STUDIES ON HAPLOID INDUCTION AND MOLECULAR CHARACTERIZATION OF INDUCED VARIANTS IN MULBERRY

Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

BY

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CERTIFICATE

This is to certify that **Ms. MADHUMITA BHOWMIK** has carried out the research work embodied in the present thesis entitled **"Studies on haploid induction and molecular characterization of induced variants in mulberry"** for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Studies on haploid induction and molecular characterization of induced variants in mulberry"** has been carried out by me under the supervision of Dr. G. Padmaja in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

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….DEDICATED TO MY PARENTS, Mr. ARUN B. BHOWMIK AND Mrs. MANJU BHOWMIK,, AND MY HUSBAND Mr. ABHIJIT BHATTACHARJEE

Acknowledgements

With high regards, I wish to express my deep sense of gratitude to my supervisor Dr. G. Padmaja for her stimulating guidance and constant encouragement throughout my tenure

My sincere thanks to Prof. A.S. Raghavendra, Dean, School of Life Sciences, Prof. A. R. Podile, Head, Department of Plant Sciences, Prof. T. Suryanarayana, former Dean and Prof. M. N. V. Prasad and Prof. P. B. Kirti, former Heads for providing necessary facilities for the research

I would like to thank Prof. Arjula R. Reddy for providing me lab facilities for conducting molecular studies

I would also like to thank Prof. R. P. Sharma for extending me lab facilities for pollen germination studies

My sincere thanks to Prof. Appa Rao Podile and DR. Ch. Venkatrammana for allowing me to use the Gel-Documentation facility

I would like to specially thank Prof. Aparna Dutta Gupta for extending the lab facilities, valuable advice, support and encouragement throughout my tenure

My sincere thanks to Prof. Prakash Babu for allowing me to conduct microscopy experiments in his laboratory

I would also like to thank Dr. Anurag Chathurvedi, Acharya N. G. Ranga Agricultural University, for helping me with pollen irradiation experiments

I wish to thank Dr. M. Sujatha, Diroctorate of Oilseeds Research , Hyderabad and Dr. Pankaj Kaushal, Indian Grassland and Fodder Research Institute, Jhansi, for helping me in Flow Cytometric analysis

My special thanks to Dr. Umesh Reddy, Associate Professor, and Dr. N. Padmavathi, Research Scientist, West Virginia State University and Prof. S. Sokka Reddy, Acharya N. G. Ranga Agricultural University for their help in AFLP marker studies

Timely and useful suggestions of the faculty members of Life sciences are sincerely acknowledged

I also want to thank all my friends and colleagues of the department for their timely help and support

I would like to thank my lab mates Haritha, Samuel and Sujatha for their timely help and also maintaining a cheerful environment in the laboratory

I wish to thank Mr. U. Kantha Rao for his help in lab and field experiments.

I would like to thank all the administrative staff of the School of Life Sciences

I want to thank ILS, DST for the financial assistance and CSIR for the award of Senior Research Fellowship

Last but not the least I would like to take privilege to thank my parents, brother, in-laws and my husband Mr. Abhijit Bhattacharjee without whose support and encouragement I would not have been able to successfully complete my Doctoral research work

MADUMITA BHOWMIK

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ABBREVIATIONS

%	:	Percentage
˚C	:	Degree centigrade
$\mu Em^{-2}s^{-1}$	:	micro Einstein per meter square per second
µg	:	micro gram
µl	:	micro litre
µm	:	micro meter
µM	:	micro molar
2,4-D	:	2, 4-Dichlorophenoxyacetic acid
AFLP	:	Amplified fragment length polymorphism
BAP	:	6-Benzyl amino purine
bp	:	Base pair
Chl	:	Chlorophyll
cm^2	:	Centimeter square
DH	:	Double haploid
dNTP	:	Deoxy nucleotide tri-phosphate
DTT	:	Dithiothretol
EDTA	:	Ethylene diamine tetra acetic acid
FDA	:	Fluorescence di acetate
Gy	:	Gray
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
hr	:	Hour
IBA	:	Indole-butyric acid
ISSR	:	Inter simple sequence repeat
K_2HPO_4	:	Di potassium orthophosphate
K_3PO_4	:	Tri potassium phosphate
kGy	:	Kilo gray
KH_2PO_4	:	Potassium dihydrogen phosphate
kR	:	Kilo rad
M	:	Molar
mg/l	:	milligram per litre
$MgCO_3$	:	Magnesium carbonate
ml	:	milli litre
mm	:	milli meter
mM	:	milli molar
MS	:	Murashige and Skoog medium
N	:	Normal
NAA	:	α-Naphthalene acetic acid
NaF	:	Sodium fluoride
NaOH	:	Sodium hydroxide

GA_3	:	Gibberellic acid
ng	:	nano gram
nm	:	nano meter
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PEG	:	Poly ethylene glycol
pH	:	Negative logarithm of hydrogen ion concentration
PI	:	Propedium iodide
PMSF	:	Phenyl methyl sulphonyl fluoride
PPFD	:	Photosynthetic photon flux density
PVP	:	Polyvinyl pyrrolidone
RAPD	:	Random amplified polymorphic DNA
RIL	:	Recombinant inbred lines
rpm	:	Revolutions per minute
SDS-PAGE	:	Sodium dodecyl sulphate
SEM	:	Scanning electron microscope
SSR	:	Simple sequence repeats
TEMED	:	N, N, N, N (Tetramethyl) Aminomethyl ethylenediamine
Tris	:	Tris (hydroxymethyl) amino methane
v/v	:	Volume by volume

INTRODUCTION

Mulberry is an important plant since its foliage constitutes the main diet for the silkworm (*Bombyx mori* L.). The silkworm is monophagous and survives solely on the mulberry leaves. Mulberry is a fast growing deciduous woody perennial plant. It is highly heterozygous and clonally propagated species. The quality of silk production is directly proportional to the quality of leaves used and therefore leaf quality is utmost important in sericulture. Most of the species of *Morus* are diploid having a chromosome number of 2n=28.

Persistent breeding efforts are required to develop mulberry varieties that are high yielding with outstanding leaf quality and durable disease/pest resistance. Being a highly heterozygous perennial plant, mulberry is not readily amenable to conventional methods of plant improvement. Homozygous plants are true breeding, and are of vital importance to plant breeders either as parental lines or as finished cultivars. The homozygous lines are useful in genetic studies, particularly in elucidating the genetic control of traits by recessive alleles, linkage mapping and analysis of quantitative trait loci (Cadalen *et al*., 1998). Production of homozygous lines through recurrent inbreeding is not useful in mulberry due to long generation cycles and high levels of heterozygosity (Hamrick *et al*., 1979). Obtaining homozygous lines *via* inbreeding or by brother x sister crosses is not possible because the genotypes are dioecious and the male and female lines are genetically diverse. Hence, production of haploid plants is the fastest and the only easy method for producing homozygous lines in mulberry.

Haploids are sporophytes with gametophytic chromosome number. The significance of haploids in genetics and plant breeding has been recognized for long time.

Spontaneous production of doubled haploids has been reported in some species but their induction frequency remained low. As a result of haploid induction followed by chromosome doubling, homozygosity can be achieved in the quickest possible way making genetic and breeding research much easier. Due to the importance of doubled haploid plants for plant breeding, their production would be especially useful in species with long generation times which make traditional breeding methods impractical. Frequency of naturally producing haploids is very low. Therefore, attempts have been made to produce them artificially through distant hybridization, delayed pollination, anther or pollen and unpollinated ovary cultures and *in situ* parthenogenesis induced by irradiated pollen.

Anther, pollen and unpollinated ovary culture techniques have been utilized successfully for production of haploids in different plant species (Don Palmer *et al.*, 2005). Many factors are involved in obtaining regeneration of plants from anther and unpollinated ovary cultures. The nutrient media, genotype, culture vessels, condition of donor plants, carbohydrate source, phytohormones, reduced nitrogen are some of the important factors that influence the success from anther and unpollinated ovary cultures (Campion *et al*., 1992; Datta, 2005). Androgenic and gynogenic haploids have been used to breed new cultivars and for obtaining homozygous lines (Germana`, 2006). Although the number of new plant varieties developed by these methods has been limited, refinement of tissue culture methods has extended the range of crop species from which haploid plants have been produced with high efficiency (Morrison and Evans, 1988).

Haploid embryos were produced in plants after pollination by distantly related species (Kasha and Kao, 1970; Laurie *et al*., 1990); the process is best documented in cereals. In most cases, normal double fertilization took place to form a hybrid zygote and endosperm. Subsequent cell division in the zygote resulted in the elimination of paternal chromosomes leaving a haploid embryo. The rapidly dividing endosperm suffered chromosomal elimination and usually aborted early in seed development; as a consequence the haploid embryo was rescued by *in vitro* culture (Forster *et al*., 2007). The first and most widely used method of this type is the 'bulbosum' method in which a high frequency of haploids of cultivated barley, *Hordeum vulgare* were obtained after crossing with the related species *Hordeum bulbosum* (Kasha and Kao, 1970).

Double haploids have contributed to breeding programs for diverse crops. The production of haploid and double haploid plants may open new dimension for genetic studies and breeding work in mulberry. Attempts have been made to produce haploids through anther and unpollinated ovary cultures in mulberry, but with limited success. Studies have been conducted on the induction of division in pollen cultures (Katagiri, 1989) and the effects of sugar and sugar alcohols on the division of mulberry pollen in tissue cultures (Katagiri and Modela, 1991). Shoukang *et al*. (1987) reported androgenic haploids in five Chinese genotypes of mulberry, but the production percentage was very low ranging from 0 to 13.6% and was genotype dependent. Sethi *et al*. (1992) reported embryo differentiation in anther cultures of Japanese genotypes of mulberry (RFS135 and RFS175). Jain *et al*. (1996) reported induction of haploid callus and embryogenesis from the cultured anthers of mulberry (var. Rainfed Selection 135).

There are a few reports of gynogenic haploids in mulberry. Sita *et al*. (1991) reported induction of gynogenic plants from ovary cultures of mulberry; however, gynogenic plants regenerated remained small and did not survive in soil. Dennis *et al.* (1999) reported an efficient method for regeneration of gynogenic haploids from unpollinated ovary cultures of *Morus alba* L. The frequency of haploid plants was very low and established plants are not known to date.

A major limitation in producing haploids is the lack of techniques for their large-scale production from any given stock and the requirement of colchicine for doubling of the chromosome. Direct production of fertile homozygous plants can help in eliminating this laborious step (Pandey *et al*., 1990).

The ability to induce the development of spontaneous parthenogenetic embryos does not appear to exist in all plant species. Pollen irradiation technique has been used to induce haploid plants through *in situ* parthenogenesis. Pollen irradiation technique has been employed in plant breeding programme for the development of haploids, overcoming incompatibility barriers (Pandey, 1974), gene transformation (Pandey, 1978) and generating targeted mutations (Yang *et al*., 2004). The genetic composition of the parental genotypes, in particular, the irradiated pollen parent as well as the dose of gamma-irradiated pollen were important factors in the parthenogenetic embryo induction by ionizing radiation (Jain *et al*., 1996). So far, there have been no reports on using pollen irradiation technique for production of haploids in mulberry.

Successful production of haploids or doubled haploids through induced parthenogenesis by irradiated pollen has been demonstrated in several species such as

barley (Subramanyan and Kasha, 1976), pear (Bouvier *et al*., 1993), apple (Zhang and Lespinasse, 1991; Witte and Keulemans, 1994), muskmelon (Cuny *et al*., 1993), carnation (Sato *et al*., 2000), kiwifruit (Pandey *et al*., 1990; Chalak and Legave, 1997) and mandarin (Froelicher *et al*. 2007). However, the number of parthenogenetic seeds produced was generally low.

Haploid pollen has unique advantages for mutagenesis, since mutations are directly passed onto the next generation in a hemizygous state and large numbers of pollen grains (haploid nuclei) can be easily mutated (Yang *et al*., 2004). Genetic and developmental changes associated with irradiated pollen include mutational changes/damages (Werner *et al*., 1984), selective gene transfer (Borrino *et al*., 1985), egg transformation *via* incorporation of fragments of male DNA after high doses of pollen irradiation (Pandey, 1978) and mentor pollen effect. Nicoll *et al*. (1987) studied the endosperm response to irradiated pollen in apples and reported nuclear abnormalities, enhanced number of polyploidy restitution nuclei, bridges between nuclei and disrupted mitotic synchrony. Mutagenesis or gene transfer of haploids, followed by chromosome doubling would enable recessive genes to be expressed and also provided useful information on inheritance of desirable characteristics, sex determination and breeding sensitivity (Chalak and Legave, 1997).

Pollination through irradiated pollen can induce haploid embryo development, but the mechanism behind the exact origin of haploid embryo still remains unknown. Cadler and Abak (1999) reported *in situ* haploid embryo induction in cucumber after pollination by irradiated pollen. Studies revealed that the higher the irradiation doses, the lower the

haploid production. Irradiation doses up to 600 Gy did not affect fruit set or seed production.

Induction of gynogenesis through irradiated pollen in cucumber was reported by Nikolova *et al*. (2001). The optimal doses for haploid embryo induction were reported to be 300 and 500 Gy. The growth of the haploid plants was slow and the flowers were smaller in size with strongly cut petals. The haploid plants were found to be sterile in nature. In pear (Bouvier *et al*., 2002) and in melon (Lotfi *et al*., 2003), spontaneous doubled haploid plants were directly generated by induced gynogenesis. Froelicher *et al*. (2007) reported induced parthenogenesis and haploid plant production in mandarin. Pollination was carried out for three genotypes of mandarin with four levels of γ-irradiated pollen (150, 300, 600, and 900 Gy). The resulting seeds were characterized by a small size. The haploid parthenogenetic origin was confirmed using microsatellite marker analysis and chromosome count.

In few of the cases induced parthenogenesis resulted in mutants in F_1 generation. Falque (1994) obtained morphological mutants with 50 Gy irradiated pollen, but no haploid was obtained. The detection of irradiation-induced mutations depends on the ability of such changes to be transmitted to subsequent generations. Most irradiation-induced mutations are deficiencies, duplications, translocations and inversions (Pfahler, 1967). Marker locus mutations can also be identified in the M_1 generation if irradiated pollen is used to cross to a homozygous recessive mutant (Yang *et al*., 2004). In some instances, problems have been encountered in the transmission of large deletions through the gametes; this is particularly true for the male gametophyte (Sears, 1952; Sigh, 1993).

Induced parthenogenesis may yield either haploid or diploid plants. Haploid plant may be produced from induced haploid cell whereas the diploid plant may arise from abnormal diploid egg cell or haploid egg stimulated to double the chromosome number, producing diploid embryo (Pandey *et al*., 1990). This may be of immense interest to the breeder, as it would give rise to homozygous fertile diploids directly. Höfer and Grafe (2003) reported homozygous lines in sweet cherry by *in situ* parthenogenesis followed by embryo and cotyledon culture. Flow cytometric analysis of the regenerants revealed their diploid nature. Four lines of the regenerants were characterized as homozygous using isozyme analysis.

Aslam *et al*. (1994) reported a sharp decline in seed production and survival rate with the increase in irradiation dose. Musial and Przywara (1998) studied the endosperm response to pollen irradiation in kiwifruit and observed that pollination with irradiated pollen yielded endosperm with low amounts of storage products and is autonomous and represented the 2n level. There were no mitotic abnormalities in the irradiated endosperm. Vizir and Mulligan (1999) studied the genetics of gamma irradiation induced mutations in *Arabidopsis thaliana* and reported that large chromosomal deletions can be rescued through the fertilization of diploid eggs. It provided a genetic tool for deletion mapping and for analysis of chromosomal regions essential for chromosome maintenance. In *Arabidopsis*, the percentage of seeds decreased linearly with the increase of the irradiation dose (Yang *et al*., 2004). Fifty percent of the seeds aborted at the dose of 400 Gy and all the seeds at 1200 Gy.

A number of methods have been developed for the detection of haploids in a population of diploids. Although the most effective means is by cytological methods for determination of chromosome number, flow cytometry, stomata size, chloroplast counts in the guard cells of leaf stomata, pollen size, and pollen abortion are the other criteria used for initial screening of the haploids (Sari *et al*., 1999; Dennis *et al*., 1999; Forster *et al*., 2007). Flow cytometric analysis of the ploidy status is easier and quicker than the chromosome counts (González Castañón and Schroeder, 2001) but the final verification is done usually by chromosome counts. Phenotypic effects can be used for identification of haploids as the vegetative and floral parts and the cell size are reduced relative to diploids. In potato (*Solanum tuberosum* sub sp. *tuberosum*), isozyme analysis and visual examination were performed independently to compare the efficiency of discriminating hybrids from haploids (Liu and Doucher, 1993). Hofer *et al*. (2008) characterized the plant material obtained by *in vitro* androgenesis and *in situ* parthenogenesis in apple using ploidy level, zygosity state using isozyme and simple sequence repeat (SSR) analysis, tree morphology, flower and fruit quality.

Molecular markers offer a powerful supplement to the morphological data and estimation of the level of genetic variation. Molecular markers have many advantages compared with morphological markers, such as robustness to environmental change, nearly unlimited number (unlike isozymes) and relative ease and rapidity of data collection (Lombard *et al*., 2000). Molecular markers enable homozygotes and heterozygotes to be distinguished. Molecular tools hold a promising way of allowing the identification of genes involved in a number of traits including polymorphism. DNA

markers are useful in both basic (phylogenetic analysis and search for useful genes) and applied research (marker assisted selection and paternity testing).

