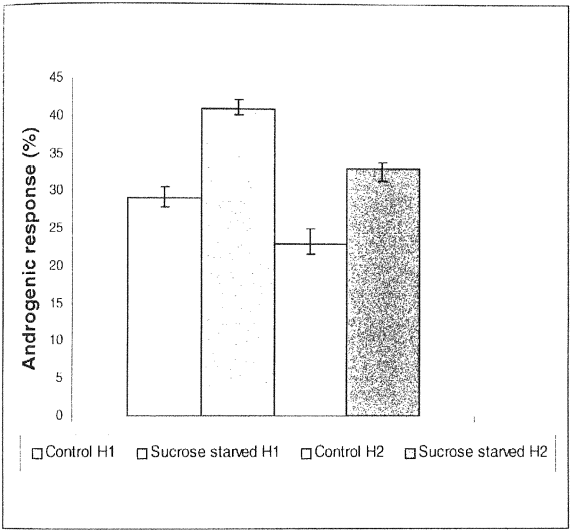


Figure 13. Effect of sucrose starvation on androgenic induction.
In both the hybrids, sucrose starved cultures showed better response than controls.

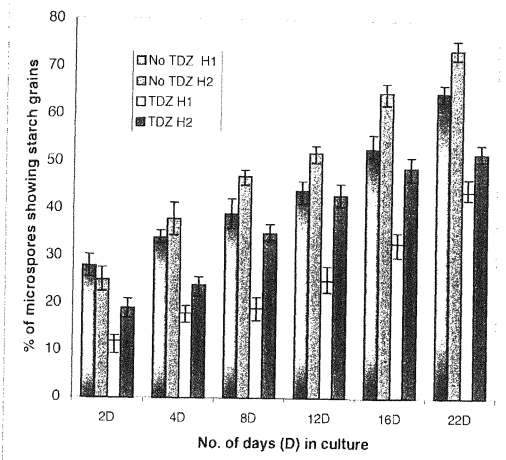


Figure 14. Effect of TDZ in arresting starch formation in developing microspores *in vitro*. In both the hybrids (H1 and H2), addition of TDZ could successfully bring down the frequency of microspores containing starch.

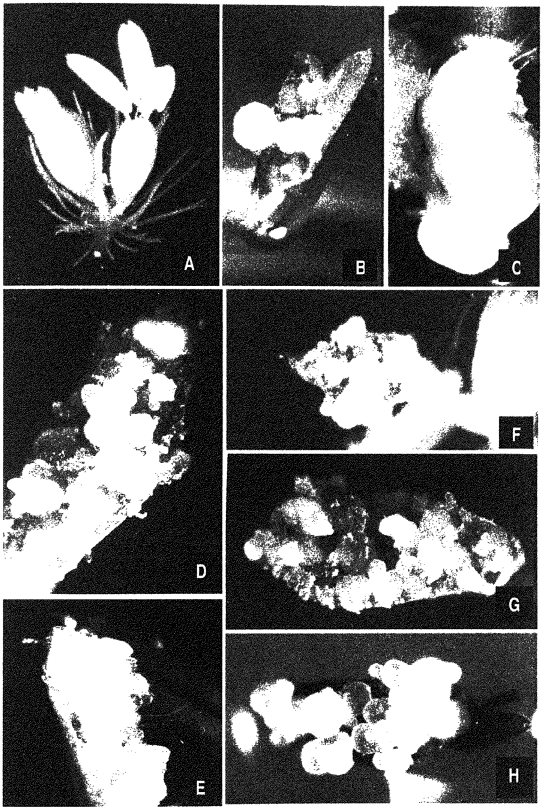


Figure 15. Effect of different plant growth regulators on androgenic embryo and calli production from *in vitro* cultured anthers. Anthers released from cultured spikelets (A). Proembryo formation from responding microspores on medium with 2 mg 2,4-D and 0.1 mg kinetin (B). Advanced stages of above proembryos (C). Both callus and embryo formation on medium with 2 mg 2,4-D and 0.5 mg kinetin (E). Predominant embryo formation and very little callus on medium with 1 mg 2,4-D and 1 mg BAP showing (D,F,G). Exclusive embryo formation on medium with 1 mg 2,4-D, 1 mg BAP and 0.5 mg TDZ (H).

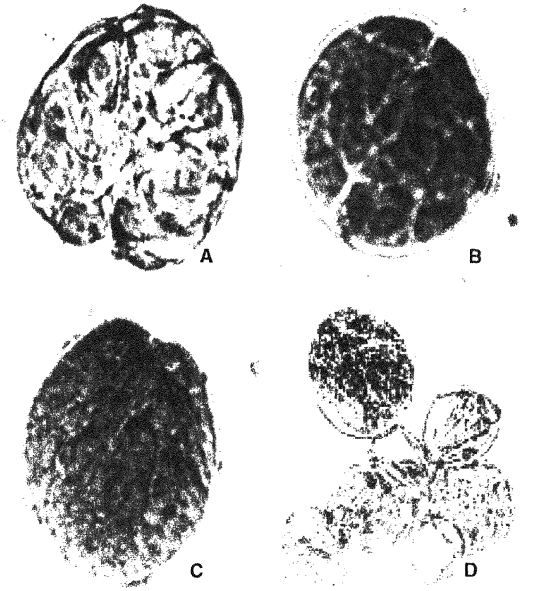


Figure 16: Various types of proembryos (stained with acetocarmine).
A) Vegetative cell derived proembryo B) An undifferentiated cell derived proembryo. C) Generative cell derived proembryo. D) A group of microspore derived proembryos

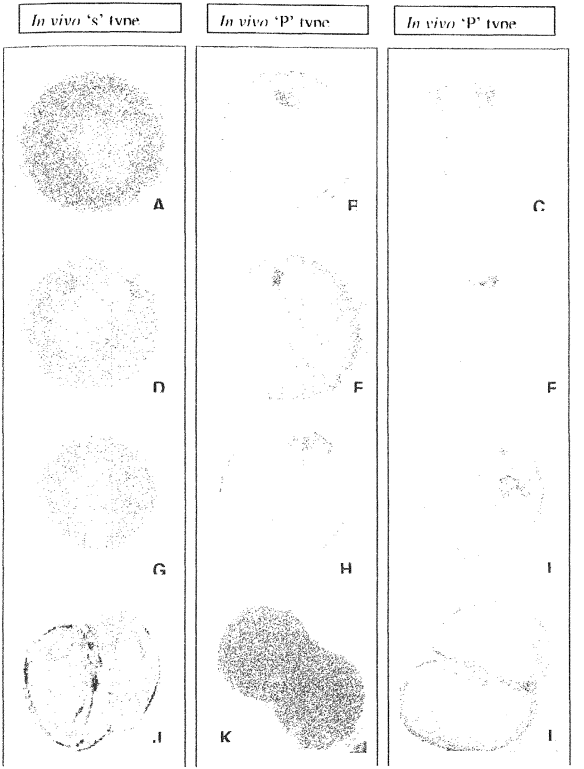


Figure 17. Pollen dimorphism as observed *in vivo* and *in vitro* in pearl millet. A densely stained uninucleate microspore ('s' type) observed *in vivo* (A, D, G, & J), corresponding stage 'p' type microspore observed *in vivo* (B, E, H & K) and same as Observed *in vitro* (C, F, I & L).

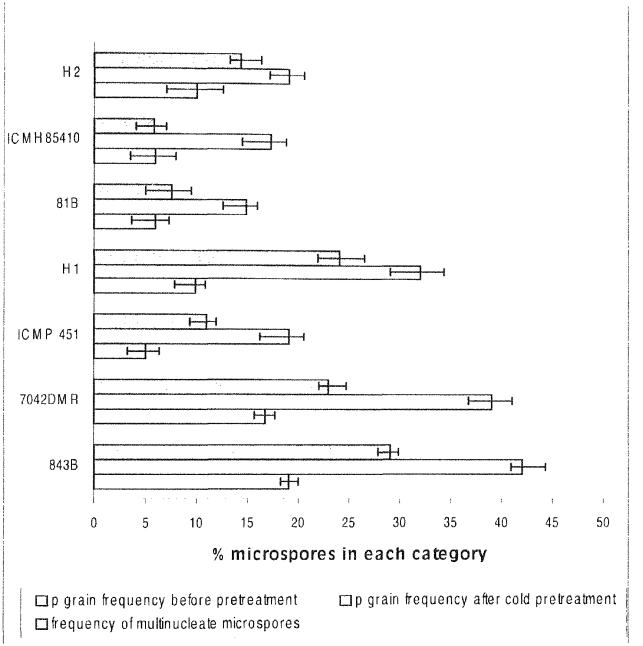


Figure 18. Correlation between cold pretreatment, p grain frequency and microspore embryo formation. In all the seven genotypes studied, p grain frequency has increased as a result of cold pretreatment. But in the genotypes, ICMH85410, 81B and ICMP451, the increase in p grain frequency did not necessarily increase the frequency of microspore embryos.

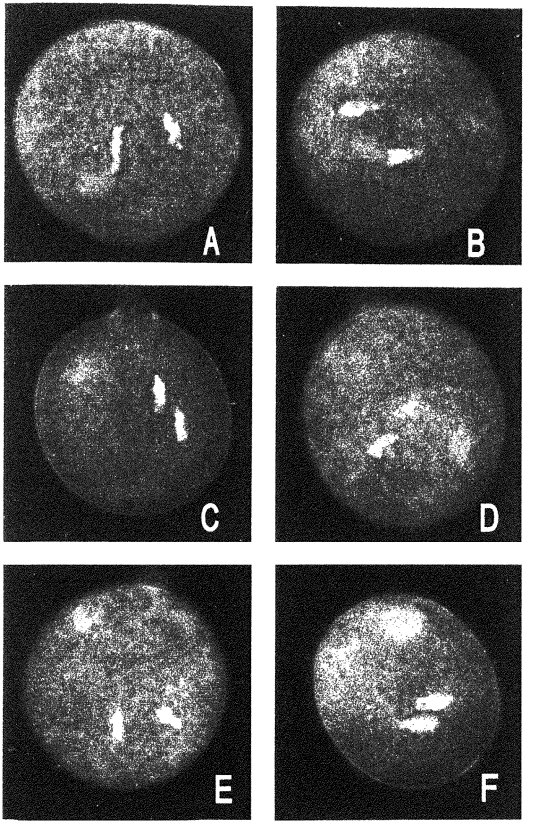


Figure 19. Different stages of *in vivo* microspores stained with DAPI showing vegetative and generative nuclei.

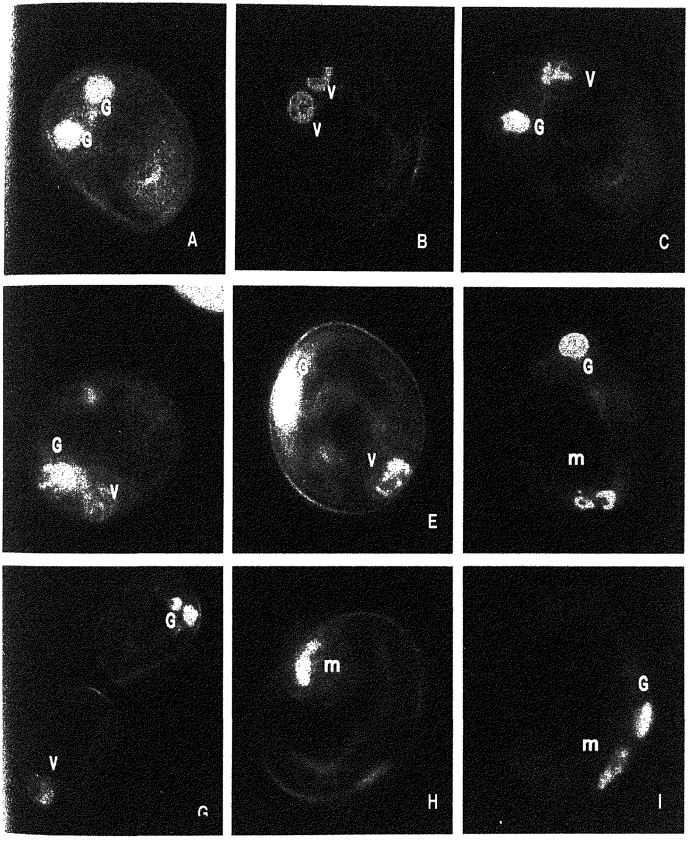


Figure 20. Different types of nuclear divions in 3 day old in vitro cultured microspores, stained with DAPI. In each picture, vegetative nucleus is labelled as V, generative nucleus as G and micro-nuclei are named as m.