A commonly used DNA marker is Random amplified polymorphic DNA (RAPD), based on the polymerase chain reaction (PCR) and arbitrary sequence primers. RAPD, as a relatively fast and low-cost technique, has been frequently used in genetic investigations of different plant species. The microspore origin of anther culture derived plants of flax was determined using ISSR and RAPD markers (Chen *et al*., 1998). Using one ISSR primer and two RAPD primers, 12 out of 16 plants were identified as being derived from microspores. Plants derived from the same callus had identical PCR patterns at five polymorphic loci and were likely to be derived from the same microspore. Kamiński *et al*. (2003) analyzed double haploid lines derived from cabbage by the use of RAPD markers for their diversity and uniformity. Eight primers yielding informative bands were used and out of the total of 83 RAPD bands scored, 16.9% were polymorphic between a set of 13 double haploid lines.

Amplified fragment length polymorphism (AFLP) analysis is a technique through which selected fragments from the digestion of total plant DNA are amplified by the polymerase chain reaction (Vos *et al*., 1995). The resulting DNA fingerprints provide a large number of genetic markers, and the multiplex ratio, defined as the number of information points analyzed per experiment, is much higher than for other types of markers, such as RFLP (Restriction fragment length polymorphism), RAPD or SSRP (Simple sequence repeat polymorphism) (Powell *et al*., 1996). The AFLP technique has been successively used to assess the genetic diversity in many plant species including

mulberry (Sharma *et al*., 2000; Botton *et al*., 2005), analysis of variation in somatic embryos (Vendrame *et al*., 1999) and somaclonal variation (Polanco and Ruiz, 2002) and for following the possible introgression of maize DNA in wheat X maize crosses (Brazauskas *et al*., 2004).

Keeping in view of the above, the present investigation aimed at production of haploids using *in vitro* androgenesis, gynogenesis and pollen irradiation technique. The pollen irradiation technique was employed for the first time in mulberry to explore the possibility of inducing haploids through *in situ* parthenogenesis. The specific objectives of the present work are:

- To study the effect of media and growth regulators on callus induction and plant regeneration from *in vitro* cultured anthers and unpollinated ovary cultures.
- To investigate the possibility of inducing haploids through irradiated pollen technique.
- Evaluation of fruit and seed set in M-5 cultivar after pollination with control and irradiated pollen of S-13 and China White cultivars separately.
- Morphological analysis of M_1 plants obtained after pollination with irradiated pollen of S-13 and China White.
- Flow cytometric and cytological analysis of M_1 plants for identification of haploids and variants.

- Molecular characterization of M_1 plants obtained after crossing M-5 with S-13 irradiated pollen using RAPD and AFLP markers.

- Molecular characterization of M_1 plants obtained through interspecific hybridization of M-5 cultivar with China White irradiated pollen using RAPD markers.

REVIEW OF LITERATURE

Mulberry is an economically important plant used for sericulture, as it is the sole food plant for the silkworm, *Bombyx mori* L. It is a perennial, deep-rooted, widely adaptable fast growing tree plant belonging to the genus *Morus* L. under the order Urticales and the tribe Moraceae (Hooker 1885). Mulberry leaf is a major economic component in sericulture industry since the quality and quantity of leaf produced per unit area has a direct bearing on the cocoon harvest. It is found in a wide range of areas around the world, ranging from tropical to sub-arctic regions and accounting for a large diversity in its genetic resources. In India, most states have taken up sericulture as an important agro-industry with excellent results. The total acreage of mulberry in India is around 282,244 hectares. Though mulberry cultivation is practiced in various climates, the major area is in tropical zone covering Andhra Pradesh, Karnataka and Tamilnadu.

Although mulberry includes mainly diploid cultivars with 28 chromosomes, natural polyploids are also known to be cultivated (Machii *et al*. 2000). In addition, mulberry breeding programs have induced artificial polyploids, which exhibit increased vigour and adaptability (Machii *et al*. 2000). There are about 69 species of the genus *Morus;* the majority of them occur in Asia, especially in China and Japan whereas it has been widely believed that mulberry species originated on the low slopes of Himalayas bordering China and India. The study of Hou (1994) suggests a multicentered origin. In India, there are many species of *Morus*, of which *Morus alba*, *M. indica*, *M. serrata* and *M. laevigata* grow wild in the Himalayas. Most of the Indian varieties of mulberry belong to *M. indica*. Plants of *M. alba* and *M. indica* are generally grown for silkworm rearing while the same from *M. macrour* Miq. and *M. serrata* Roxb. are mostly grown as ornamental plants due to the rough, hairy and thick leaves, which are not suitable for

silkworm feeding (Tikader and Dandin, 2001). A few species of mulberry are valued for their edible fruits (*M. alba* and *M. laevigata*) and timber (*M. laevigata* and *M. serrata*). As mulberry fruit has been increasingly evaluated as desirable for use in fresh and processed food and drink such as jam and mulberry wine, opportunities for using the mulberry trees as fruit source have increased. Interest in development of mulberry varieties suitable for the production of mulberry fruits has increased in the recent past.

Though mulberry is basically a tree, in sericulture it is being maintained as small bushes through repeated pruning and training. The chief mode of propagation of this crop in tropical countries like India, Pakistan, and Bangladesh is through stem cuttings while in temperate countries the seed is the major source of propagation (Vijayan *et al*. 2004). Mulberry bears different sex types *i.e.* male, female and bisexual flowers on the same plant (monoecious) or on different plants (dioecious). Normally, mulberry flowers between January and April, and flowering can also be induced by mechanical injury, like pruning at any time of the year. Various reports show that expression of sex often depends on several physiological and biochemical factors (Das and Mukherjee, 1986; Jaisawal and Kumar, 1980). Minamizawa (1963) reported that high temperature, long day and full day light have favoured predominated female flowering in mulberry. They observed the development of different sexes on right and left side of the same shoot in S799.

Most of the mulberry species are dioecious and can cross-pollinate among themselves to produce fertile hybrids. Due to high degree of cross-pollination, lot of heterozygosity is present in the mulberry species. In India, the cost of mulberry leaf

production is reported to be covering nearly 60% of the total expenditure of silkworm cocoon production. Therefore, attempts have been made regularly to improve the leaf productivity per unit area. As a result, a number of mulberry varieties have recently been developed in India for its cultivation at farmer's level. Most of the varieties are suitable to specific regions rather than for wider locality with varying climatic conditions as India experiences wide range of agro-climatic conditions. Hence, persistent breeding efforts are needed to develop varieties with wider adaptability and higher yield potential and resistance to diseases and pests to sustain the profitability of sericulture industry in these areas (Chakraborti *et al*. 1999). As mulberry has very high heterozygosity and a long juvenile period, developing inbred lines, F_2s and back cross progenies is time-consuming and labour-intensive. Also the production of homozygous lines of mulberry by inbreeding is not possible because of its dioecious nature and male and female lines being genetically diverse. The homozygous lines can be utilized for F_1 hybrid production with potential for high, stable and predictive yields. The homozygous lines could be useful in genetic studies, particularly in elucidating the genetic control of traits by recessive alleles and analysis of quantitative trait loci (Cadalen, 1998). Therefore, production of haploid plants through anther and/or unpollinated ovary cultures or pseudo-fertilized ovule cultures would be of immense use in producing homozygous lines rapidly for utilization in breeding programs.

Plant haploid research began with the discovery that sporophytes can be produced in higher plants carrying the gametic chromosome number (n instead of 2n) and that their chromosome number can subsequently be doubled up by colchicine treatment. In conventional breeding programme, homozygous lines are obtained after several

generations of selfing. Haplo-diplodization through gametic embryogenesis allows single-step development of complete homozygous lines from heterozygous plants (Germaná, 2006).

Dorothy Bergner was the first to describe the natural occurrence of sporophytic haploids in the weed species *Datura stramonium* (Blakeslee, 1922). This was followed by similar reports in tobacco (Clausen and Mann, 1924), wheat (Gains and Hannah, 1926) and subsequently in several other species (Kimber and Riley, 1963; Riley, 1974). It was in the year 1964; Guha and Maheshwari reported the first *in vitro* cultured androgenic haploids from *Datura*. This event added a new dimension to the plant breeders by shortening the time needed to produce homozygous lines. Since then haploids have been reported in several species and doubled haploids have been routinely used in breeding programmes for new cultivar development in many crops (Veilleux, 1994; Forster *et al*. 2007).

A rapid expansion in research ensued with further development of chromosome doubling techniques that converted sterile haploids into fertile, homozygous doubled haploid plants (Jensen, 1974). A milestone was set with the release of the first doubled haploid crop plant, the cultivar Maris Haplona of rapeseed (*Brassica napus*) in the early 1970s (Thompson, 1972) followed by Mingo in barley (*Hordeum vulgare*) in 1980 (Ho and Jones, 1980) although doubled haploid lines in maize (*Zea mays*) have previously been successful for commercial production using spontaneously occurring haploids (Chase, 1969).

Doubled haploids provide an excellent material to geneticists and plant breeder for development of true breeding lines. In some species, doubled haploid production proved troublesome and time consuming. However, scientific and technological innovations contributed to the development of doubled haploids in a wide range of species. Now a days, haploids and doubled haploids have been reported in more than 200 plant species belonging to almost all families of plant kingdom, from *Aconitum carmichaeli* to *Zingiber officinale* (Maluszynski, 2003) using various spontaneous and induced (*in vitro*) methods to obtain information on the location of major genes and QTL's for economically important traits (Khush and Virmani, 1996).

Androgenesis:

Androgenesis is the most widely used method for producing haploids in crop plants (Sopory and Munshi, 1996). This system provides an unparallel opportunity to shorten the breeding cycle and to fix agronomic traits in the homozygous state, such as recessive genes for disease resistance. The principle of androgenesis is to arrest the development of male gametophytes and to force them towards a somatic pathway. *In vitro* androgenesis can be achieved from the microspores, leading to the formation of haploids either by direct embryogenesis or *via* callus formation. In general, the plants regenerated from the microspores are haploids and require chromosome doubling treatments. A few species, such as barley, regenerate a large number of doubled haploids as a result of induced chromosome doubling during early cell division of microspores (Kasha, 2005). Although the application of anther culture is widespread, the processes involved are poorly understood. Investigations have been hampered by the presence of

the sporophytic anther wall that prevents direct access to the microspores contained within. Isolated microspore culture is a simple modification of anther culture in which anthers are stimulated to dehisce and release their microspores, usually into a liquid medium. Anther and isolated microspore cultures have been successfully used to produce haploids in a large number of species (Forster *et al*. 2007).

There are several factors affecting androgenic haploid development. In most of the species, the mid-uninucleate or the early bi-nucleate stage was most appropriate for haploid induction. The effect of genotype, growth regulators and preconditioning of donor plants on callus induction in anther culture of flax was investigated (Burbulis *et al*. 2005) and the results suggested that specific combinations of growth regulators must be designed for each genotype. Temperature conditions, nutritional or osmotic stress played an important role in simulating the exact condition required by the microspores to undergo embryogenesis. The levels of different hormones and the type of carbohydrate source also play an important role. The stress may be physical *i.e.* by wounding the anther wall, thermal (by subjecting the anthers to cold or heat treatment), chemical, water stress and starvation (Germaná, 2006). Stress treatment is needed to switch efficiently and the developmental fate of microspores differed and greatly depended on the plant species and the species genotype. It has been established that the nitrogen composition of the culture medium played a significant role in androgenesis. Increasing glutamine and decreasing ammonium nitrate enhanced embryo development in many cereal species (Datta *et al*., 1990; Jähne and Lörz, 1995; Hunter, 1987). Pre-culturing the anthers on medium containing 15% sucrose for 2-7 days before transferring to the same medium with 6% sucrose for 28 days resulted in increased regeneration of shoots in flax (Chen

and Dribnenki, 2004). This result suggested the beneficial role of high sucrose concentration in the medium on initial microspore survival and cell division.

Attempts have been made to produce haploids from anther cultures in mulberry but with limited success rate. Katagiri (1989) studied the induction of division in pollen cultures. Katagiri and Modala (1991) reported the effects of sugar and sugar alcohols on the division of mulberry pollen in tissue cultures. Shoukang *et al*. (1987) reported androgenic haploids in five Chinese genotypes of mulberry but the production percentage was very low ranging from 0 to 13.6% and was genotype dependent. Sethi *et al*. (1992) reported the differentiation of embryos in anther cultures of two diploid elite genotypes of mulberry (*Morus* spp.) namely, RFS135 and RFS175, and a locally grown Japanese genotype Goshoerami. The percentages of embryogenic cultures ranged from 0.23 to 1.82% in different genotypes. They observed the development of normal leafy shoot but not a root, although the radical was well developed in the embryos. Jain *et al*. (1996) examined the influence of temperature and kinetin pretreatment on induction of androgenic callus from *in vitro* cultured anthers of mulberry (*Morus indica*). Pretreatment with cold at 4˚C was found to induce divisions in microspores. Twenty four hours cold pretreated anthers, cultured on medium with 8% sucrose, showed the maximum percentage (9.2%) of anthers inducing callus. Anther culture has not been very successful in producing haploids in this tree crop.

Gynogenesis:

Efforts have been made by different investigators to produce haploids from unpollinated ovary cultures as an alternative to anther cultures. In gynogenesis, haploid

cells of the female gametophyte (usually the unfertilized egg cell) are stimulated to develop into an embryo in an induced process similar to parthenogenesis. The success rate of gynogenesis is lower in comparison to androgenesis owing to the fact that microspores are more abundant, developmentally more synchronized and easier to manipulate. Gynogenesis was used successfully in different species. Maternally derived haploids may be the only efficient means of haploids in dioecious species. The culture response was genotype dependent (Alan *et al*., 2003; Bohanec *et al*., 2003) and culture media composition played an important role. In some members of Chenopodiaceae, Liliaceae and Cucurbitaceae, gynogenesis is the main route to double haploid production (Don Palmer *et al*., 2005). In barley, maize, rice and wheat, gynogenic haploids have been produced even when anther culture is a success (Don Palmer *et al*., 2005). Genetic stability of the doubled haploids and the absence of albinism are the main advantages of this technique. The studies of Bossoutrot and Hosemans (1985) during *in vitro* gynogenesis in *Beta vulgaris* have shown that the embryos formed from oosphere or the antipodals underwent secondary proliferation resulting in callus due to auxin like impurities contained in the culture medium. Van Geyt *et al*. (1987) obtained haploid plantlets from male fertile and male sterile sugarbeet plants at frequencies up to 2.2% using ovule culture of sugar beet (*Beta vulgaris* L.). Metwally *et al*. (1998) indicated that in unpollinated ovule cultures of *Cucurbita pepo*, embryogenesis occurs in the embryo sac cells especially egg cell and the embryo grows directly into haploid plantlet.

In onion (*Allium cepa*), haploid plants have been obtained from embryos developed directly from ovules without callus formation, although the percentage of ovules producing embryos remained very low at 0.28% (Campion and Alloni, 1990).

Michalik *et al*. (2000) were successful in inducing gynogenesis in onion using flower bud or ovary culture. No particular stress pre-treatment was used although the growth temperature of the donor plant before flowering was crucial. All regenerated plants were haploid and required chromosome doubling treatments. The use of doubled haploids in onion breeding not only produced recombinant inbreds but also saved time. The method used in sugarbeet (*B. vulgaris*) was similar although a cold treatment of inflorescences (8°C for one week) combined with high temperatures (30°C) during the induction phase improved the response (Wremerth-Weich and Levall, 2003).

Haploids as well as diploids were induced from unpollinated ovary cultures of mulberry by Sita *et al*. (1991) although the induction frequencies remained low. In order to rule out the possibility of obtaining diploids from the fertilized ovules, Dennis *et al*. (1999) developed a reproducible protocol for the production of gynogenic haploids of mulberry, *Morus alba* L. They observed maximum gynogenic response when excised ovaries from inflorescence segments were cultured on MS medium with BAP (8.5 μM) and 2,4-D (4.5 μM) followed by transfer to MS medium supplemented with 2,4-D (4.5 μM), glycine (6660 μM) and proline (1738 μM). The gynogenic plants produced were found to be haploids as well as aneuploids. Although the efficiency of haploid induction is low in gynogenesis in different species, the value of doubled haploids in species that do not respond to more efficient techniques makes the method worthwhile.

***In situ* Parthenogenesis induced by irradiated pollen:**

Pollen mutagenesis offers an opportunity of combining single-cell selection procedures with the advantages of haploid system. It also includes the ability to treat large numbers of pollen grains, the absence of chimerism and direct expression of

targeted alleles in the M_1 generations (Yang *et al*., 2004). The detection of irradiation-induced mutations in plants largely depends on transmission of these changes to the subsequent generations (Pfahler, 1967). Fertilization with irradiated pollen may sometimes result in zygote embryo abortion due to the production of dominant lethals. Swaminathan and Murty (1959) reported that pollen irradiation increased the pollen tube growth and frequency of hybrids in interspecific crosses of *Nicotiana* whereas in *Tradescantia* there were no detectable changes in pollen germination and pollen tube growth (Koller, 1943). In higher plants, chromosome aberrations induced by radiation have been utilized for many years in classical genetic studies (Mc Clinctok, 1984) and have provided starting material for gene isolation and mapping (Liharska *et al*., 1997, Bhatt *et al*., 2001). Sniezko and Visser (1986) studied the embryo development and fruit-set in pear induced by untreated and irradiated pollen. They observed that after 6 weeks after pollination only about 21% of the seeds induced by the irradiated pollen contained normal embryos.