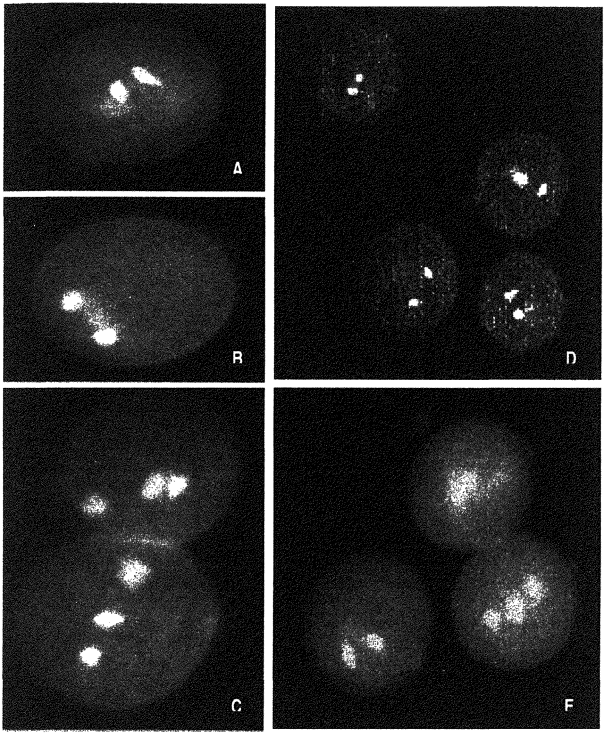


Figure 21. Different types of nuclear divisions (resembling in vivo pattern) in *in vitro* cultured non-responding microspores. Microspores at the end of 6 th day with two generative nuclei (A&B), at the end of 6 th day showing two generative nuclei and a vegetative nucleus (C), group of microspores at the end of 10 th day showing same number of nuclei as in A and B, without any further divisions but faint cytoplasmic contents (D), group of microspores at the end of 10 th day showing same number of nuclei as in C, without any further divisions but dense cytoplasmic contents (D).

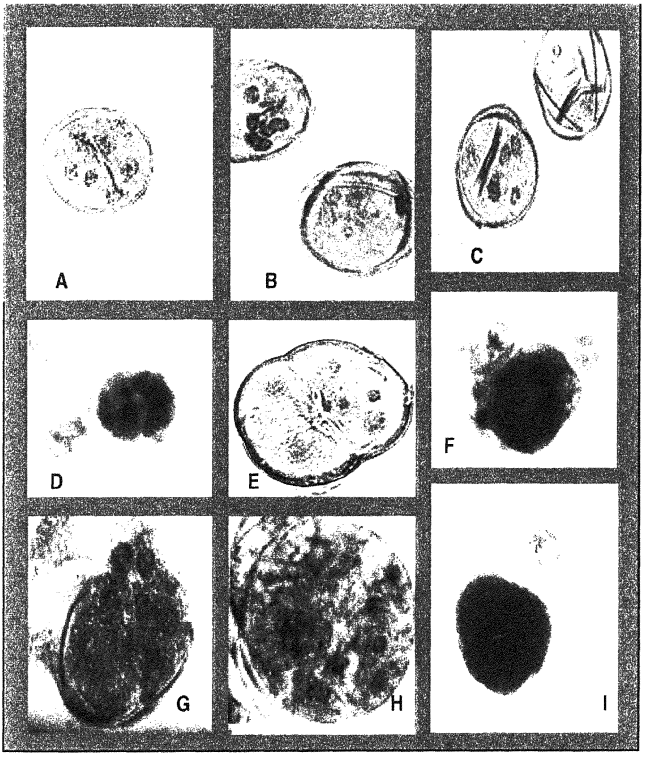


Figure 22. Different pathways of microspore embryogenesis observed in microspore cultures of pearl millet. Six nucleate vegetative cell derived (A) six nuceate generative derived (B) microspore showing divisions from both vegetative and generative cells (C, D and E). Multi-nucleate icrospore showing multiple divisions in generative cell (left intensely stained region in F) but no divisions in vegetative cell (right non stained region in F) ; advanced stage embryos from microspores following A pathway (G), B pathway (H) and C pathway (I).

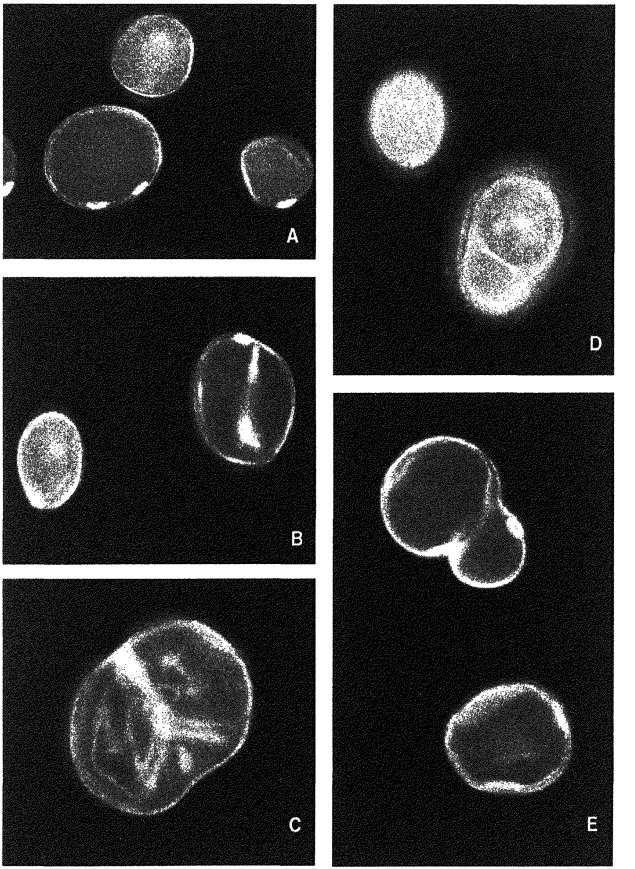


Figure 23 (A-E). DAPI stained dividing microspores showing various phases of cell wall formation showing centripital ingrowths. Microspore ready to divide showing two germinationpores (A). Dividing microspore with one round of cytokinesis (B) and after second round (C) and further stages of cell wall formation (D and E).

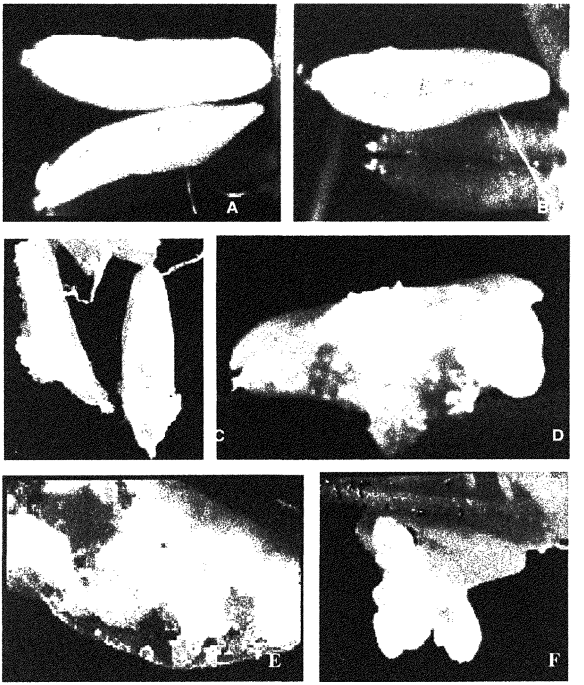


Figure 24: Microspore derived calli and somatic embryogenesis from heat shocked and sucrose starved anthers. Anthers showing developing proembryos and calli after a heat shock of 30°C for 36 h (A). Developing proembryos in sucrose starved anthers (B). Advanced stages of proembryos and calli from heat shocked anthers (C & E). Advanced stages of proembryos from sucrose starved anthers (D & F).

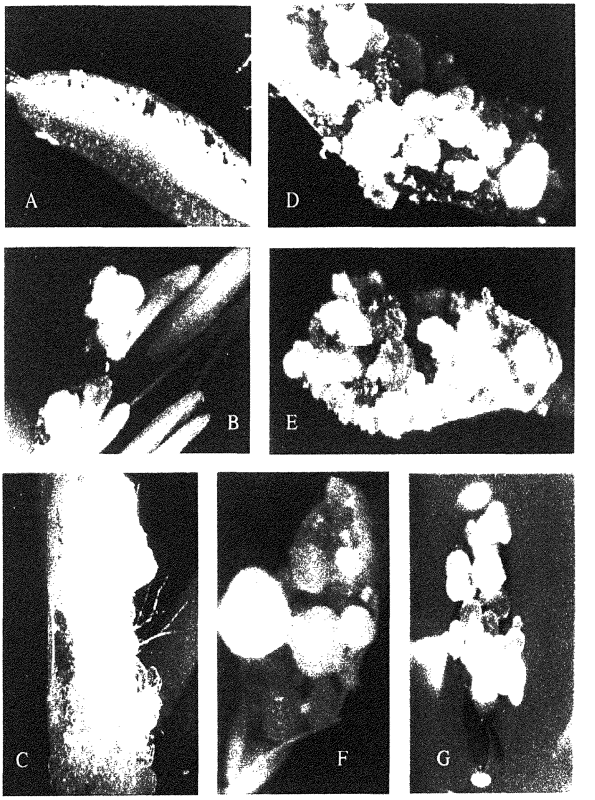


Figure 25. Various kinds of androgenic responses in liquid and solid media. In liquid media, depending on phytohormones, anthers produced either embryos (A, B, F and G) or predominantly calli (C). On solid media, anthers produced a both calli and embryos (D and E).

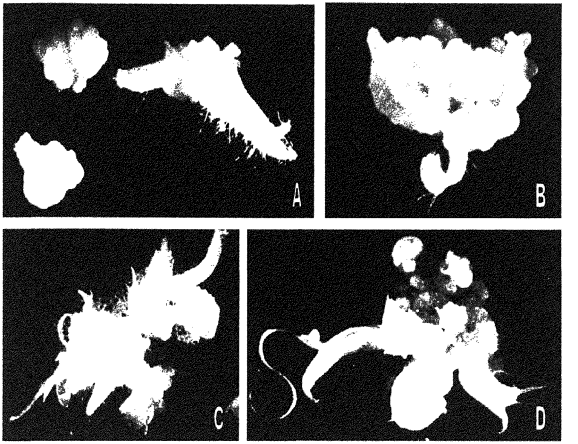


Figure 26 (A-D). Progressive stages of differentiation from microspore-derived embryos.

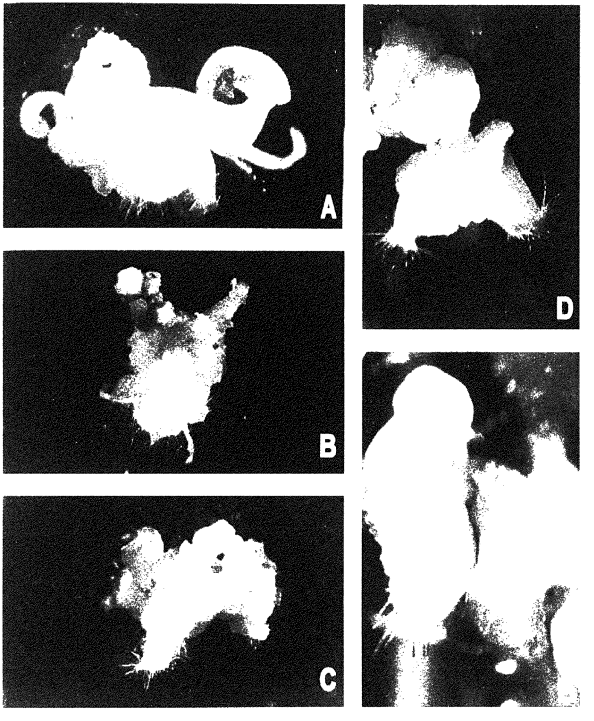


Figure 27 A-E. Various stages of differentiation from microspore derived embryogenic calli.

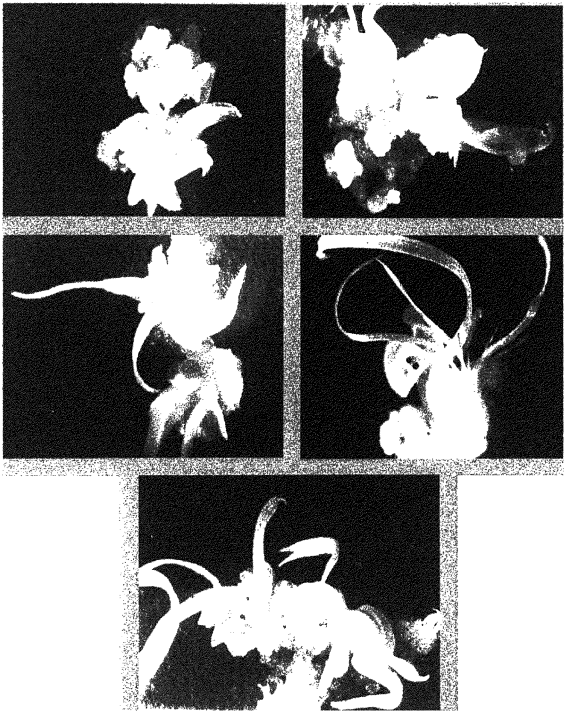


Figure 28. Various stages of plant regeneration from anther derived embryogenic callus

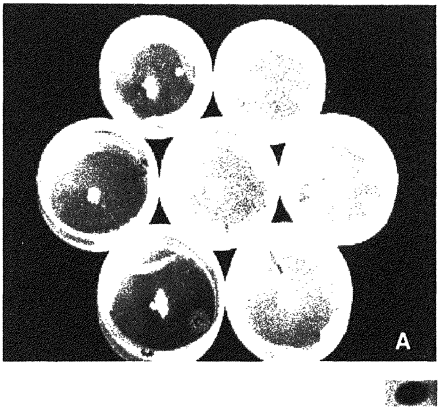


Figure 29. Plant regeneration from anther derived callus.
A) Anther derived embryogenic callus, subclutured onto regeneration medium. B) Formation of albino plantlets from embryogenic calli.