Pollen irradiation technique has been applied to several crops but parthenogenic seed production was generally very low. In order to increase seed production, pollen irradiation has been combined with *in vitro* cultures of ovules (Raquin, 1985), seeds or embryos (James *et al*., 1985; Pandey *et al*., 1990; Zhang and Lespinasse, 1991; Cuny *et al*., 1993; Witte and Keulemans, 1994; Falque, 1994; Meynet *et al*., 1994; Sari *et al*., 1994). Several studies have shown that the irradiated pollen can germinate on the stigma, elongate within the style and reach the embryo sac but cannot fertilize the egg cell and the polar nuclei. Mutation occurs at lower dose of irradiation whereas at higher doses the frequency of parthenogenetic embryo development increases, which can be explained by

'Hertwig effect'. The 'Hertwig effect' was also observed in apple (James *et al*., 1985; Zhang and Lespinasse, 1991), muskmelon (Cuny *et al*., 1993) and rose (Meynet *et al*., 1994). Genetic and developmental events associated with irradiated pollen also include selective gene transfer (Shizukuda *et al*., 1983; Snape *et al*., 1983; Borino *et al*., 1985), 'egg transformation' *via* incorporation of fragments of male DNA after high pollen irradiation dose (Pandey, 1978) and the mentor pollen effect used to overcome incompatibility barriers (Wolf and Van Tuly, 1984). Fertilization resulting only in the endosperm development after pollination with irradiated pollen is known as autonomous endosperm. Autonomous endosperm development was observed in apple (James *et al*., 1985; Nicoll *et al*., 1987; Zhang and Lespinasse, 1991), cacao (Falque, 1994), and cucumber (Le Denuff and Sauton, 1994).

Gynogenesis was also induced by using irradiated pollen (Winton and Stettler, 1974). This has been achieved in trees by irradiating the pollen of a related species, which is then used to pollinate the desired parent to induce gynogenetic haploid production. Gynogenic haploid embryo induction and plant production has been successful through irradiated pollen technique in several species *viz*., melon (Sauton and Dumas de Vaulx, 1987; Sari *et al*., 1992; Maestro-Tejada, 1992; Cuny, 1992; Abak *et al*., 1996), cucumber (Truong-Andre, 1988; Sauton, 1989; Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Cağlar and Abak, 1999) and watermelon (Gürsöz *et al*., 1991; Sari *et al*., 1994).

Parthenogenesis may produce either haploid or diploid plant. Pandey (1980) proposed that "pseudo-fertilization" by irradiated pollen causes partial induction of the

cell cycle leading to DNA replication and chromosome division but not cell division. This produces a diploidized egg cell, and may, under certain circumstances, give rise to a parthenogenetic diploid embryo. Pandey *et al*. (1990) reported the induction of parthenogenic development of seeds in kiwifruit. A total of 479 pollinations were carried out from which 413 fruits were obtained. Irradiation dose of 700 Gy yielded the highest number of germinating seeds and thirty one haploid plants were produced. According to Zhang and Lespinasse (1991), irradiation doses of 250 to 500 Gy were most suitable in apple. A total number of 11 haploid plants were obtained. Haploid plants showed short internodes, small and pointed leaves and less vigour. Attempts on rooting the haploids showed that haploid plants were rather difficult to root. Plants obtained above 200 Gy showed no expression of a marker gene present in the pollen parent. Falque *et al*. (1992) reported that irradiation dose up to 1000 Gy had no effect on pollen viability and germination in cacao but the fruit set was strongly inhibited. High irradiation dose resulted in all-or-none response in the embryo sac, either creating abnormal embryos and/or endosperms which were aborted. Callus produced from excised endosperm derived from 100 krad pollen differed in genome size.

The effect of irradiation on parthenogenetic haploid production in muskmelon was studied by Cuny *et al*. (1993). Fruit set and number of seeds per fruit were comparable to control regardless of irradiation dosage. Haploid embryo production was genotype-dependent. In contrary to other plant species, pollen irradiation in muskmelon did not yield any hybrids with abnormal or sterile phenotypes. Irradiation led to the formation of two abnormal sperm cells leading to the induction of *in situ* parthenogenesis.

Pollen irradiation method has not always yielded haploid plants. In cacao, no haploid was obtained (Falque, 1994). Morphological mutants were obtained from one clone with irradiation dose of 50 Gy and some of them had inherited paternal alleles of enzyme markers without showing the axil-spot dominant character. Many of the mutants were too weak to survive. Witte and Keulmans (1994) successfully produced haploid plants from apple. The green plant production was strongly influenced by irradiation dose, picking time and quality of the irradiated pollen. Irradiation dose of 250 Gy was found to be more efficient for haploid production and delayed fruit picking had a significant positive effect.

Musial and Przywara (1998) studied the development of seeds following pollination with irradiated pollen at 700 and 900 Gy in *Actinidia deliciosa* (kiwifruit). They observed that the proportion of seeds containing endosperm only was almost ten-fold higher than those containing both embryo and endosperm. The induction of parthenogenesis was higher following gamma ray doses of 900 Gy than 700 Gy, which suggested the 'Hertwig effect'. In cucumber, seasonal effect played an important role in haploid embryo induction. The hot period between April and October was found to be the best season (Cadlar and Abak 1999). Musial and Przywara (1999) reported that ovule culture following pollination with irradiated pollen offered a better chance to produce haploids than undisturbed pollination in kiwifruit. Doubled haploid plants were obtained by pseudofertilized ovule culture in carnation (Sato *et al*., 2000). Plantlets obtained were morphologically different from the mother plants which indicated their origin from the maternal somatic cells. The R_0 plants were fertile and the selfed seeds obtained from R_0

plants were morphologically uniform and identical to the R_0 plants indicating that the R_0 plants were doubled haploids.

Todorova *et al*. (1997) reported double haploid production in sunflower irradiated pollen induced parthenogenesis. In total, 2279 embryos were cultivated *in vitro* of which 1107 plants were obtained and 582 of them produced seeds after selfing. The ploidy level of the regenerants was evaluated at the two-three leaf stage and 296 of the plantlets obtained were haploids. Some of them underwent spontaneous diploidization; the others were treated with colchicine solution for chromosome doubling. Aslam (2000) studied the effect of pollen irradiation and utilization of this technique for improvement of *G. hirsutum* L. It was observed that the mutants selected from M_2 generation had higher yield potential, big boll size and showed segregation of different economic traits in M_3 generation. Höfer and Grafe (2003) reported homozygous lines in sweet cherry by *in situ* parthenogenesis followed by embryo and cotyledon culture. Flow cytometric analysis of the regenerants revealed their diploid nature. Four lines of the regenerants were characterized as homozygous using isozyme analysis.

Peixe *et al*. (2000) reported abnormal embryo development in European plum. Abnormalities of the embryo development were helpful for understanding the causes of the early ovule death after irradiation. Yanmaz *et al*. (2000) reported the effects of gamma irradiation on pollen viability and haploid plant formation in *Cucumis melo*. *In vitro* pollen viability was higher in pollen irradiated with 300 Gy doses than with 350 Gy. Pollen viability decreased with increase in irradiation doses. Haploid embryos and plants were observed in *Cucurbita pepo* (Kurtar *et al*., 2002). Different shapes and stages of

embryos were obtained from seeds issued after pollen irradiation. Haploid production was strongly influenced by gamma ray doses, embryo stages and genotypes. Kumar and Rai (2006) reported the pleiotropic effects of γ-irradiation on *in vitro* pollen germination and fertility in soybean. The study revealed that at the 400 Gy and 500 Gy doses most of the pollen grains, though viable did not germinate. The pollen morphology and pollen tubes were also found affected at higher doses of 300, 400 and 500 Gy.

Naess *et al*. (1998) reported ploidy reduction in tetraploid blackberry through pollen irradiation. Ploidy reduction was obtained following pollinations with 100 and 150 kR gamma irradiated pollen. Most of the seedlings obtained were aneuploid. Pollen irradiation at 150 kR was most efficient method of obtaining dihaploids from tetraploid blackberries. Twenty percentage of the seedlings obtained following this treatment were dihaploids.

Faris *et al*. (1999) reported haploid induction in cucumber through irradiated pollen. It was reported that 100 Gy stimulated the development of higher number of haploid embryos. Induction of gynogenesis through irradiated pollen in cucumber was also reported by Nikolova *et al*. (2001). The optimal doses for haploid embryo induction were reported to be 0.3 and 0.5 kGy. The growth of the haploid plants was slow and the flowers were smaller in size with strongly cut petals. The haploid plants were sterile in nature. In squash, different shapes and stages of embryos were obtained after pollination with irradiated pollen (Kurtar *et al*., 2002). All the embryos with point shape, globular shape, arrow tips and stick shape developed into haploid plants whereas cotyledon and amorphous shaped embryos produced only diploid plantlets. The highest embryo number was observed in between May and June with gamma ray dose of 25 Gy.

Kitamura *et al*. (2003) analyzed the chromosome constitution of the interspecific hybrids between *Nicotiana gossei* Domin and *N. tabacum* L., wherein the pollen of male parent was exposed to helium ion beans or gamma rays. They detected chromosomal changes in structure or constitution in the hybrids.

Wide Hybridization:

Wide hybridization method of haploid production, through the elimination of all the chromosomes of the pollinating parent of a wide cross, is sometimes referred to as the bulbosum method as it came into prominence with the recovery of haploid *Hordeum vulgare* in a cross with *Hordeum bulbosum* as the pollinating parent (Kasha and Kao, 1970). After fertilization, there is usually endosperm failure and the embryo must be rescued and cultured *in vitro*. Doubled haploids are recovered by treating either the embryo or the plantlet with colchicine. This approach is now widely used in cereals. Wheat and maize crosses yielded wheat haploids with high efficiency (Laurie and Bennett, 1986; Kisana *et al*., 1993; Mujeeb-Kazi and Riera-Lizarazu, 1996; Khush and Virmani 1996). The advantages of this method are genotype independence, drastic reduction in albinism and absence of gametoclonal variation. Doubled haploid lines produced by this method compared favorably with those produced by anther culture and by single seed descent (Guzy-Wrobelska and Szarejko, 2003). However, instances of reduced fertility and pollinating parent chromosome retention have been reported (Riera-Lizarazu *et al*. 1996).

Doubled haploids in crop improvement:

Sari and Yetisir (2002) studied the agronomical characteristics of the doubled haploids produced by irradiated pollen in comparison to the parental diploids in melon. Doubled haploid lines showed similar results to that of the original diploid lines. Great variations were observed in respect of fruit weight and fruit length. It was concluded that homozygosity did not cause any adverse effect on plant vigour, yield or quality in melons. Drumeva *et al*. (2005) conducted studies on the doubled haploid origin of sunflower lines developed through gamma irradiation induced parthenogenesis. Two co-dominant loci were characterized in the progeny of the analyzed diploid plants. In both investigated loci, the allele specific for the pollen source was not observed in the analyzed lines, which is evident that the pollen did not participate with its own genetic material in the formation and development process of these plants.

Chase (1949) exploited a spontaneous parthenogenesis system to produce the first maize doubled haploid inbreds. Selected doubled haploids were used to produce commercial hybrids (Chase, 1974). Doubled haploid cultivars are now a feature in many crop species. The doubled haploids are used prominently as parents for F_1 hybrid seed production.

It is estimated that for barley (*H. vulgare*), 50% of contemporary cultivars in Europe are produced *via* a doubled haploid system. There is currently much interest in expanding doubled haploids for F_1 hybrid production to high value crops. Doubled haploids for F_1 production has the potential to make significant advances in providing high, stable and predictive yields (Forster *et al*., 2007). An exciting development has

been the production of fertile doubled haploid lines in species such as rye (*S. cereale*) (Immonen and Antilla, 1996) and forage grasses, Festuca and Lolium (Nitzche, 1970) that suffer from inbreeding depression. In out-pollinating species, genetic analysis was simplified by using at least one doubled haploid parent in the initial cross to produce a segregating population. This has been successful in vegetables such as the *Brassica oleracea* complex, namely cabbage, cauliflower, broccoli and brussels sprouts (Sebastian *et al*., 2000). Therefore, homozygous doubled haploids provide new opportunities for genetic studies and plant breeding in these species (Forster *et al*., 2007).

Molecular characterization of doubled haploids and diversity analysis of genotypes by molecular markers:

Molecular tools hold a promising way of allowing the identification of genes involved in a number of traits including polymorphism. In recent years, DNA-based markers allowing direct comparison of the genetic material of two individual plants have been used extensively (Bhattacharya *et al*., 2005). Molecular techniques like polymerase chain reaction (PCR) have been used extensively to amplify genomic DNA to study the phylogeny and evolution of species and to characterize germplasm to select desirable genotypes for breeding and other utilization purposes, due to their superiority over the conventional methods (Tosti and Negri, 2002).

Allozyme markers have been used to unambiguously identify species and hybrids providing many advantages over morphological methods (Adams 1983; Ayala 1983; Rajora 1990). However, allozyme studies are limited by the number of enzymes and loci

that can be resolved, and reveal only genetic changes in coding regions of the genome that have resulted in changed amino-acid sequences.

Molecular markers are based on naturally occurring polymorphisms in DNA sequences (*i.e.* base pair deletions, substitutions, additions or patterns). Molecular markers are superior to other forms because they are relatively simple to detect, abundant throughout the genome even in highly bred cultivars, completely independent of environmental conditions and can be detected at virtually any stage of plant development Molecular markers must be polymorphic, co-dominantly inherited, randomly and frequently distributed in the genome, easy and cheap to detect and reproducible. Molecular markers offer a powerful supplement to the morphological data and allow the identification of variation at the genomic level (Mondal and Chand, 2002). The PCR-based RAPD technique (Williams *et al*., 1990) is a simple and effective tool that has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements). They permit detection of genetic mutation induced by mutagens as well as those induced during *in vitro* culture (Sanchez-Teyar *et al*. 2003; Khawale *et al*. 2007). RAPD is a semi-quantitative method which has been used in genetic mapping, taxonomy and phylogeny, pedigrees, construction of genetic maps, identification of cultivars and detection of somaclonal variation (Atienzar and Jha, 2006).

Doubled haploid (DH) lines derived from cabbage were analyzed by the use of RAPD markers for their diversity and uniformity by Kamiński *et al*. (2003). Eight primers yielding informative bands were used and out of the total of 83 RAPD bands

scored, 16.9% were polymorphic between a set of 13 DH lines. Guzy-Wróbelska and Szarejko (2003) carried out multiloci RAPD and AFLP assays in wheat doubled haploid lines to compare the genetic fidelity of the maize pollinated and anther culture derived lines and simultaneously to check whether any selection pressure is exerted by the maize pollination or anther culture methods. Analysis of gametoclonal variation revealed that both wheat DH populations compared showed very high levels of genetic fidelity, as shown by the very low frequency of DNA changes leading to altered banding patterns.

Kiss *et al*. (2001) studied the morphological and RAPD analysis of poplar trees of anther origin and out of the 48 primers tested, 6 primers were able to detect polymorphism. No differences were observed between the DNA patterns of the haploid and aneuploid trees obtained from cultured anthers. Laura *et al*. (2006) established anther cultures from the elite cultivars of *Anemone coronaria*. RAPD-based DNA fingerprinting showed that all the regenerants tested differed genetically from their anther donor, confirming their androgenic origin.

Molecular investigations on the doubled haploid origin of sunflower lines developed through gamma irradiation induced parthenogenesis were carried out by Drumeva *et al*. (2005). Two co-dominant loci were characterized in the progeny of the analyzed diploid plants. In both investigated loci, the allele specific for the pollen source was not observed in the analyzed lines, which shows that the pollen did not participate with its own genetic material in the formation and development processes of these plants. Kiss *et al*. (2001) studied the morphological and RAPD analysis of poplar trees of anther origin and out of the 48 primers tested, 6 primers were able to detect polymorphism. No

differences were observed between the DNA patterns of the haploid and aneuploid trees obtained from cultured anthers. Laura *et al*. (2006) established anther culture from the elite cultivars of *Anemone coronaria*. RAPD-based DNA fingerprinting showed that all the regenerants tested differed genetically from their anther donor, confirming their androgenenic origin. The banding patterns detected by RAPD markers permitted the identification of multiple microspore origins inside each anther. In mulberry, RAPD markers have been used for assessing the genetic diversity and relationships among different species including those differing in ploidy level (Vijayan *et al*., 2004; Awasthi *et al*., 2004).

Studies on molecular diversity of mulberry genotypes:

The haploid origin of the plants produced either from anther, unpollinated ovary cultures or pollen irradiation methods can be determined using molecular markers. Several attempts have been made to assess the genetic diversity amongst the different species of *Morus* through molecular markers. Bhattacharya *et al*. (2005) assessed the molecular variation amongst nine varieties of mulberry using RAPD and DAMD (Directed amplification of minisatellite DNA) profiles. The varieties were analyzed using 23 arbitrary sequence decamer primers for RAPD and 3 minisatellite core sequence primers for DAMD reactions. The results showed that the triploid varieties were found to be most similar to each other using RAPD analysis, while the varieties S13 and S34 were more similar using DAMD analysis. Nearly 85% of the RAPD bands and 91% of the DAMD bands were polymorphic across the nine varieties.