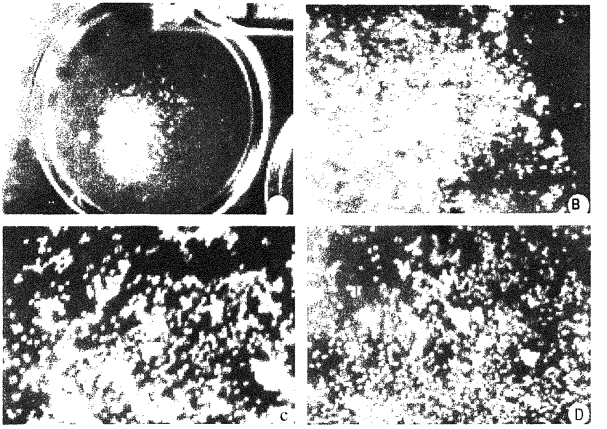


Figure 30. Initiation of microspore cultures from precultured anthers.
Freshly isolated anthers (A), and microspores at 2 weeks (B), 3 weeks (C) and 5 week at different developmental stages.

Figure 31. Embryogenesis in isolated microspore cultures.
Microspores isolated from precultured anthers (A), 6 day old microspore cultures showing multicellular microspores (B), Microspore derived calli at two developmental stages (C and D), Microspore derived embryos (E), magnified view of an embryo (F), scanning electron micrographs of microspore derived embryos (G and H).

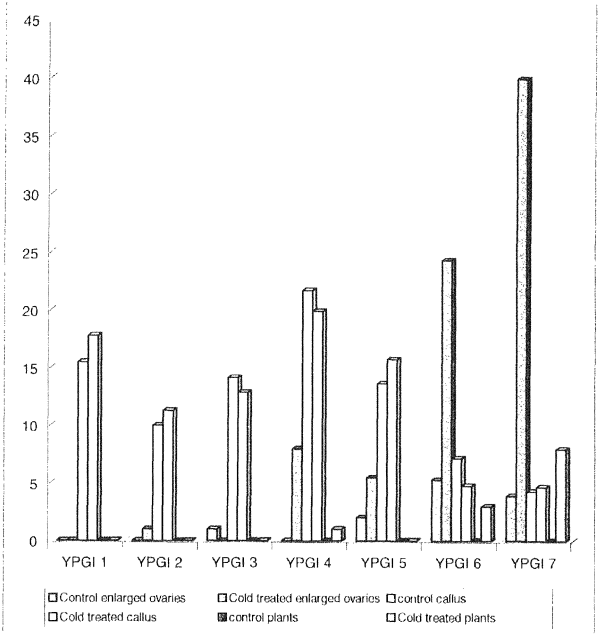


Figure 32. Effect of cold pretreatment on gynogenic induction.
Cold treatment has increased frequency of enlarged ovaries. With favorable media combinations like YPGI 6 and YPGI 7, cold treated spikelets produced gynogenic plants.

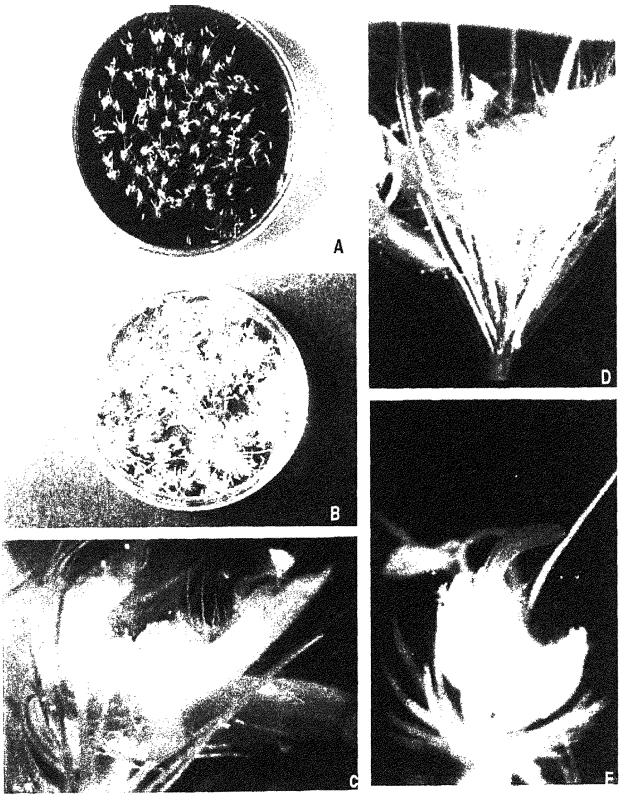


Figure 33. Gynogenic induction in cultured spikelets. Spikelets cultured on solid (A) and liquid (B) YP medium. Spikelets showing callus formation in Liquid medium (C) and solid medium (D) after 3 weeks of *in vitro* culture. Plantlet emergence in 8 weeks old spikelet cultured on solid medium (E).