Awasthi *et al*. (2004) also analyzed the genetic diversity and relationships in mulberry using RAPD and ISSR markers. RAPD analysis using 19 random primers generated 128 discrete markers ranging from 500–3000 bp in size. One-hundred-nineteen of these were polymorphic (92%), with an average of 6.26 markers per primer. Chatterjee *et al*. (2004) reported morphological and molecular variation of *Morus laevigata* in India. Amplification on genomic DNA of 29 accessions and 13 selected RAPD primers generated a total of 71 prominent fragments, resulting 139 from 5.4 markers per primer and also revealed a very high degree of polymorphism (94%). Analyses revealed high degree of genotypic similarity of collection from Himalayan foothill (West Bengal) with those from Andaman Islands. Burgess *et al*. (2005) estimated the magnitude and direction of hybridization between red mulberry (*Morus rubra* L.), an endangered species in Canada, and the introduced and more abundant white mulberry (*Morus alba* L.) using nuclear (randomly amplified polymorphic DNA) and cytoplasmic (chloroplast DNA sequence) markers. The parental and hybrid taxa differed with respect to all of the morphological traits. Sixty-seven percent of all hybrids contained more nuclear markers from *M. alba* than *M.rubra* (hybrid index = 0.46); among populations, the degree of *M. alba* bias was correlated with the frequency of *M. alba.* In addition, the majority of hybrids (68%) contained the chloroplast genome of white mulberry.

Vijayan (2003) elucidated the genetic relationships among 18 mulberry genotypes collected from India and Japan using 15 Inter simple sequence repeat (ISSR) and 15 Random amplified polymorphic DNA (RAPD) primers. The ISSR primers generated 81.13% polymorphism while the RAPDs generated 71.78% polymorphism. From the

study, it was concluded that though morphologically Japanese and Indian mulberry genotypes show little divergence, genetic analysis using DNA markers could unravel significant genetic variation between these two groups. Vijayan *et al*. (2004) used 17 random amplified polymorphic DNA (RAPD) and 11 inter-simple sequence repeat (ISSR) primers for determining the genetic relationships among high yielding mulberry varieties. The RAPD and ISSR primers revealed more than 75% polymorphism among the varieties. The dendrogram constructed from these markers grouped the varieties into three major groups comprising the low yielding, medium yielding and high yielding. Srivastava *et al*. (2004) evaluated eleven genotypes of *M. alba*, collected from Japan, India, Philippines and Italy for their genetic relationships. DNA polymorphism of 60.75 and 74.13% could be detected among the genotypes using 10 RAPD and 10 ISSR primers. UPGMA analysis grouped the genotypes into 2 distinct clusters. The first cluster comprised genotypes from China and India, while the second comprised genotypes from Japan, Italy and a single genotype from the Philippines.

Single primer amplification reaction methods revealed that exotic and indigenous mulberry varieties are similarly diverse (Bhattacharya *et al*., 2005). The genetic diversity among 27 mulberry varieties were carried out using four minisatellite core sequence primers for directed amplification of minisatellite DNA (DAMD), three SSR motifs as primers for ISSR and 20 arbitrary RAPD primers. All the three methods revealed wide range of distances supporting a wide range of mulberry genetic diversity. The result indicated that exotic varieties of mulberry were found to have slightly greater diversity than the indigenous ones.

The first genetic linkage map of mulberry was constructed with 50 F_1 full-sib progeny by Venkateswarlu *et al*. (2006) using RAPD (100 primers), ISSR (42 primers), SSR markers and pseudotestcross mapping strategy. The primers amplified 517 markers out of which 188 showed test-cross configuration, corresponding to the heterozygous condition in one parent and null in the other.

AFLP technique (Vos *et al*., 1995) allows the identification of a greater number of polymorphisms than RFLP or RAPD analysis and is relatively easy to perform, is highly reproducible, and uses small amounts of DNA. The AFLP technique detects restriction fragments by PCR amplification and its versatility arises from the fact that, as RAPD, it does not require prior knowledge of sequence. A virtually unlimited number of restriction fragments can be detected in complex genomes. There are only two reports on AFLP analysis of mulberry. Sharma *et al*. (2000) estimated the genetic diversity in 45 mulberry accessions from different eco-geographic regions of Japan and other parts of the world. They observed a high degree of polymorphism using five primer combinations that ranged from 69.7 to 82.3% across all the genotypes studied. The results obtained by AFLP analysis of mulberry accessions supported to a large extent the genetic relatedness established among them by conventional methods. Botton *et al*. (2005) using AFLP-based fingerprints as a tool for estimating genetic variability within as well as among three different mulberry species (*M. alba* L., *M. latifolia* Poir and *M. bombycis* Koidz). A high level of polymorphism (72.2%) was found over all the 48 accessions analyzed. Although there are many reports on characterization of genetic diversity in mulberry species, no information is available on utilization of molecular markers for characterization of plants generated using androgenesis, gynogenesis and pollen

irradiated methods. The primers used by earlier investigators for RAPD analysis provided a basis for characterization of the variants generated using pollen irradiated approach in the present study.

MATERIALS & METHODS

1. Anther and unpollinated ovary cultures:

1.1 Plant material:

Economically important cultivars of *Morus indica* L. v*iz*., M-5, S-36, S-13 and V-1 were used for studies on anther and unpollinated ovary cultures. The important attributes of these cultivars are given in the Table 1.

Saplings of the above cultivars were established in Plant Culture Facility of University of Hyderabad. Originally, these cultivars were procured from Department of Sericulture, P_2, L. R. Seed Farm, Kammadanam, Mahboobnagar, Andhra Pradesh.

1.2 Source of anthers and unpollinated ovary cultures:

The cultivars, S-13 and V-1 were used for studies on androgenesis whereas M-5 and S-36 were used for gynogenesis. Anthers and unpollinated ovaries were obtained from catkins collected from the plants established in the field during flowering season (January-March, 2003) and from catkins that were induced throughout the year by pruning the plants or *in vitro* sprouted axillary buds. The catkins were surface sterilized with 70% alcohol for 2 min followed by 0.1% bavistin for 3 min and rinsed 4 times with sterile double distilled water. This was followed by treatment with 0.1% $HgCl_2$ for 10 min and rinsed 4 times with sterile distilled water. Cold treatment was given to these catkins by placing on MS (Murashige and Skoog, 1962) basal medium and storing them for 1-7 days at 4^oC.

1.2.1 Sprouting of axillary buds *in vitro:*

Nodal explants of 3-4 cm were collected from field grown plants and washed thoroughly under running tap water for 30 min and then treated with 0.1% bavistin for 10 min under sterile conditions. The explants were then surface sterilized in 70% alcohol for 1 min followed by 0.1% $HgCl_2$ for 15 min and finally rinsed 4-5 times in sterile distilled water with duration of 5 min each. The sterilized nodal explants were cultured on MS medium supplemented with 0.3 mg/l 2,4-D for inducing axillary bud sprouting. The cultures were maintained at 25 ± 2^{o}C under a photosynthetic photon flux density of 83.6 $\mu Em^{-2}s^{-1}$ provided by white fluorescent tubes. Male catkins induced from the axillary buds of S-13 and V-1 cultivars and female catkins induced from the axillary buds of M-5 and S-36 cultivars were used as source of anthers and unpollinated ovaries, respectively. The effect of cold treatment on induction of androgenesis was tested by placing the cultures of sprouted axillary buds for varying durations (1-7 days) at 4^{o}C.

1.3 Anther culture:

The staminate flowers of S-13 and V-1 cultivars were collected from the catkins when most of the microspores were at uninucleate stage. The stage of microspore development in the anthers was ascertained by squashing the anthers in 2% acetocarmine. Pollen viability of staminate flowers of *in vitro* induced catkins and those obtained from field grown plants was evaluated using aceto-carmine method and expressed as the proportion of pollen grains that stained red. Anthers were removed aseptically and cultured on MS, N6 or B5 media supplemented with 2,4-D, IAA, NAA, BAP and TDZ in different combinations and concentrations for induction of androgenesis. The effect of biotin, L-ascorbic acid, casein hydrolysate, L-glutamine, silver nitrate, maltose and

fructose on androgenesis was tested by addition in MS medium. In another experiment, the staminate flowers were cultured on the medium and the anthers were separated and cultured on fresh medium. Anthers (8-10) were inoculated in glass test tubes (25 x 150 mm) containing 15 ml of media whereas 20-25 anthers were placed in sterile disposable petriplates containing 25 ml of medium. The response from the anthers was monitored periodically under stereomicroscope.

1.4 Unpollinated ovary culture:

A two-step protocol was used for culturing the unpollinated ovaries of M-5 and S-36 cultivars. In the first step, individual pistillate flowers were separated from the catkins and cultured on MS medium containing 2,4-D, BAP and NAA in different combinations. After few days of enlargement, the unpollinated ovaries were isolated and cultured on the same medium or on a medium containing different growth regulators and the response from the cultured ovaries was recorded at regular intervals.

1.4.1 Anatomical studies of ovules cultured *in vitro*:

The ovules were taken either at the culture initiation stage or after 30 days of culture on medium containing 2 mg/l 2,4-D, 0.5 mg/l BAP and 3% sucrose and fixed in absolute ethanol and acetic acid (3:1) for 24 hrs and subsequently transferred to 70% ethanol. They were then dehydrated with successive changes of ethanol (70-100%), then with butanol and embedded in paraffin. Paraffin was then removed from serial sections (10-20 μm in thickness) after drying at 37^{o}C for a week by giving two changes of xylene with 10 min each, mixture of ethanol and xylene (1:1) for 10 min, two changes of 100 % ethanol with 10 min each. The sections were stained in hemotoxylin and eosin, and viewed and photographed with Nikon microscope.

2. Studies on Pollen Irradiation:

2.1 Plant Material:

M-5 cultivar (*Morus indica* L.) was used as a female parent whereas S-13 cultivar (*M. indica* L.) was used as pollen parent. Nodal explants having the male catkins were collected at anthesis stage and irradiated with gamma rays.

2.2 Pollen irradiation:

The nodal explants along with the male catkins were collected from the field-grown plants of S-13 cultivar and placed in tissue culture bottles containing 20 ml of sterile water to prevent the staminate flowers from drying. Gamma irradiation was carried out by exposing the male catkins to cobalt 60 source at Acharya N.G. Ranga Agricultural University. The pollination experiments were carried out in the year 2004 and 2005. For the experiments conducted in the year 2004, the pollen of S-13 cultivar (*Morus indica* L.) was irradiated at different doses of 50, 80, 100, 200 and 500 Gy and used to pollinate the clones of M-5 cultivar. In addition to the doses mentioned above, the pollen of S-13 cultivar was irradiated at higher doses of 1000, 2000 and 3000 Gy and used for pollination experiments in 2005. The pollen was isolated from the catkins and used for pollinating the pistillate flowers of M-5 cultivar. Pollinations carried out with non-irradiated pollen were treated as controls.

2.3 Pollination procedure:

The crosses were made during normal flowering season *i.e.* January-March in 2004 and 2005. The shoots with female catkins of same size were enclosed in pollination

bags at least one week prior to their receptive period and, when the pistillate flowers were fully receptive as evidenced by their white color they were pollinated separately with control and irradiated pollen of S-13 cultivar. The pistillate flowers were hand pollinated by brushing the receptive stigma with irradiated as well as control pollen separately. After pollination, the flowers were bagged again to avoid any alien pollination. A few of the female catkins were kept bagged throughout the experiment to record any event of spontaneous parthenogenesis. For the crosses made in 2004, 15 female catkins were pollinated with control or irradiated pollen. For the experiments conducted in 2005, 30 female catkins were pollinated with control or irradiated pollen. The fruits formed after pollination with control and irradiated pollen were collected after 20-25 days of pollination and the seeds were separated and stored at 4^{o}C until use.

2.4 Pollen viability:

The effect of gamma irradiation on pollen viability S-13 cultivar was determined by staining them with FDA and PI stains using standard protocol (Oparka and Read, 1994). Three replicates of 100 pollen grains were scored for viability along with controls. Pollen was isolated in 1 ml of 20 mM phosphate buffer saline (PBS) of pH 7.5 from the anthers just after irradiation and kept in the stain for 15 min in dark. After the incubation, the pollen was centrifuged at 1000 rpm and the pellet was redissolved in phosphate buffer saline (PBS) and was observed under fluorescence microscope.

2.5 Pollen germination *in vitro*:

The effect of different media on *in vitro* pollen germination was tested. The *in vitro* germination response of irradiated as well as control pollen was tested by culturing them on four different media. The pollen was incubated in 1 ml of germination medium at different temperatures in the dark and germination percentage was recorded after 48 hrs under microscope. The medium that gave the best results was then used to study the effect of gamma irradiation on *in vitro* pollen germination. To follow the sperm nucleus movement into the pollen tube and to determine any abnormality in its formation after irradiation with gamma rays, the pollen was stained with DAPI for 1 hr in dark and then observed under fluorescence microscope.

2.6 Pollen germination *in situ*:

The *in situ* germination capacity of the untreated (control) and irradiated pollen was carried out to compare the differences in germination pattern of control and irradiated pollen. The pistils were fixed in ethanol and acetic acid in 3:1 (v/v) ratio for 24 hrs, after 24 hrs of pollination. The pistils were washed in 0.1M K_3PO_4 and subjected to hydrolysis in 2N NaOH for 1 hr; again washed in 0.1M K_3PO_4 and stained in 0.1% aniline blue. Samples were viewed under fluorescence microscope after overnight staining. Five pistil squashes were analyzed per treatment for recording the observations on pollen germination.

2.7 Seed germination:

The fruits were collected from the plants after 20-25 days of pollination. The seeds extracted from the fruits were collected and stored at 4^{o}C until further use. The seeds obtained from control and irradiated pollen experiments were surface sterilized in 70% ethanol for 3 min followed by 0.4% bavistin for 15 min and then in 0.1% $HgCl_2$ for 5 min and rinsed 5-6 times with sterile distilled water. The germination response of the seeds was evaluated by culturing them in Woody Plant Medium (Lloyd and McCown, 1980) supplemented with 3 mg/l GA_3. The frequency of germination, shoot length (cm) and root length (cm) was recorded after 4 weeks of culture.

2.8 Shoot multiplication:

The seedlings obtained from crosses of M-5 with irradiated pollen of S-13 cultivar in 2004 were multiplied *in vitro* by placing the shoot tips of 2-3 cm on MS medium supplemented with 0.5 mg/l BAP. The shoots subcultured for two times on shoot multiplication medium were established in soil. The effect of gamma irradiation on shoot multiplication was determined after 5 subcultures at 30-day intervals. The average number of shoots induced per explant as well as the length of shoots was determined in plants obtained after pollination with control and irradiated pollen.

2.9 Root induction:

The shoots (3-4 cm) obtained from *in vitro* propagated plants or seedlings were cultured on MS medium supplemented with 0.1 mg/l IBA for root induction. The data on the frequency of root induction, number of roots and length of roots was recorded along

with controls. The *in vitro* raised seedlings were placed on MS medium with 0.1 mg/l IBA for one month for shoot and root development before transfer to soil.

All the cultures were maintained at 25 $\pm$ 2^{o}C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 µE m^{-2} s^{-1} provided by white fluorescent tubes. The data was analyzed by one-way ANOVA followed by Student-Newman-Keul's method for multiple comparisons.

2.10 Acclimatization of plants:

The *in vitro* raised plants of irradiated pollen experiments along with controls and parents were transferred to plastic pots containing red soil, sand, vermiculite and organic manure (3:1:1:1 v/v) and kept in the greenhouse (26$\pm$ 2^{o}C) for 15-20 days. The humidity was maintained by covering with polythene covers. The plants were transferred to earthen pots containing soil and organic manure (3:1) and kept in the greenhouse for 2 months and then transferred to field.

2.11 Interspecific hybridization with control and irradiated pollen:

China White cultivar of *Morus alba* L. was used as pollen parent. China White is valued for its broad leaf, dark green foliage and is suitable for cultivation in temperate regions. It is a monoecious plant and has low rooting ability. It has been introduced into India from China. The pollen was irradiated with gamma rays at different doses of 50, 80, 100, 500, 1000 and 2000 Gy and used for pollination. Untreated pollen was used as a control. The female flowers of M-5 cultivar were pollinated separately with control and gamma irradiated pollen. The fruits were collected after 20-25 days of pollination. The

seeds were isolated from the fruits and surface sterilized and thereafter placed on Woody Plant Medium with 3 mg/l GA_3 as described above (Section 2.7).

2.12 Cytological studies:

The root tips obtained from M_1 plants were fixed between 10.30-11.00 AM and pretreated in 8-hydroxyquinoline (Sigma make) for 3.5 hrs at 4^0C and transferred to ethanol: acetic acid (3:1 v/v) for 24 hrs at room temperature. Subsequently, they were transferred to 70% ethanol. The root tips are washed in double distilled water and hydrolyzed in 1N HCl for 5 min and stained with 2% aceto-orcein for 1 hr. The root tip squashes were prepared in 2.0% aceto-orcein and observed under light microscope (Olympus make).

2.13 Morphological analysis of M_1 plants obtained after pollination with irradiated pollen

The *in vitro* propagated plants or the seedlings obtained after pollination with control and irradiated pollen along with parents (M-5 and China White cultivars) were transferred to soil in the field. In case of S-13 cultivar which is a male plant, the nodal explants with axillary buds were cultured on MS medium with 0.3 mg/l 2,4-D for inducing axillary bud sprouting. The shoots that differentiated from the axillary buds were placed on MS medium with 0.1 mg/l IBA for root induction and then established in soil. The observations on height of the plant, leaf length and width, color, shape, margin, thickness of the main stem and internodal distance was recorded in four-month-old plants. The data on parental plants and control hybrids was an average of ten plants.

2.14 Stomatal length:

Stomatal length was determined in M_1 plants obtained after pollination with irradiated pollen along with control plants. The lower epidermis was peeled off and observed under light microscope. The leaf samples were gold coated and then observations were recorded in scanning electron microscope (SEM).

2.15 Chloroplast number per stomata:

Thin leaf epidermal peals were taken from control and M_1 plants and the number of chloroplasts per stomata was determined under a light microscope.

2.16 Flow Cytometric analysis:

The ploidy level of M_1 plants obtained after pollination with irradiated pollen was evaluated using Flow Cytometer for identification of haploids. The leaves (third from shoot apex) were taken and a small amount of leaf tissue (approx. cm^2) was chopped with a sharp razor blade in ice-cold nuclei extraction buffer (Solution A of the High Resolution Kit for Plant DNA, Partec, Munster, Germany). The ploidy level of seeds obtained from bisexual and female florets of mixed catkins of Plant 40 was determined along with M-5 seed. Single seeds from bisexual and female florets of Plant 40 and M-5 cultivar were taken and chopped with a sharp razor blade in ice-cold extraction buffer. The suspension prepared from the leaf tissues or seeds were filtered through a 50 μm nylon sieve, and stained using the solution B of the High Resolution Kit for plant DNA, Partec, Munster, Germany. Analyses were performed with a PAS Flow Cytometer (Partec). A minimum of 10,000 particles were analyzed for each sample. To determine the standard peak position of 2C cells, the 2C peak from nuclei isolated from control

plants was adjusted to the channel 50-position. Three replications were done using the samples taken from different regions of the same plant and the DNA measurements were made twice for each sample.

2.17 Protein Extraction:

Protein was extracted from the leaf samples of four-month-old M_1 plants obtained after pollination with irradiated pollen along with M-5, S-13 and control hybrid. The leaves (third from the shoot apex) were used to extract crude protein. The leaf samples were weighed 100 mg each and ground in a pre-chilled motor and pestle in 1ml of extraction buffer consisting of 50 mM Tris HCl buffer (pH 7.5) with 5 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 5 mM DTT, 2 mM KH_2PO_4, 2% PVP, 20% glycerol, 10 mM NaF, 10 mM β-mercaptoethanol and 2 mM PMSF. After homogenization, the samples were centrifuged at 4ºC for 20 min at 12,000 rpm. The supernatant was taken and soluble protein content was estimated by Lowry's (1951) method with minor modifications.

2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE):

The protein profiles of M_1 plants along with M-5, S-13 and control hybrids were analyzed using one-dimensional gel electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) with minor modifications. The separation of proteins was performed in 5% stacking gel and 12% resolving gel. Equal amount of protein (25 µg) was loaded for each sample. The experiment was repeated thrice at three different times. The medium range (97.4-14.3 kDa) protein molecular weight marker was used as standard protein and was procured from Bangalore Genei.

2.19 Silver staining:

The proteins were detected in the gel by silver staining procedure according to Blum *et al.* (1987). The gels were fixed for 1 hr in a fixative containing 50% methanol, 7.5% acetic acid and 0.5 ml of 37% formaldehyde per litre. The gels were washed with 50% ethanol for 3 times at 20 min interval. The gels were pre-treated with 0.002% sodium thio-sulphate solution for exactly 1 min and rinsed three times at 20 sec interval with distilled water. The pre-treated gels were impregnated for 20 min in 0.2% silver nitrate solution containing 0.02775% formaldehyde. The gels were rinsed two times with distilled water and developed for the proteins with the solution containing 6% sodium carbonate, 0.0185% formaldehyde and 0.0004% sodium thio-sulphate. The gels were placed in fixative solution for 10 min for the development of protein spots in appropriate intensity and subsequently stored in 50% methanol at 4^0C.

3. Molecular characterization of M_1 plants by PCR based markers:

The M_1 plants obtained after pollination with irradiated pollen were characterized at the molecular level along with M-5, S-13 cultivars and control hybrid using RAPD and AFLP markers. The procedure for DNA isolation, RAPD and AFLP methods is described below.

3.1 DNA Isolation:

Leaves were collected from the morphological variants along with M-5, S-13 cultivars and control hybrid (obtained after crossing M-5 cultivar with untreated pollen of S-13 cultivar) established in the field. Genomic DNA was isolated from leaf tissues using mini-prep method. About 300 mg of leaf tissue was quick frozen in liquid nitrogen

and ground to fine powder using micro pestle in 1.5 ml eppendorf. The fine powder was thoroughly extracted with 700 µl of extraction buffer (75 mM Tris [pH 8.0], 10 mm EDTA [pH 8.0], 1M NaCl, 1% SDS, 0.5% PVP and 10 mM β-mercaptoethanol and incubated at 65˚C for 30 min. The solution was kept at room temperature for 15 min and to this solution 300 µl of 5M potassium acetate solution was added, mixed gently and was kept on ice for 20-30 min. The solution was then centrifuged at 10,000 rpm for 10 min at 4˚C. The supernatant was transferred to a fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed gently and was centrifuged at 10,000 rpm for 10 min at 4˚C. The supernatant was removed carefully without disturbing the inter-phase and to the supernatant 600 µl of ice cold isopropanol was added and incubated for 20-30 min at -20˚C and then centrifuged at 11,000 rpm at room temperature for 15 min. The pellet was then washed with 70% ethanol; air-dried and was dissolved in 250 µl of water. The DNA was quantified using nano drop method.

3.2 RAPD analysis:

PCR amplifications of the genomic DNA with RAPD primers were conducted according to Williams *et al.* (1990) on Thermal Cycler using 20 µl of reaction mixture containing 1X PCR buffer; 0.2 mM deoxyribonucleotide triphosphates (dNTPs); 2 mM MgCl2, 0.25 µm primer; 1.0 unit of Taq polymerase and 25 ng genomic DNA. DNA was amplified in a thermal cycler that was programmed as follows: after preheating for 3 min at 94˚C for 1 min, 37˚C for 1 min, 72˚C for 2 min and a final extension at 72˚C for 7 min that was followed by cooling to 4^{o}C. The PCR products were separated on a 1.2% agarose gel. The primers used for detecting polymorphism in M_1 plants are given in the Table 2. Three replications were done for each primer. The amplification data obtained

from 21 morphological variants for different RAPD markers were scored as 1 (presence) and 0 (absent). The 0-1 matrix thus obtained was used to calculate the genetic similarities among the variants using Jaccard's similarity co-efficient. Based on similarity co-efficients, an UPGMA dendrogram was constructed using numerical package (Rohlf, 2002).

3.2.1 Interspecific hybridization with control and irradiated pollen:

The M_1 interspecific hybrids obtained after crosses of M-5 cultivar with control and irradiated pollen of China White cultivar were characterized using RAPD method. The procedure for isolation of genomic DNA and RAPD is same as described above (Section 3.1 and 3.2). The primers used for detecting polymorphism in M_1 interspecific hybrids are given in the Table 3.

3.3 AFLP analysis:

For AFLP analysis, DNA was extracted following Qiagen Extraction Kit. Genomic DNA was isolated from 8 leaf samples *viz*., M-5, S-13 and China White cultivars, control hybrid obtained after crossing M-5 cultivar with untreated pollen of S-13 cultivar, and four M_1 plants obtained after crossing M-5 cultivar with irradiated pollen of S-13 cultivar. A modified AFLP (Vos *et al*. 1995) procedure was used to assess the genetic variability in M_1 plants. The AFLP procedure was carried out in 3 steps: 1) DNA template preparation 2) DNA template preamplification 3) AFLP selective amplification.

3.3.1 DNA template preparation: Restriction digestion was completed using 6 µl of quantified genomic DNA (100 ng) that was incubated with *ECo*RI and *MSe*I restriction endonucleases for 1 hr at 37^{o}C in solution containing 6.0 µl 5X R/L buffer, 0.6 µl of

*ECo*RI (12U @ 20U/µl), 2.0 µl of 8 U/µl *MSe*I enzyme and autoclaved nanopure H_20. Five µl of ligation mixture containing 0.5 µl of 5X T4 DNA ligase, 0.5 µl of 5 pmoles/µl *ECo*RI adapter, 0.5 µl of 50 pmoles/µl *MSe*I adapter, 0.5 µl ATP (10 mM, pH 8.0) and 2.0 µl autoclaved nanopure water was added to the restriction digestion product and was incubated overnight at 37^{o}C using Thermocycler; the ligation mixture was diluted each in 1: 10 with autoclaved nanopure water.

3.3.2 Pre-amplification of DNA template: A master mix of pre-amp primer mix containing 3.0 µl of 10X PCR buffer, 2.4 µl dNTP mixture (2.5 mM), 1.0 µl of *Eco*R1 adapted primer (@50 ng/µl), 1.0 µl of *Mse*1 adapted primer (@50 ng/µl), 0.4 µl of Taq polymerase (@ 5U/µl), 19.2 µl of autoclaved nanopure water were added to each reaction tube containing diluted ligation product and amplified using 28 PCR cycles of 15 sec at 94^{o}C, 30 sec at 60^{o}C, 60 sec + 1 sec/cycle at 72^{o}C, 1 cycle of 2 min at 72^{o}C final extension and hold at 4^{o}C. The oligonucleotide primers in the pre-amp primer mix are complementary to the adapter/restriction site with *Mse*1 primer and *Eco*R1 primers containing one selective nucleotide each. Each preamplification product was diluted with nanopure water.

3.3.3 AFLP selective amplification: Eight primer pair combinations *viz.*, EAAC/MCAA, EACC/MCTC, EAAC/MCAG, EACC/MCTT, EACG/MCAA, EACA/MCTC, EACG/MCAG and EACA/MCTT were used in the study. A selective amplification master mix was made using 2 µl of diluted preamplification product, 9.73 µl of autoclaved nanopure water, 2.0 µl of 10X PCR buffer, 1.6 µl of dNTP mixture (2.5 mM), 0.83 µl IRD-labeled *ECo*R1-primer (@ 6 ng/µl), 0.6 µl of *Mse*1 primer (@50 ng/ µl) and 0.24 µl of Taq polymerase (@ 5U/µl).

The "Touchdown" PCR program: 1 cycle of 10 sec at 94°C, 30 sec at 65°C, and 60 sec at 72°C, 12 cycles of 10 sec at 94°C, 30 sec at 65°C (lowering the annealing temperature by 0.7°C) and 60 sec at 72°C, 25 cycles of 10 sec at 94°C, 30 sec at 56°C, 60 sec at 72°C, plus 1 sec. per cycle, 1 cycle of 2 min at 72°C final extension and then hold at 4°C was used for selective amplification. Reactions were stopped prior to loading the selective amplification product on polyacrylamide gels by adding 2.5 µl stop solution (LI-COR) to each reaction tube, then holding at 95°C for 3 min, then cooling to 4°C prior to loading the PAGE gel. Electrophoresis through KB^{Plus} 6.5% ready-to-use gel matrix (LI-COR) was used to separate the DNA and the bands were detected by a LI-COR Gene Read IR 4200 sequencer.

AFLP bands were evaluated using an IRD-700 labeled 50-700 bp marker as a reference and scored manually for their presence (1) or absence (0) in the plants analyzed. A binary data matrix was used to estimate genetic similarity using the Jaccard index through the SIMQUAC procedure using NTSYSPC (Rohlf, 2000). Dendrograms were constructed to illustrate genetic similarity, following the methodology described by Sneath and Sohal (1973). Two-dimensional scatter plot or principal coordinate analysis of the plants was performed according to Kruskal *et al.* (1965). Bootstrap analysis was used as a way of testing the reliability of the dataset using BOOD-P software ver 3.1 (Coelho, 2001).

RESULTS

In the present study, an attempt has been made to produce haploids through *in vitro* androgenesis and gynogenesis. The results on the factors affecting callus formation from anther and unpollinated ovary cultures are presented. As the efforts to produce haploids through anther and unpollinated ovary cultures were not successful, the technique of pollen irradiated approach has been used for the first time in mulberry to produce haploids through *in situ* parthenogenesis. The M-5 plants of mulberry were pollinated separately with control and irradiated pollen of S-13 and China White cultivars and the effect of pollen irradiation on fruit and seed formation, and seed germination was investigated. The ploidy status of M_1 plants resulting from the above crosses was determined using flow cytometry and cytological analysis. These plants were characterized using morphological and molecular analysis and the data is presented.

Axillary bud sprouting and *in vitro* induction of catkins

Nodal explants (3-4 cm) of M-5, S-36, S-13 and V-1 cultivars were placed on MS medium with 0.3 mg/l 2,4-D for inducing axillary bud sprouting. Sprouting occurred in 8-10 days with a frequency of 78.4%, 73.4%, 68.3%, 75.6% in M-5, S-36, S-13 and V-1 cultivars, respectively. Shoots as well as catkins were induced from the sprouted axillary buds of all the cultivars irrespective of the season during which the nodal explants were collected (Fig. 1, 2 & 3). The response from anthers and unpollinated ovaries obtained from the catkins induced from *in vitro* cultured axillary buds as well as from the catkins collected from field grown plants was evaluated.

Anther culture:

The staminate flowers of S-13 and V-1 cultivars collected from catkins of 0.8 -1.0 cm had high frequency anthers at uninucleate microspore stage (Fig. 4). Significant differences were not observed in pollen viability from *in vitro* and *in vivo* induced catkins (Table 4). The frequency of viable pollen was 88.2% and 90.2% in S-13 and V-1 cultivars, respectively collected from field grown plants in comparison to *in vitro* induced catkins which exhibited a pollen viability of 81.4% and 81.8% in S-13 and V-1 cultivars (Table 4). The source of anthers from which they were collected did not have any significant effect on callus formation on the media tested. Anthers isolated from the staminate flowers were cultured on MS, N6 or B5 media containing different combinations of growth regulators. The anthers of both the cultivars turned brown within 10 days of culture and no favourable response in terms of callus induction or embryogenesis was observed even at the end of 60 days of culture (Fig. 5). The second experiment was conducted by culture of staminate flowers of S-13 and V-1 cultivars on MS, N6 and B5 media containing 2,4-D, NAA, BAP and TDZ in different combinations. The staminate flowers opened and the anthers liberated out within 10-12 days of culture irrespective of the media and growth regulators used (Fig. 6 & 7). Cold pre-treatment for 4 days was found to be effective for induction of callus from the anthers of S-13 cultivar. On MS medium supplemented with 8 mg/l 2,4-D, 0.1 mg/l NAA and 6% sucrose, 6.8 % of the anthers responded as evidenced by increase in size and swelling (Table 5; Fig. 8). Hard callus developed from the anthers upon subculture to fresh medium (Fig. 9). Rhizogenesis was induced in the callus following transfer to MS medium containing 1.0 mg/l 2,4-D, 0.1 mg/l NAA and 3% sucrose with induction of 9-10 roots at the end of 30

days of culture (Table 5; Fig. 10 & 11). Anthers dehisced from the staminate flowers of S-13 cultivar cultured on MS medium supplemented with 10 mg/l 2,4-D, 1 mg/l KN and 6% sucrose, and callus developed with a frequency of 3.3% from the anthers after 60 days of culture. The callus turned brown immediately after subculture to the fresh medium. Among the different media tested, MS medium supplemented with 4 mg/l 2,4-D, 0.1 mg/l TDZ and 6% sucrose favoured callus induction with 3.8% frequency from anthers of V-1 cultivar that were given cold pretreatment for 6 days (Table 5; Fig. 12). In both the cultivars, the anthers underwent swelling initially followed by callus induction, indicating the possibility of its origin from anther wall and pollen. Addition of various compounds such as casein hydrolysate, L-glutamine, L-ascorbic acid, biotin, silver nitrate, maltose and fructose in MS medium did not evoke any response from anthers of S-13 and V-1 cultivars. Attempts to induce plant regeneration from the callus induced from anthers of S-13 and V-1 cultivars have not been successful.

Unpollinated ovary culture:

A two-step procedure was adopted for unpollinated ovary cultures of M-5 and S-36 cultivars. In the first step, individual pistillate flowers were separated from female catkins of M-5 (Fig. 13) and S-36 cultivars and cultured on MS medium with 2,4-D, BAP and sucrose (3-12%). The response from the flowers varied depending on the stage at which they were cultured and the growth regulators used. The pistillate flowers isolated from catkins of 1.0 cm long turned red and ripened within 2 weeks of culture whereas pistillate flowers of 0.5 cm long remained green and showed enlargement on medium with 2.0 mg/l 2,4-D, 0.5 mg/l BAP and 3% sucrose (Fig. 14). In order to induce callus from the unpollinated ovaries, the pistillate flowers were separated from catkins after a

period of enlargement, and individual ovaries were excised and cultured on fresh medium. Slightly friable callus was induced with a frequency of 4.5% from unpollinated ovaries of M-5 cultivar after 30 days of culture (Fig. 15) whereas no response was observed in S-36 cultivar. Green, nodular structures were induced from the callus when subcultured on medium with 2 mg/l BAP and 3% sucrose (Fig. 16).