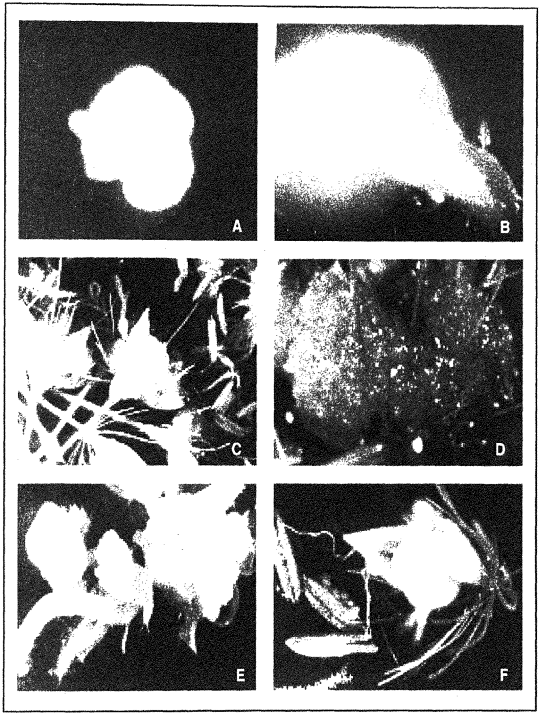


Figure 34: **Two different types of gynogenic responses (calli, and enlarged ovaries) in *in vitro* cultured spikelets and isolated ovaries.** (A) Enlarged ovary in 6 week old sequential ovary cultures. (B) Germination of a gynogenic embryo from enlarged ovaries. Non-embryogenic callus formation from 4 week old (C) and 8 week old (D) spikelets in liquid medium. (E) Embryogenic callus formation from 8 week old spikelets on solid medium. (F) Both androgenic and gynogenic responses from spikelets cultured on solid medium (***note:*** *well developed androgenic embryos along side of the split anther in F. The connection between the spike and anther is still intact*).

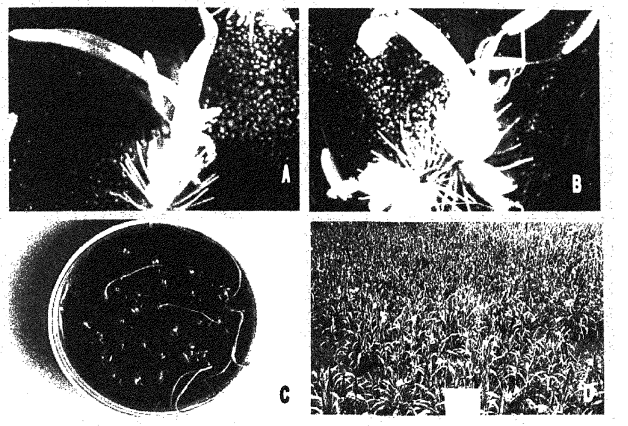
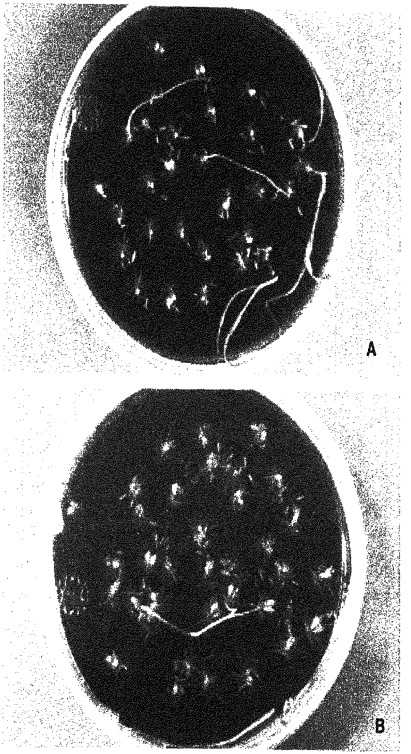


Figure 35. Plant regeneration from spikelet cultures. Direct plant emergence from cultured spikelets (A-B), Gynogenic plants in a petridish (C) and progeny of gynogenic doubled haploid regenerant growing in the field (D).



igure 36: Gynogenic plants in petridishes. A) Five gynogenic plants (all green) seen in petridish. B) An albino gynogenic regenerant emerging from cultured spikelet.

Figure 37. Acclamatization of gynogenic regenerants from petridishes (A) to pots (B, C, and D) greenhouse conditions. Seed set on a gynogenic regenerant (E).

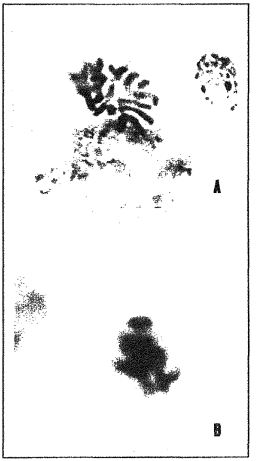


Figure 38. Mitotic chromosomes in a doubled haploid (A) and a haploid (B) gynogenic regenerants.

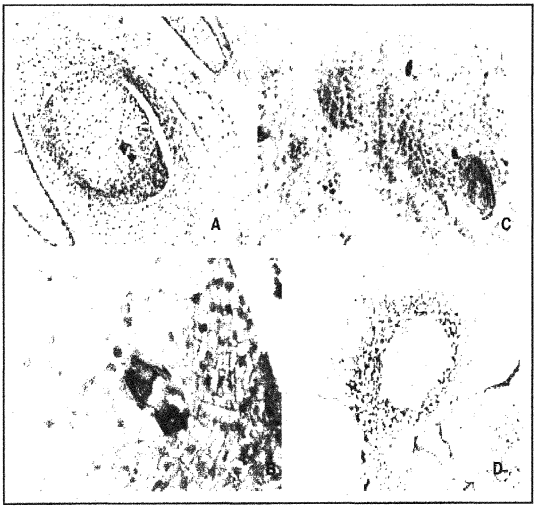


Figure 39. Anatomy of the spikelet during culture initiation (tetra-nucleate stage) and anatomy of the developing (callusing) spikelet after 4 weeks of *in vitro* culture.

A) Anatomy of the spikelet at the time of culture initiation showing ovaries with multi-nucleate embryosacs.

B) Magnified view of embryosac. C) Somatic embryogenesis from ovary.

D) Embryogenic callus formation from ovary and surrounding tissues.

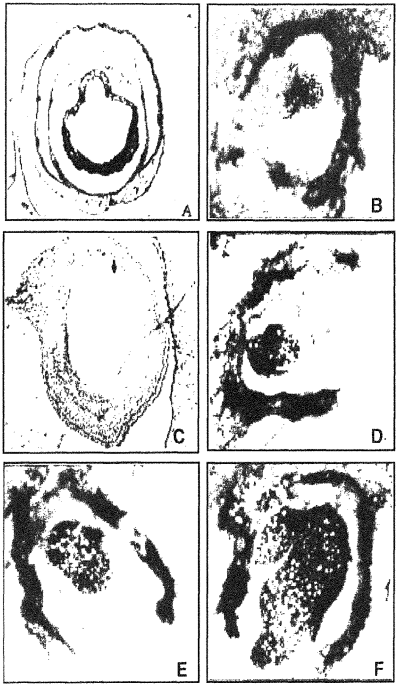


Figure 40. Anatomy of various stages of developing gynogenic embryos inside the ovary. (A) Transverse section of a 4 week old *in vitro* cultured spikelet. (*Note: hollow ovary, indicating deterioration of gynogenic tissue*) no gynogenic was seen in such ovaries (B) longitudinal section of a 4-week-old *in vitro* cultured spikelet showing developing gynogenic callus inside the ovary. (c) 6 week old *in vitro* cultured spikelet showing thickened ovary walls but no gynogenic structures developed inside. (d) 6 week old *in vitro* cultured spikelet showing developing gynogenic embryo. (e-f) Anatomy of 8 and 10 week old *in vitro* cultured spikelets showing progressive stages of gynogenic embryos.