Anatomical sections of the unpollinated ovaries of M-5 cultivar cultured for 30 days on MS medium with 2.0 mg/l 2,4-D, 0.5 mg/l BAP and 3% sucrose showed the presence of developing embryos in 6.6% of the cultures, although callus formation was not seen in these cultures (Fig. 17). Further development of embryos was not observed even when left on the same medium for 2 months.

Pollen irradiated approach for induction of haploids:

The pollen irradiation approach was employed in mulberry in order to evaluate the possibility of inducing haploids through *in situ* parthenogenesis. The pollen of S-13 cultivar was irradiated at different doses of 50, 80, 100, 200, 500, 1000 and 2000 Gy and used to pollinate the female clones of M-5 cultivar (Fig. 18). The seed obtained after pollination with control and irradiated pollen was germinated *in vitro* and the germination percentage was recorded.

Effect of gamma irradiation on pollen viability:

The viability of S-13 pollen decreased with the increase in the dose of irradiation. The viability of pollen irradiated at 50-2000 Gy ranged from 60.4-9.8% in comparison to control (non-irradiated) pollen that exhibited 96.2% viability (Table 6; Fig. 19 & 20). High doses of irradiation (3000 Gy) resulted in complete loss of viability.

The medium for *in vitro* pollen germination was optimized. The best germination response was observed on medium containing 10 mg/l EDTA, 100 mg/l H_3BO_3, 400 mg/l $Ca(NO_{3)2}$ and 10% sucrose compared to other media tested. On this medium, the pollen started germinating after 6-7 hours of incubation and the germination percentage of 59.6% was observed after 48 hrs of incubation (Table 7; Fig. 21 a-c). Irradiation of pollen affected the germination capacity and decreased (27.7-4.9%) with increase in the dose of irradiation from 50 to 1000 Gy. A very low germination percentage (4.9%) was observed at 1000 Gy irradiation dose (Table 8). The effect of gamma irradiation on sperm nucleus movement was studied after staining with DAPI for 1 hr and observing under fluorescence microscope. The sperm nuclei could be tracked along the pollen tube in controls (Fig. 22 a-d). In one of the pollen irradiated at 1000 Gy, the sperm nuclei could not be tracked in the pollen tube possibly due to the disintegration (Fig. 23 a-c).

In situ pollen germination was carried out to compare the differences, if any, between the germination pattern of control and irradiated pollen. The results showed that pollen germination capacity *in situ* was affected by irradiation. The pollen germination decreased (50.5-12.3%) with increase in the dose of irradiation (50-1000 Gy) with complete loss of germination ability at 2000 Gy whereas control pollen germinated at 81.9% frequency (Table 9; Fig.24 a-d). This result showed similarity with those observed with *in vitro* experiment. This may be the reason behind the failure of fertilization of the female catkins and eventually withering of the fruits at higher doses of irradiation.

Anatomical studies:

Anatomical studies of 10-day-old ovules following pollination of pistillate flowers with control (non-irradiated) and irradiated pollen revealed embryo development in the control as well as in the irradiated samples (50-100 Gy) whereas no embryo development was observed in the ovules of unpollinated flowers (Fig. 25). Embryo and endosperm development was observed in the ovules pollinated with control pollen (Fig. 26). The presence of two developing embryos within a single embryo sac was observed in the ovules of 50 Gy irradiated pollen indicating the possible induction through parthenogenesis (Fig. 27). Normal embryo and endosperm development was observed in one of the ovules after pollination with 80 Gy (Fig. 28). A degenerate embryo with disintegrated endosperm was observed in one of the ovules pollinated with 100 Gy (Fig. 29). From these results, it is clear that pollination with gamma irradiated pollen caused abnormalities in embryo and endosperm development at a dose of 100 Gy although at a low percentage. However, a detailed study involving more number of samples would help in assessing the developmental changes occurring following pollination with irradiated pollen.

Fruit and seed formation following fertilization with irradiated pollen:

The fruit and seed formation in M-5 cultivar following pollination with S-13 irradiated pollen was evaluated. Fruit development was not observed from the unpollinated flowers continuously bagged ruling out the chances of wind or insect pollination. Experiments conducted in the year 2004 revealed that irradiation of pollen at 50-200 Gy did not have any significant effect on fruit size and average weight of the single seed. However, fruit weight decreased (240.2 mg) with increase in the dose of

irradiation to 500 Gy in comparison to 368.0 mg observed in the control (Table 10). Pollen irradiated at 500 Gy resulted in production of parthenocarpic fruits or fruits containing only 2.4 seeds per fruit.

The experiments on pollen irradiation were conducted in the year 2005 and a large number of pollinations were carried out for all the doses (50-2000 Gy) in order to increase the chances of induction of haploids through parthenogenesis. For the treatments of 50 to 1000 Gy, no significant differences were observed with respect to fruit size, fruit weight and number of seeds per fruit in comparison to pollinations carried out with control pollen (Table 11). The average fruit size varied from 1.0-1.1 cm for different treatments and control. The fruit weight of control was 397.1 mg and it ranged from 289.7-443.0 mg for different treatments. In contrast to the observations recorded in the year 2004, seed set was observed at 1000 Gy irradiation dose. The number of seeds per fruit varied from 18.3-26.4 for different treatments in comparison to control that had 29.7 seeds per fruit (Table 11). It was observed that 15-20% of fruits obtained at higher doses (500 and 1000 Gy) did not contain any seeds or contained very few seeds compared to control. For all the doses tested, the seed weight was lower (0.4-0.6 mg) than that of control seed (0.9 mg). Probably, the differences observed with respect to seed set frequency in year 2004 and 2005, could have been due to more number of pollinations carried out in the year 2005. Pollinations with 2000 kGy resulted in withering of female catkins without any seed set, which may be due to the failure of pollen germination. These results are in accordance with *in situ* pollen germination experiments where the pollen irradiated at 2000 Gy failed to germinate when placed on stigma.

Influence of media on M-5 seed germination:

Seeds of M-5 cultivar germinated with high frequency (91.5%) on McCown woody plant medium (WPM) containing 3 mg/l GA_3 compared to MS basal medium or MS medium with 3 mg/l GA_3 (Table 12). Germination commenced within 8-10 days on GA_3 supplemented medium in contrast to 12-14 days observed on basal medium. Shoots of 3.2 cm and roots of 4.1 cm were induced after 30 days of culture on WPM medium supplemented with 3 mg/l GA_3.

Germination capacity of M_1 seeds obtained after pollination with S-13 irradiated pollen in the year 2004:

Seed germination was not affected as reflected by percent germination, shoot and root length from those derived by 50 Gy irradiated pollen (Table 13). With increase in the dose of irradiation from 80 to 200 Gy, the percentage of germination decreased. Seeds issued from pollen irradiated at 100 Gy germinated with a frequency of 58.3% with development of shoots of 2.3 cm and roots of 1.3 cm after 30 days of culture. A further decrease in germination percentage (46.7%) was noticed from the seeds obtained from 200 Gy irradiated pollen and the growth of the seedlings was slow with shoots of 1.6 cm and roots of 0.9 cm whereas control seedlings had shoots of 3.2 cm and roots of 4.0 cm.

Germination capacity of M_1 seeds obtained after pollination with S-13 irradiated pollen in the year 2005:

It was observed that the germination frequency of seed decreased with increase in the dose of irradiation as compared to control (Table 14; Fig. 30). The seeds obtained from 50-500 Gy irradiated pollen germinated with a frequency of 71.8 -24.9 %. The seed obtained with 1000 Gy irradiated pollen germinated with a very low frequency of 9.4%.

Two seeds obtained from higher doses of irradiation (1000 Gy) germinated but immediately died indicating the induction of lethals (Fig. 31 a-d). Two seedlings that germinated from seeds derived from 1000 Gy irradiated pollen lacked shoot meristems although root development was normal.

Shoot multiplication:

A study was conducted in 2004 to determine the rates of shoot multiplication of control seedlings and those obtained from different treatments of 50, 80 and 100 Gy. The shoot multiplication rates were examined after 5^{th} subculture (Table 15). A clear decrease in the length of the shoots and leaf size was observed in two plants, Plant 23 and 36 whereas for other plants no differences in shoot multiplication were noticed. The average number of shoots was 5.2 and 8.8 for Plants 23 and 36, in comparison to control that had 11.4 shoots per culture (Fig. 32). The average shoot length was 3.4 and 2.3 cm in Plant 23 and 36 respectively, whereas the shoot length of control was 9.6 cm. The average leaf length was 0.9 and 0.6 cm in Plants 23 and 36 respectively, whereas the average leaf length of control was 2.4 cm.

The rooting capacity of the shoots derived from irradiated pollen experiments was compared with control (Fig. 33). There were no significant differences in rooting frequency from the shoots derived from the control and irradiated ones except for plants 23 and 36. About 3-5 roots were induced in these shoots with average root length of 3.4 cm whereas in control shoots, 12-14 roots were induced with average root length of 8.2 cm (Fig. 34). The *in vitro* rooted plants of different treatments along with control hybrid and parents were acclimatized and established in soil with a high success rate of 90-95% (Fig. 35 a-f).

Morphological analysis of M_1 plants obtained from S-13 irradiated pollen:

Fifty M_1 plants obtained from the experiments conducted in 2004 were examined for variation in morphological traits. Variations in various morphological traits such as height of the plant, leaf length and width, shape and margin, thickness of the main stem and internodal distance were observed in 21 plants derived from crosses of S-13 irradiated pollen in comparison to parents (Table 16; Fig. 36 a-f). These plants were derived out of pollination with pollen irradiated at 50, 80 and 100 Gy doses. The height of the variants ranged from 54.0-170.0 cm in comparison to 142.3 cm and 132.7 cm observed in M-5 and S-13 parents, respectively. The leaf length ranged from 9.0-22.0 cm in comparison to 15.4 and 16.5 cm observed in M-5 and S-13, respectively. The leaves of control hybrid were ovate with crenate margin similar to M-5 parent. Five variants were found to be superior to control diploid plants with respect to leaf size. The other prominent changes observed in the variants were elliptical shaped leaves, pale green leaves, five lobed leaves and bicrenate leaf margin (Fig. 37 a-e).

Morphological observations have been recorded in the M_1 plants obtained after crossing M-5 with S-13 irradiated pollen during 2005 (Table 17). Out of the forty M_1 plants analyzed, ten plants showed variations with respect to height of the plant, leaf length and width, shape and margin, thickness of the main stem and internodal distance in comparison to parents. Three variants *viz*., N15, N21 and N53 were characterized by large leaf size which ranged from 19.0-21.0 cm whereas the leaf length of M-5 and S-13 was found to be 13.4 and 15.5 cm, respectively. Four variants *viz*., N17, N18, N34 and N60 exhibited slow growth with reduced height ranging from 76.0-97.0 cm. Variants

with five lobed leaves, cordate leaf base, acute leaf apex, small curly leaves and biserrate leaf margin have been recovered from these experiments.

Flow cytometric studies:

The ploidy level of morphological variants obtained through irradiated pollen approach in 2004 and 2005 was analyzed by flow cytometric analysis. Flow cytometric of morphological variants showed a peak corresponding to diploid similar to that of control hybrid and parents and no differences in ploidy level were observed in any variant (Fig. 38 a-c).

Cytological studies:

Cytological analysis in the root tips of morphological variants was carried out along with control hybrid and parents. The chromosome number in morphological variants was found to be diploid (2n = 28) similar to that of control hybrid and parents (Fig. 39 a-b).

Scanning electron microscopy studies:

Scanning electron microscopic studies of leaf surface of 21 morphological variants obtained after pollination with S-13 irradiated pollen were carried out (Table 18; Fig. 40 a-b). The stomatal length of control hybrid was 31.8 µm whereas it ranged from 17.6-25.4 µm in M_1 plants obtained after pollination with different doses of irradiation. It was interesting to observe that Plant 40 with large leaf size had a stomatal length of 17.6 µm which was almost half of the stomatal length of parents (28.5-32.2 µm).

Chloroplast number:

The chloroplast number in the stomata was determined in plants obtained after pollination with control and irradiated pollen (Table 18; Fig. 41 a & b). The parent plants and control hybrid had 9.4-10.4 chloroplasts per stomata. The chloroplast number in the morphological variants varied from 5.8-9.6. Plant 40 had 5.8 chloroplasts per stomata.

Analysis of protein profiles in leaves of M_1 plants:

SDS-PAGE protein analysis of leaves of M-5 and S-13 cultivars, control hybrid and M_1 plants was carried out to identify the differences in expression of proteins. A major protein band of 55 kDa and at least 14 minor protein bands were identified in all the plants. Analysis of protein patterns of the morphological variants showed a clear increase in the content of 47 kDa and 18 kDa proteins in Plants 15, 40 and 52 compared to M-5 and S-13 cultivars (Fig. 42 a-d). The banding pattern of other variants was more or less comparable to the parent cultivars.

Sex expression studies:

Fifteen M_1 plants established in soil exhibited flowering in 2 years whereas no flowering was observed in the control plants during this period. Ten M_1 plants produced female catkins and one M_1 plant produced male catkins. The parent plant M-5 cultivar produced 100% female catkins out of which 99% ripened into mature fruits (Table 19). Four plants *viz.*, Plant 14, Plant 15, Plant 40 and Plant 45 produced male catkins and mixed catkins with bisexual florets and female florets (Table 19; Fig. 43 a-c). The percentage of male catkins and mixed catkins were 80% and 20%, respectively in Plant

40. The male catkins and mixed catkins were induced at a frequency of 78% and 22%, respectively in Plant 14. In Plants 15 and 45, the percentage of male catkins were 84% and 76%, respectively whereas mixed catkins were produced at 16% and 24%, respectively. A few florets of mixed catkins of Plant 45 had elongated styles (Fig. 44 a & b).

The fruit size (3.1 cm) of the mixed catkins of Plant 40 was found to be larger than M-5 cultivar (1.4 cm) whereas it varied from 1.4-1.6 cm in Plants 14, 15 and 45 (Table 20; Fig. 45 a-c). The fruit weight (931.6 mg), the fruit thickness (2.8 cm) and the peduncle length (0.9 cm) of Plant 40 were significantly higher than M-5 fruits. The number of bisexual florets was found to be higher than female florets in the mixed catkins of Plants 14 and 40 (Table 21). The seeds from bisexual florets were smaller than those obtained from female florets. The seed weight from female florets was found to be higher than that of bisexual florets in mixed catkins of all the four bisexual plants (Table 21).

The germination percentage of the seeds obtained from the female florets of mixed catkins of M_1 plants ranged from 81.4-95.4% in contrast to the seeds of bisexual florets which germinated with a low frequency ranging from 16.7-27.5% (Table 22). Flow cytometric analysis interestingly revealed two peaks at 2C and 4C in one bisexual seed and three peaks at 2C, 3C and 4C in another bisexual seed obtained from Plant 40 in contrast to 2C observed in M-5 seed (Fig. 46 a-c). One bisexual seed of Plant 40 did not show any peak showing that it is non-viable (Fig. 46 d). The plants obtained from bisexual seeds were weak and exhibited low vigour when compared to those raised from

the seeds of female florets (Fig. 47 a-c). One of the plants raised from the bisexual seed exhibited flowering after six months of transfer to the field which produced catkins with bisexual florets showing the inheritance of this trait to the subsequent generation (Fig. 48).

Studies on interspecific hybridization with control and irradiated pollen:

In the present study, pollen irradiation technique was extended to interspecific hybridization to determine the possibility of producing haploids through *in situ* parthenogenesis. There were no significant differences observed with respect to fruit size and single seed weight of control and different treatments (Table 23). The fruit size and single seed weight varied from 0.9-1.3 cm and 1.4-2.1 mg respectively for different treatments whereas the control fruit size was 1.4 cm and single seed weight was 1.9 mg. It was interesting to note that the fruit weight (633.2 mg) of 1000 Gy irradiated pollen was equivalent to control (631.1 mg) whereas for doses of 50-500 Gy it ranged from 276.2-424.6 mg. Pollen irradiation had a significant effect on seed set frequency and resulted in lower number of seeds per fruit. A variable response was observed with respect to the number of seeds formed per fruit for different treatments. The number of seeds varied from 5.6-13.8 in comparison to control that had 22.0 seeds per fruit.

The M-5 seeds obtained from crosses using control and irradiated pollen of China White cultivar were germinated *in vitro*. The M-5 seed obtained after pollination with China White pollen germinated with a frequency of 82.2 % (Table 24). Pollen irradiation decreased the germination frequency of seed to 63.9% even for the lowest dose of irradiation (50 Gy) tested. The germination frequency of the seed decreased to 31.6%

with 1000 Gy treatment. The seed obtained from 1000 Gy showed no signs of germination after 10 days of culture whereas control seed started germinating within 10 days of culture. Germination was discernible after 15-17 days of culture in case of seed obtained from 1000 Gy irradiated pollen. One seed obtained from higher doses of irradiation (1000 Gy) germinated but died subsequently indicating the induction of lethals (Fig. 49 a-b).