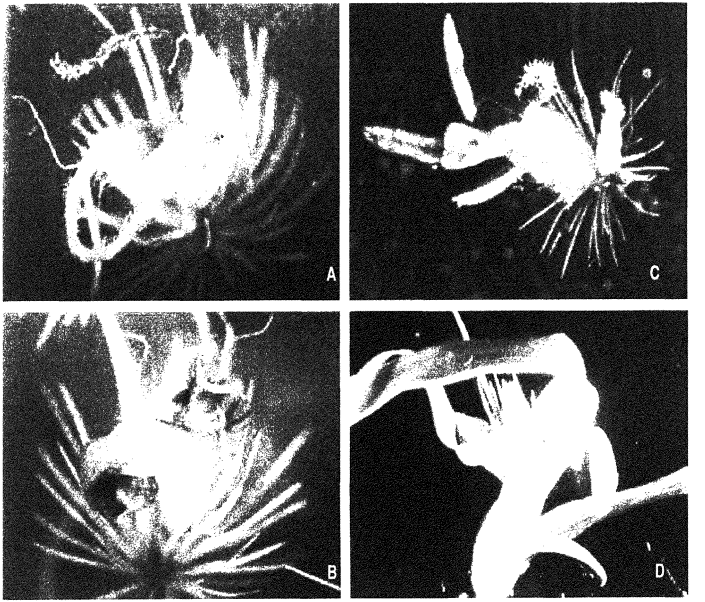
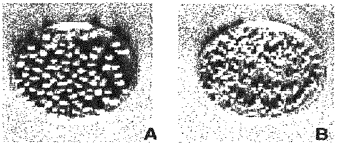


Figure 41. Effect of charcoal on gynogenic plant emergence.

Gynogenic plantlet emergence from a 6 week-old (A) and 8-weeks old spikelets (B) subcultured onto regeneration medium without charcoal. Gynogenic plantlet emergence from a 6 week old (C) and 8 weeks old (D) spikelets subcultured onto regeneration medium with charcoal.



Seeds from a DH plant (A) and a haploid plant (B).

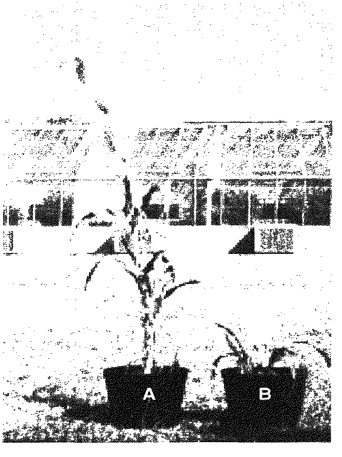


Figure 42. A doubled haploid plant (*left)* and haploid plant (*right*) at flowering stage

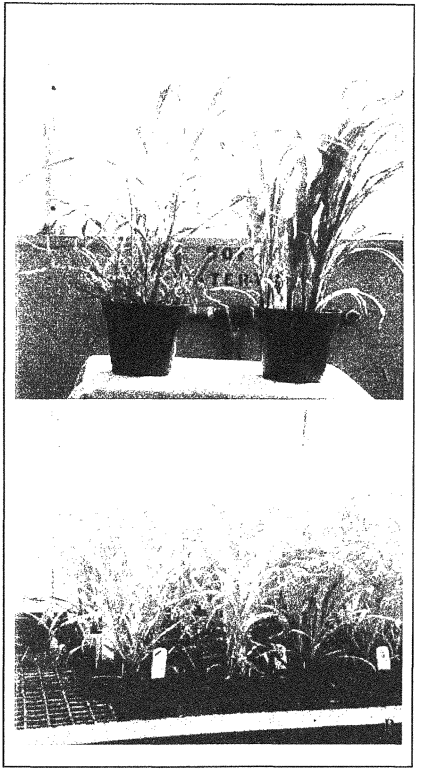


Figure 43. Glasshouse screening of a DH progeny against downy mildew pathogen. (A) Comparison of parental genotypes (of F_1 used for *in vitro* haploid production) for DM resistance, ICMP451 is susceptible (left) while 843B is moderately resistant (right). (B) DH progeny screened in greenhouse for DM resistance. The symptoms and resistance pattern in the progeny resembled as that of 843B.

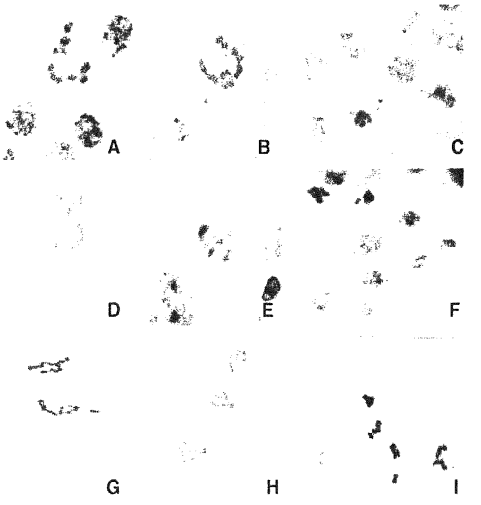


Figure 44. Abnormal meiotic configurations in haploid microspres. Pollen mother cells showing chromosome clumping (A,C and D). Single bivalents and five univalents (B). Migration of single chromosomes to poles instead of pairs of chromosome (E). Formation of anaphase bridge (F). Abnormal configurations of haploid univalents (G and H). Products of first division showing three chromosomes in some of the cells and four chromosomes in other cells (I).

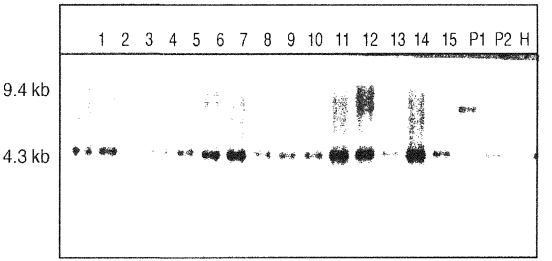


Figure 45. RFLP profile of progeny of a gynogenic regenerant (B). Genomic DNA was restriction digested with the enzyme *EcoR-I* and probed with *psm* 410. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 9.41 Kb was generated in parent 1 and hybrid. A band of 4.3 Kb was generated in parent 2 and hybrid. No polymorphism was observed among lanes 1 to 15, all the progeny lines contained only single band (4.3 Kb) as that of parent 2.

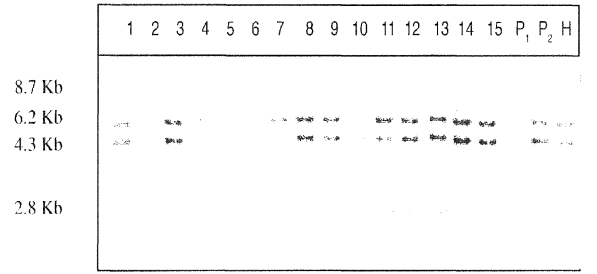


Figure 46. RFLP profile of progeny of a gynogenic regenerant (D). Genomic DNA was restriction digested with the enzyme *EcoR*-V and probed with *psm* 412. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 8.7 Kb was generated in parent 1 and hybrid. A band of 4.3 Kb was generated in parent 2 and hybrid. No polymorphism was observed among lanes 1 to 15, all the progeny lines showed the band (4.3 Kb) as that of parent 2, but the 8,7 Kb band from parent 1 is absent in progeny lines. Additionally, two bands (6.2 and 2.8 Kb each) were seen in all the samples.

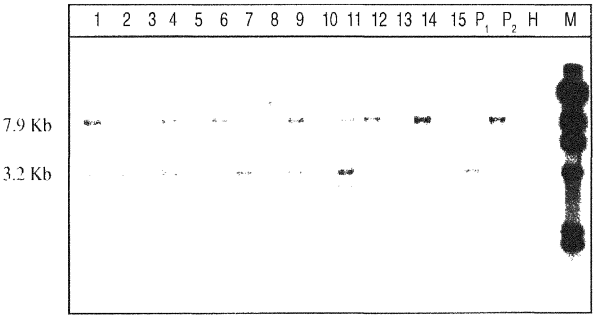


Figure 47. RFLP profile of progeny of a gynogenic regenerant A. Genomic DNA was restriction digested with the enzyme *EcoR-I* and probed with *psm* 356. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 7.9 Kb was generated in parent 2 and hybrid. A band of 3.2 Kb was generated in parent 1 and hybrid, but was absent in parent 2. All the progeny lines contained resembled hybrid.

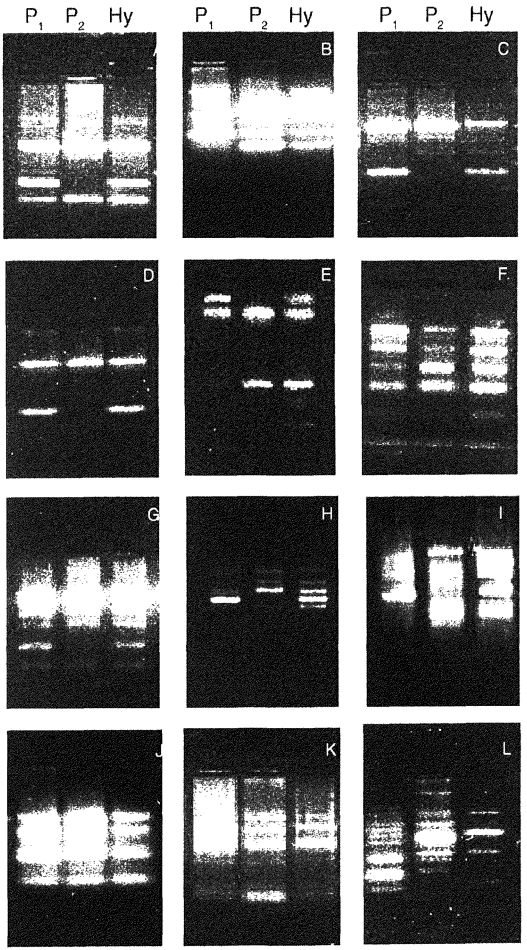


Figure 48. Screening of random primers (for polymorphism between two parents and hybrid) for further RAPD analysis of DH progenies.

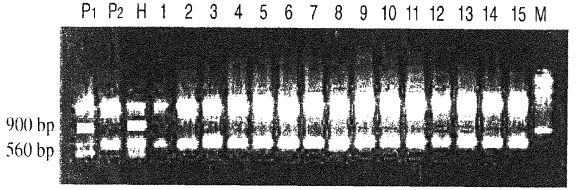


Figure 49. RAPD profile of progeny of a gynogenic regenerant E. Genomic DNA was amplified with OPG 8. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 900 bp was generated in parent 1 and hybrid. A band of 560 bp was generated in parent 2 and hybrid. No polymorphism was observed among lanes 1 to 15, all the progeny lines contained banding pattern as that of parent 2 and did not show the unique band of parent 1.

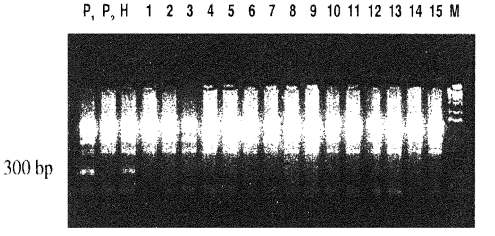


Figure 50. RAPD profile of progeny of a gynogenic regenerant E. Genomic DNA was amplified with OPD 2. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 300 bp was amplified in parent 1 and hybrid and that was absent in parent 2. No polymorphism was observed among lanes 1 to 15, all the progeny lines contained banding pattern as that of parent 2 and did not show the unique band of parent 1.

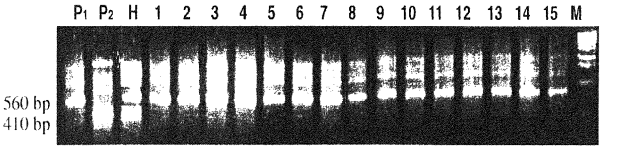


Figure 51. RAPD profile of progeny lines of a gynogenic regenerant E. Genomic DNA was amplified with OPD 11. Lanes 1-15 indicate progeny; P_1 indicates parent 1; P_2 indicates parent 2; H indicates hybrid. A band of 560 bp was amplified in parent 1 and hybrid. This band of 560 bp was absent in parent 2 while it showed a band of 410 bp that was present in hybrid but absent in parent 1 . No polymorphism was observed among lanes 1 to 15, all the progeny lines contained banding pattern as that of parent 1 and did not show the unique band of parent 2.