Forty plants issued from interspecific cross of M-5 with irradiated pollen of China White cultivar were acclimatized in the greenhouse and established in the soil along with control hybrids and parents. Morphological changes were observed with respect to height of the plant, leaf length and width, leaf shape and margin, thickness of the main stem and internodal distance (Table 25; Fig. 50 a-d, Fig. 51 a-d). The leaf size of two variants *viz.*, C32 and C77 was larger than control hybrid and M-5 parent but smaller than China White cultivar. The leaf of control hybrid was similar to that of M-5 parent. The height of the morphological variants ranged from 57.0-148.0 cm in comparison to M-5 and China White cultivars which exhibited a height of 135.3 and 112.7 cm, respectively. Six variants *viz*., C2, C14, C20, C21, C51 and C52 exhibited low vigour with reduced height, thin stem and small leaves. Variants with dentate leaf margin, cordate leaf base, caudate leaf apex and acuminate leaf apex have been recovered in these experiments.

The stomatal length of M_1 interspecific hybrids ranged from 20.0-27.6 µm in comparison to 32.1 µm and 28.6 µm observed in M-5 and China White, respectively. A decrease in chloroplast number per stomata was observed in the variants in comparison to control. The number of chloroplasts per stomata ranged from 6.5-9.3 in M_1 interspecific

hybrids in comparison to parents and control hybrids which showed 10.4-11.4 chloroplasts per stomata (Table 26; Fig. 52 a-b).

Flow cytometric analysis of the variants obtained after pollination with China White irradiated pollen showed a peak corresponding to diploids similar to that of parents (Fig. 53 a-c).

The leaf protein profiles of M_1 interspecific hybrids obtained after pollination with China White irradiated pollen were analyzed by SDS-PAGE and compared with the control hybrid and parents. The leaf protein profiles showed a major band of 55 kDa and at least 14 minor bands in all the plants analyzed. An increase in intensity of 47 kDa and 18 kDa proteins was observed in six morphological variants *viz*., Plant C11, C20, C22, C33, C34 and C35 in comparison to the parents (Fig. 54 a-e).

Molecular characterization of morphological variants obtained after pollination with S-13 irradiated pollen:

For RAPD analysis, genomic DNA was extracted and amplified with decamer oligonucleotide primers to determine the similarities and relatedness of the morphological variants to the parents, M-5 and S-13 cultivars. Nineteen primers were selected for the study based on their ability to detect polymorphism between M-5 and S-13 cultivars. These primers generated DNA polymorphism which ranged from 33.3-100% among the plants analyzed (Table 27). Among the primers used, OPA-04, OPA-11, OPA-13, OPG-05, OPW-03 and OPY-03 revealed 100% polymorphism whereas lowest polymorphism of 33.3% was obtained with the primer OPY-06. The primer OPY-03 produced highest number of polymorphic bands (104) in the plants analyzed. The size of the amplified

products ranged from 500 bp to 3000 bp with 3-6 bands for different primers. Typical results obtained with the primer, OPY-10 are shown in Fig. 55 a & b.

The primers, OPY-03, OPY-06 and OPY-10 amplified fragments specific to M-5 and S-13 cultivars whereas the other primers produced bands specific to M-5 cultivar but absent in S-13 cultivar. In total, 35 M-5 specific bands and 3 S-13 specific bands were produced by above primers. Three morphological variants *viz*., 33, 34 and 57 showed highest number of M-5 specific bands (27-29) with 1-2 S-13 specific bands. The number of M-5 specific bands ranged from 16-29 whereas S-13 specific bands ranged from 1-3 in the remaining variants. When the primers OPA-02, OPA-04, OPY-03 and OPY-05 were used, unique bands (1-2) were observed in 10 morphological variants.

UPGMA cluster analysis was used to determine the relationships among the morphological variants and parents. The cluster analysis obtained from the RAPD profiles divided the variants into three major groups and five outliers (Fig. 56). The group A comprised of four sub-groups. The first sub-group comprised of Plant 33, Plant 34 and Plant 57 along with M-5 cultivar while S-13 was clustered separately within this sub-group. The Plants 33, 34 and 57 showed highest similarity towards the female parent. The second sub-group consisted of Plant 46, Plant 32 and control hybrid, while the third sub-group consisted of Plant 40 and Plant 45. The fourth sub-group consisted of Plants 63, 23, 27 and 28. The group B comprised of Plant 37, 16 and 20. The group C consisted of Plants 15 and 22.

RAPD technique was used to characterize the genetic variability of 18 M_1 interspecific hybrids obtained after crosses of M-5 cultivar with China White irradiated

pollen in relation to the parent cultivars, M-5 and China White. Ten decamer primers that distinguished M-5 and China White were selected for RAPD analysis. These primers generated polymorphism ranging from 56.3-100% among the plants analyzed (Table 28). Out of the ten primers tested, six primers showed 100% polymorphism. Using the ten selected primers, 48-85 bands were scored, ranging in size from 500 to 3000 bp. Each of the ten RAPD primers generated 3-7 informative bands. The primer OPW-01 produced highest number of polymorphic bands (85) in the plants analyzed. An example of the patterns obtained by the RAPD analysis using the primer OPW-04 is shown in the Fig. 57 a & b.

The primers OPG-05, OPW-04, OPY-11 and OPY-16 produced bands specific to M-5 and China white cultivars. The other primers amplified fragments specific to China White cultivar whereas fragments specific to M-5 cultivar were not detected. In total, 6 M-5 specific bands and 25 CW specific bands were produced by different primers. The control hybrid showed 3 M-5 specific and 11 CW specific bands. The number of M-5 specific bands ranged from 3-6 whereas China White specific bands ranged from 7-22 in the variants. It was noted that unique bands were produced in all the variants in the presence of primers OPG-16, OPW-03, OPY-11, OPY-15 and OPY-16 which ranged from 1-6.

Cluster analysis based on Jaccard's coefficient using UPGMA showed considerable genetic variation in the morphological variants. The morphological variants were grouped into two major groups and an outlier (Fig. 58). The major group A was resolved into three sub-groups while M-5 was clustered separately within the group. The

major group B was further sub-divided into two sub-groups. While the variants, C33, C20 and C14 comprised the first sub-group and clustered with China White, the variants C22, C25 and C30 belonged to the second sub-group. The variant C11 exhibited least similarity to M-5 cultivar.

AFLP studies:

In the present study, AFLP based fingerprint was used as a tool for characterizing 4 morphological variants obtained after pollination with S-13 irradiated pollen. These variants produced male catkins as well as mixed catkins with female and bisexual florets. Eight primer combinations amplified fragments ranging from 233 to 381 across all the eight plants which included M-5, S-13, China White, control hybrid and four M_1 plants *viz*. 14, 15, 40 and 45 (Table 29). The size of the amplified fragments ranged from 50-700 bp. On an average, 37 fragments per primer combination were scored per plant. A high level of polymorphism ranging from 77.0-97.6% was found over all the plants analyzed for different primer combinations. The highest number of polymorphic fragments (333) was produced with primer combination EACC/MCTC and lowest number of polymorphic fragments (201) was produced with primer combination EACA/MCTC.

AFLP method resulted in production of many fragments specific to the parents (Fig. 59 a-d; Fig. 60 a-d). Out of 8 AFLP primer combinations tested, seven were found to produce fragments specific to M-5, S-13 and China White cultivars (Tables 30 a & b). The primer combination EACG/MCAA produced fragments specific to S-13 and China White cultivar but did not produce any fragments that were specific to M-5 cultivar. The

primer combination, EACA/MCTT produced the highest number of fragments (14) specific to M-5 cultivar. In case of S-13 cultivar, highest number of fragments (8) was produced by the primer combination EACC/MCTT. The highest number of fragments specific to China white cultivar was found to be 4 for the primer combinations, EAAC/MCAA and EACC/MCTC. On the whole, eight primer combinations produced 39 bands specific to M-5 cultivar, 33 bands specific to S-13 cultivar and 20 bands specific to China White cultivar (Fig. 59 a-d; Fig. 60 a-d). Among all the primer combinations tested, EACA/MCTT was found to be most efficient in distinguishing M-5 and S-13 cultivars. In contrast, EAAC/MCAG was found to produce lowest number of polymorphic fragments with one each for M-5 and S-13 cultivars. Thus the results showed the usefulness of the eight primer combinations for DNA fingerprinting of mulberry cultivars.

The hybrid obtained from the cross of M-5 cultivar with control (non-irradiated) pollen of S-13 cultivar showed 22 bands specific to M-5 cultivar and 26 bands specific to S-13 cultivar. Interestingly, Plant 40 showed 14 bands specific to M-5 cultivar and 3 bands specific to S-13 cultivar (Tables 30 a & b). In case of Plant 45, the number of M-5 specific and S-13 specific bands was found to be 19 and 4, respectively. The Plant 14 showed 23 M-5 specific bands and 11 S-13 specific bands. The Plant 15 had 24 bands specific to M-5 cultivar and 10 bands specific to S-13 cultivar. It was observed that 262 novel AFLP fragments not amplified in M-5 or S-13 parental plants were produced in the morphological variants whereas control hybrid did not show any novel fragments (Fig. 59 a-d; Fig. 60 a-d). The Plant 40 showed highest number (135) of unique fragments. Thus

the Plants 40 and Plants 45 possessed most of the AFLP fragments specific to M-5 cultivar and very few S-13 specific bands apart from many unique fragments.

AFLP analysis revealed 85% similarity between M-5 and S-13 whereas the control hybrid exhibited 85% similarity with M-5 and 92% similarity to S-13 cultivar. Cluster analysis was used to construct a neighbor joining dendrogram displaying a high level of genetic variation detected among the morphological variants. The cluster analysis showed that two variants (Plant 40 and Plant 45) got entirely separated from the tree (Fig. 61). This was in agreement with the PCA analysis which showed that the four variants showed a distinct pattern and were genetically different from the parent cultivars (Fig. 62). On the whole, a high level of genetic variation was observed among the morphological variants. Bootstrap analysis showed that the parents M-5 and S-13 and the control hybrid were supported with very high bootstrap values *i.e.* 100% whereas China White is added to this cluster with a bootstrap value of 51 indicating its instability within the group. The variants formed a totally different cluster with a bootstrap value of 100%.

DISCUSSION

The main objective of the present study is to induce haploids through anther and unpollinated ovary cultures. In addition, pollen irradiated approach was employed to investigate the possibility of inducing haploids through *in situ* parthenogenesis. The ploidy level of the M_1 plants obtained from crosses of M-5 plants with S-13 and China White irradiated pollen were determined. The study resulted in production of useful variants which were characterized at molecular level.

Androgenesis:

The success of callus induction and plant regeneration from anther cultures depends on a number of factors such as the genotype, stage of pollen development, pretreatment of anthers, culture media, growth regulators and culture conditions (Keller and Armstrong, 1977; Bajaj, 1990; Anderson *et al*. 1990; Osolnik *et al*. 1993; Gorecka *et al*. 2005**)**. The purpose of applying physical pretreatment before plating anthers in the medium is to bring about the stress needed to change the developmental pathway of immature pollen grains (Maheshwari *et al*. 1982). In the present experiment, cold treatment was found to be necessary for induction of callus from anthers of S-13 and V-1 cultivars of mulberry. Cold treatment of anthers for 4 days was required for callus induction in S-13 cultivar whereas in V-1 cultivar, a 6-day cold treatment was found to be essential. The beneficial effect of cold treatment is well established for many species (Nitsch and Norreel, 1973; Nitsch, 1977; Sunderland and Wildon, 1979; Osolnik *et al*. 1993) perhaps due to an increase in the number of microspores in viable condition with similar nuclei (Nitsch, 1974). The results of Jain *et al*. (1996) revealed that 24h cold pretreatment increased the percentage of anthers forming callus in mulberry.

In the present study, the requirement of growth regulators for induction of callus from the anthers varied with the cultivar. The callus induction from cold-treated anthers was very low, at a frequency of 3.8% on MS medium with 4 mg/l 2,4-D, 0.1 mg/l TDZ and 6% sucrose in V-1 cultivar. Medium supplemented with 8 mg/l 2,4-D, 0.1 mg/l NAA and 6% sucrose resulted in callus induction with a frequency of 6.8% from anthers of S-13 cultivar. Rhizogenesis was induced from callus of S-13 cultivar upon culture on medium with 1 mg/ 2,4-D, 0.1 mg/l NAA and 3% sucrose whereas no rhizogenesis was observed in V-1 cultivar. This could be due to the use of TDZ in callus induction medium in V-1 cultivar or could be due to variation in endogenous growth regulators in two cultivars. Efforts to induce embryogenesis and plant regeneration from callus induced from the anthers of two cultivars of mulberry have been unsuccessful despite some success reported by earlier investigators. Sethi *et al*. (1992) observed embryo differentiation in three genotypes of mulberry and the percentage of embryogenic cultures remained very low ranging from 0.23-1.82%. Jain *et al*. (1996) observed the development of embryogenic calluses from *in vitro* cultured anthers of mulberry on MB (modified Bourgin) medium supplemented with NAA (0.5 mg/l) and BA (1.0 mg/l) using 8% sucrose. However, the embryos induced from anther cultures germinated precociously without developing cotyledons and formed elongated shoots.

Gynogenesis:

Induction of haploids through *in vitro* gynogenesis offers an alternative when androgenesis proves unsuccessful or not possible in cases of female and male sterile plants. Gynogenesis is the only possible approach to produce haploids for the female

clones of mulberry where anther culture and inbreeding are not feasible. In the present study, a two-step approach involving culture of pistillate flowers followed by unpollinated ovary culture resulted in induction of soft callus at a frequency of 4.5% on MS medium with 2.0 mg/l 2,4-D, 0.5 mg/l BAP and 3% sucrose in M-5 cultivar. The anatomical observations revealed the presence of developing embryos, although they failed to differentiate into plants. Different growth regulators have been tried to induce plant regeneration in the present study without any success. Earlier attempts made by Sita *et al*. (1991) using unpollinated ovary cultures in mulberry resulted in induction of haploids although the induction frequency remained low. In order to exclude the possibility of obtaining diploids from the fertilized ovules, Dennis *et al*. (1999) used *in vitro* developed inflorescence as a source for ovaries for producing haploids in mulberry. The gynogenic plants produced were found to be haploids as well as aneuploids. The present studies on anther and unpollinated ovary cultures of mulberry have not been very encouraging in terms of inducing haploids. Hence, the pollen-irradiated approach was used for the first time in mulberry for producing haploids.

Although haploids have been raised mainly from anther and unpollinated ovary cultures of different species, this technique has not proved successful in respect of all genotypes of plant species. The reasons for low frequency callus induction and lack of plant regeneration from anther and unpollinated ovary cultures of mulberry could be that the suitable medium and growth regulators required for androgenesis are not provided or it is possible that the genotypes used are recalcitrant in nature.

Pollen irradiated approach for production of haploids:

In pollen-irradiated approach, the irradiated pollen may stimulate the female gametes to develop into parthenogenetic embryos resulting in haploids. Prior information of the effect of irradiation on pollen is required for carrying out pollination experiments with irradiated pollen (Stettler, 1968; Pandey, 1974). The response of pollen to irradiation varies greatly and is plant genotype and dose dependent (Brewbaker and Emery, 1962). The dose of gamma irradiation that affects pollen viability and germination is not known in mulberry. In the present study, a sharp decrease in pollen viability to 9.8% was observed with the increase in the dose of the irradiation to 2000 Gy in S-13 cultivar. Similar observations have been recorded by Peixe *et al*. (2000) in European plum where all the levels of irradiation tested had a significant effect on pollen viability. In the present study, the germination capacity of S-13 pollen decreased *in vitro* upon irradiation and this was found to be dose-dependent. Pollen germination decreased from 27.7-4.9% at 50-1000 Gy irradiation doses and completely affected at 2000 Gy. Similarly, Cuny *et al*. (1993) reported that higher dose of irradiation (1.6 kGy) reduced the germination of muskmelon pollen *in vitro*. On the contrary, gamma irradiation levels up to 1000 Gy had no significant effect on pollen germination of apple (Zhang and Lespinasse, 1991). Musial and Praywara (1998) showed that pollen irradiation at doses of 700 and 900 Gy had little effect on *in vitro* pollen germination in kiwifruit.

The present study revealed that pollen irradiation affected the germination capacity of the pollen *in situ*. Pollen tube growth was seen in control, decreased with increase in the dose of irradiation and was completely affected at 2000 Gy. It might be

possible that gamma rays have altered protein composition which could have affected the physiology of the pollen grains or it affected the protein signal recognition required for the tube growth (Kumar and Rai, 2006). Visser and Oost (1981) reported that hampered protein synthesis is a secondary and cell damage a primary cause for a reduced germination after irradiation.

In the present study, gamma irradiation of S-13 and China White pollen up to 1000 Gy did not have any significant effect on fruit development whereas higher dose of irradiation (2000 Gy) affected fruit and seed development. It was observed that about 15-20% of the fruits formed from 500 and 1000 Gy were found to be parthenocarpic or contained very few seeds. These observations are in accordance with pollen germination studies that revealed lack of pollen germination at doses above 1000 Gy. Thus the fruit growth may be controlled by endogenous auxin produced by the fertilized ovules that in turn depend on the amount of germinated pollen. Zhang and Lespinasse (1991) observed a tendency towards parthenocarpic fruit formation with the increase in the level of pollen irradiation in apple. These fruits resulted from the late degeneration of the ovules. Cuny *et al*. (1993) observed that high dose of irradiation (3.6 kGy) retarded the pollen tube growth of muskmelon and thus when the pollen tubes were unable to reach the ovule the fruit setting was lost because of a hormonal imbalance. Witte and Keulemans (1994) reported that the fruit and seed set reduced after pollination with irradiated pollen in apple. They interpreted that lower fruit set after pollination with irradiated pollen could be due to the lower sink activity of induced seeds compared to fertilized seeds.

The consequences of irradiation need not be exactly the same for a given dose and depends upon whether irradiation occurs before or after DNA synthesis. In the present

study, variable results were obtained with respect to fruit weight, seed set and single seed weight when the experiments of S-13 pollen irradiation were conducted in 2004 and 2005. In contrast to the observations of 2004, seed set was observed with 1000 Gy although the single seed weight decreased. These differences could be possibly due to increased number of pollinations made in the year 2005. This observation is further substantiated by the fact that few of the fruits formed at 500 and 1000 Gy in 2005 were parthenocarpic whereas others contained seeds comparable to controls. These results are in agreement with Pandey *et al*. (1990) who reported that some fruits formed after pollination with irradiated pollen in kiwifruit had only empty seed coats, whereas other fruits contained more than 100 apparently normal seeds. Similarly, in apple higher levels of pollen irradiation had a tendency to stimulate the formation of parthenocarpic fruits (Zhang and Lespinasse, 1991). On the other hand, Cuny *et al*. (1993) evaluated the effects of gamma ray exposures on muskmelon pollen and observed that regardless of doses of between 0.15 and 1.6 kGy, fruit set and number of seeds per fruit were comparable to those of the control.

The present studies on interspecific crosses of M-5 cultivar with China White irradiated pollen revealed no significant differences with respect to fruit size and single seed weight for all the doses examined. Significant differences with respect to number of seeds per fruit were observed after pollination with China White irradiated pollen whereas no differences were observed when pollinated with S-13 irradiated pollen. The decrease in number of seeds per fruit might be a consequence of the irradiation that might have affected the process of fertilization and thus seed development. These results implied that the genotype of the pollen parent along with gamma irradiation has an effect

on fruit and seed formation in mulberry. In majority of the species studied, an increase in the irradiation dose resulted in a significant decrease in the number of seeds per fruit (Chyi *et al*., 1984; Sanford *et al*., 1984). However, Cuny *et al*. (1993) noted that gamma ray doses between 0.15 and 0.16 kGy, did not have any significant effect on number of seeds per fruit in muskmelon (*Cucumis melo* L.). Chalak and Legave (1997) reported that the genotype of male had a marked effect on the number of seeds formed although the lowest dose of 200 Gy produced a drastic decrease in the number of full seeds in Hayward kiwifruit. Yang *et al*. (2004) also reported that the percentage seed set of M_1 plants pollinated with gamma irradiated pollen in Arabidopsis decreased linearly as irradiation dose increased. For 400 Gy at the mature pollen stage, 50% of seeds aborted, while complete seed abortion was observed for a dose of 1200 Gy.

The present study showed that pollen irradiation caused normal as well as abnormalities in embryo and endosperm development. In one case at 50 Gy, twin embryos were observed within a single embryo sac although the origin of such embryos is not known. Abnormal embryo development along with endosperm disintegration was noticed in another case at 100 Gy. Nicoll *et al*. (1987) reported that high dose of pollen irradiation (100 krad) in apple generated an all-or-nothing response in the embryo sac, either creating highly abnormal embryos and/or endosperms which aborted or showing relatively normal development. Falque (1994) revealed that embryo development in cacao was altered by irradiation whereas the endosperm development was normal. Delayed picking increased the proportion of large seeds in apple cultivar, suggesting that pollination with irradiated pollen retards embryo development (Witte and Keulemans, 1994). Berzonsky *et al*. (2003) reported twin embryo formation in wheat after pollination

with maize. Kurtar *et al*. (2002) obtained seeds with differently formed embryos in the same fruit with doses of only 25-50 Gy in squash (*Cucurbita pepo* L.).

Chalak and Legave (1997) obtained the trihaploid seedlings of kiwifruit without any *in-vitro* culture whereas Pandey *et al*. (1990) used *in vitro* sowing for recovering the haploid seedlings. For experiments involving pollen irradiation, *in vitro* culturing is essential for enhancing the rate of seed germination. The medium for *in vitro* seed germination of mulberry was optimized. It was found that Woody Plant medium supplemented with 3 mg/l GA_3 was found to be superior for seed germination compared to MS basal medium with or without GA_3. The increase in percentage of germination observed in the presence of GA_3 could be attributed to breakage of dormancy by reducing ABA level or inhibiting ABA synthesis (Ali-Rachedi *et al*. 2004).

In the present study, the seed obtained after pollination with irradiated pollen were germinated *in vitro* and the effect of pollen irradiation on seed germination was recorded. The germination percentage declined with increase in irradiation dose (1000 Gy) to as low as 9.4%. The results obtained from intervarietal cross of M-5 with S-13 irradiated pollen were more or less in accordance with interspecific cross of M-5 with China White irradiated pollen although a difference in the percentage of seed germination percentage was noticed at 1000 Gy. The main cause of germination reduction could be attributed to the occurrence of seeds without completely developed or abnormal embryos as was reported in pear (Sniezko and Visser, 1986) and cacao (Falque, 1994).

In apple, most of the embryos arising from irradiated pollen treatments were not viable, *i.e.* did not germinate or died soon after germination (Zhang and Lespinasse,

1991). The death of haploids was attributed to accumulation of deleterious recessive genes as apple is allogamous and vegetatively propagated species. In the present study, two seedlings obtained from 1000 Gy irradiated pollen of S-13 died immediately after germination possibly due to the expression of deleterious recessive alleles. The irradiated pollen affected embryo development, as the shoot meristem was absent in two seedlings. The lack of shoot meristem could be due to the abnormalities occurring during embryo development. Pandey *et al*. (1990) also observed poor germination rate from seeds obtained from irradiated pollen that reflected poor development of embryo or endosperm. In *Arabidposis*, mutations in two genes resulted in the specific failure of plants to form and maintain a shoot meristem. Embryogenesis in plants homozygous, for *stm-1* was normal in the cotyledons, hypocotyls, root and root meristem developed properly, but the mature embryos lacked a shoot meristem (Clark, 1997).

In the present study, pollen irradiation affected the shoot multiplication of two plants *viz*., 23 and 36, out of 50 plants analyzed. There appeared to be no correlation between the doses of irradiation tested (50-100 Gy) and shoot multiplication. The experiments on rooting response from the plants derived from irradiated pollen did not reveal any significant differences with respect to root number and length of the roots except for Plants 23 and 36 which showed reduced root number (3-5) and root length (3.4 cm) as compared to control. Mishra *et al*. (2007) found that the individual shoot survival in banana declined with increase in irradiation dose. In contrast, Liu *et al*. (2007) showed that pollen treatment with electrostatic field not only improved pollen germination but also increased the multiplication coefficient of embryo-induced shoots.

In the present study, analysis of ploidy level of plants obtained after intervarietal and interspecific hybridization with irradiated pollen by Flow Cytometric method revealed no differences in ploidy level. Since a low percentage of seeds induced by irradiation at higher doses germinated, *in vitro* culture of immature seeds presumably may help in recovering haploids, which were lost during seed development. Further experiments need to be conducted to test this possibility. The experience of various investigators working on pollen irradiation technique in different plant species shows that this method will not always yield haploids. Literature data demonstrated both the regeneration of haploids and diploids *via* parthenogenesis in apple (Chevreau *et al*. 1985; Zhang and Lespinasse, 1991) and the exclusive development of diploids (Kenis and Keulemanns, 2000). When pollen irradiated method is used there, is also a possibility of recovering variants. In a clonally propagated plant like mulberry, it appears that pollen irradiation approach is the best method for recovering a large number of variants as mutagenesis of seeds or cuttings leads to chimerism of resulting tissues and would require screening on a large scale to identify the resultant mutations.

The mutations induced by gamma irradiation in the male genome might have an effect on the morphology of M_1 plants. The M_1 plant arising from pollination with mutated pollen is non-chimeric and will be hemizygous for any uniquely induced mutations (Yang *et al*. 2004). In the present study, variations in morphological characters such as the height of the plant, leaf length/width, leaf shape and margin, thickness of the main stem and internodal distance were noticed among the plants obtained through the use of irradiated pollen as compared to parents indicating a broad spectrum of mutations induced by gamma rays. Twenty one variants in the year 2004 and ten variants in the

year 2005 were produced from intervarietal crosses. The underlying causes for the morphological variants are unknown. It is possible that pollen irradiation might have caused alteration of the genes giving rise to hemizygous plants expressing some recessive maternal genes, as reported with *Oryza sativa* (Chin and Gordon, 1989). Falque (1994) observed various abnormalities in the morphology, often with long and irregular leaves in M_1 plants of cacao obtained after pollination and fertilization of irradiated pollen. These apparent mutants were found with a rate of about 20% among the progeny from 50 Gy irradiated pollen whereas in our experiments the changes in morphology were observed for all the doses examined. It is essential to study the inheritance of these traits in order to understand the nature of mutations. It is highly likely that undetected mutants remained because of deficient screening. Further screening and analysis on a large scale will yield additional variants. Sanamyan *et al*. (2003) reported plants with reduced fertility and a haploid plant in M_1 cotton plants and suggested that diverse forms observed in M_1 after pollination with irradiated pollen is determined by elimination of some chromosomes and also by interchromosomal rearrangements. Low and medium doses of gamma rays caused relatively high proportion of useful mutants with normal yielding properties in miniature tomato (*Solanum lycopersicum* L.) cultivar 'Micro-tom'. Gamma ray mutagenesis produced severe phenotypic mutations because it causes large-scale deletions and occasionally, chromosome reconstitution (Matsukura *et al*., 2007).

In the present study, fifteen M_1 plants obtained after pollination with S-13 irradiated pollen showed a considerable decrease in height ranging from 54.0-97.0 cm compared to parents (125.6-145.3 cm). Researchers studying other crop species have also reported a decrease in height on exposure to the mutagens. Khawale *et al*. (2007)

observed various types of abnormality symptoms like curling/scorching of leaves, albino leaves, stunted root/shoot growth, chimera formation in chemical mutagen-induced grapevine mutants. Mutations impairing the biosynthesis or sensitivity of gibberellins (GA_3), indole-3-acetic acid and brassinosteroids have resulted in dwarf mutants as they are known to control cell and plant size (Lanahan and Ho, 1988; Nadhzimov *et al*. 1988; Sponsel *et al*. 1997; Fukuta *et al*. 2004).

In the present study, eight variants with large leaf size and seven variants differing with respect to leaf shape and margin were identified and isolated in M_1 generation from S-13 irradiated pollen experiments. In some instances, the mutation affected more than one trait such as small leaf variants exhibited a great reduction in plant height in comparison to the parents. Tani *et al.* (2003) observed incisions in the leaves of hybrid seedlings between *M. boninensis* and *M. acidosa* and suggested that such distinctive leaf morphology could be due to dominance effects in the hybrids. Thus, it can be envisaged that irradiation is an excellent tool for creating genetic variability in mulberry and recovering promising mutants.

The cytological studies using root tip squash method did not reveal any differences in chromosome number in the morphological variants as compared to parents. However, a decrease in stomatal size was observed in many morphological variants compared to parents. It was observed that Plant 40 obtained from S-13 irradiated pollen with large leaf size exhibited vigorous growth and had lowest stomatal length (17.6 µm) which was almost half as that of parents (28.5-32.2 µm). The chloroplast number per stoma was also the lowest (5.8) in Plant 40 in comparison to parents (9.8-10.4). The

superiority of the Plant 40 in terms of leaf size and growth compared to the parents may be the result of favourable gene interaction for expression of these characters than parents. The results of Kurtar *et al*. (2002) indicated that stomatal size; number of stoma per mm^2 and chloroplast number of guard cells could be used as an alternative criterion to determine the ploidy level in squash plants. In contrast, our results showed that stomatal size cannot be used as a means for identifying the ploidy status of mulberry as the variants with the same ploidy level showed a decrease in stomatal length.

The present studies on interspecific hybridization of M-5 with control and irradiated pollen of S-13 cultivar attains significance as it investigated the possibility of recovering haploids as well as studied the possibility of transferring the large leaf trait into widely adapted M-5 cultivar. The study resulted in isolation of 18 M_1 interspecific hybrids which displayed differences with respect to leaf size, shape and margin, height of the plant, stem thickness and internodal distance. Flow cytometric analysis of these plants did not reveal any differences in ploidy level. While two M_1 interspecific hybrids exhibited larger leaf size than M-5 cultivar, eight variants exhibited slow growth with small leaves. Thus the variability observed in the M_1 interspecific hybrid could be the result of gene mutations, deletions, transpositions and genome rearrangements induced in the male genome by gamma irradiation. Further efforts are needed to determine the nature of such mutations. Tikader and Dandin (2001) obtained F_1 hybrids from crosses of wild species, *M. laevigata* and *M. serrata* with *M. indica* with high biomass, vigorous growth, profuse fruit formation and timber yield.

The genetic variation of the leaf protein profile is poorly understood in mulberry. Hirano (1982) found no apparent differences of leaf protein profiles of 17

varieties of mulberry by means of SDS-urea PAGE method. The combined method of 2D-PAGE and silver staining, however, revealed varietal differences in the protein profiles. In the present study, SDS-PAGE analysis of the leaf proteins of the morphological variants was carried out for identifying the changes in protein profiles in comparison to the parent cultivars. A major protein band of 55 kDa was observed in all the variants apart from 14 minor bands. The major protein band may represent large sub-unit of Rubisco as it has expected Mol. Wt. of 55 kDa observed in green plants (Parry *et al*. 1987). The leaf protein profiles of the morphological variants showed variation in the expression of 47 kDa and 18 kDa proteins and no new protein bands were detected. An increase in the content of 47 kDa and 18 kDa proteins was noticed in three variants induced from S-13 irradiated pollen *viz*., Plants 15, 40 and 52, and six variants *viz*., Plant C11, C20, C22, C33, C34 and C35 induced from China White irradiated pollen in comparison to the parents which is an indication of differential gene expression. However, no correlation was observed between the expression of this proteins and specific morphological types. The reasons for the increase in the intensities of 47 kDa and 18 kDa proteins in these morphological variants remain to be determined. It would be of interest to purify and characterize the proteins from these variants for determining their potential function. Kavyashree *et al*. (2006) observed a decrease in protein bands in the leaves of haploid clones as compared to *in vitro* diploid plants confirming the ploidy status of the gynogenic haploid.

Bisexual flowers were observed in female clones of mulberry by treating with silver nitrate and ethrel (Dennis Thomas, 2004) and cucumber (Stankovic and Prodanovic, 2002). The interesting observation in the present study is the induction of

male and mixed catkins with female and bisexual florets in four M_1 plants. The parent plants, M-5 and S-13 cultivars never produced mixed catkins or bisexual florets in the environmental conditions studied. The induction of mixed catkins with bisexual florets and female florets could to be due to changes in the expression of floral determining genes although the exact mechanisms involved in sex expression are unknown. Schaffner (1919) observed sex reversal in staminate tree of *Morus alba* which proved its potentialities for both sexes. He opined that this is brought about by establishment of physiological gradient in the zygote that is reflected in the advanced ontogeny of the individual. Jolly *et al*. (1986) reported differences in sex expression in 124 mulberry varieties collected from different sources which consisted of 65 dioecious and 44 monoecious types and monoecious male, female and mixed type flowers.

In the present study, a few florets of mixed catkins of Plant 45 were characterized by the presence of long-style. Katsumata (1972) showed that long-style trait of cv. Hachijougowa is incompletely dominant to the short style trait of cv. Kairyonezumigaeshi in mulberry. The fruit size of the mixed catkins of Plant 40 isolated in the present study was found to be larger (3.1 cm) than the fruits (1.4 cm) of M-5 cultivar. The seeds formed in the bisexual florets were smaller with a reduction in seed weight. The reduced germination observed from the seeds of bisexual flowers could be due to reduced sink capacity of the seed. Thus the Plant 40 was found to be promising in terms of leaf size, increased vigour and sex expression.

Seed flow cytometric analysis gives high-resolution histogram and developmental variations in cell-cycle behaviour can be avoided which is present in differentiated tissue (Sliwinska *et al*., 2005). In the present study, flow cytometric

analysis of one of the bisexual seed obtained from Plant 40 interestingly revealed peaks at 2C, 3C and 4C in contrast to only 2C peak observed in control plants. The presence of 3 peaks indicates the mixoploid status of the cells of the bisexual seed. Deltour (1985) reported that mature embryos of some species contain both G_1 and G_2 nuclei while other species contain G_1 nuclei only. Sliwińska and Pedersen (1999) reported significant higher G_2/G_1 ratio was found in dry mature seeds of lower vigour. It was suggested that this could be due to a high proportion of undeveloped seeds that are characterized by high cell cycle activity in the embryo and lower germination capacity. Matzk *et al*. (2000) used the flow cytometric seed screen method for exploiting the differences in the embryo/endosperm ploidy ratio of the seed progeny generated by various reproduction pathways (parthenogenesis, pseudogamy *versus* autonomous apomixes). According to Matzk *et al*., (2000), obligatory sexuals or apomicts yield two peaks corresponding to the embryo and endosperm nuclei, respectively.

Molecular characterization of variants by PCR based markers:

RAPD method has been used successfully in characterizing the mulberry species by different investigators (Vijayan *et al*. 2003; Vijayan *et al*. 2004; Awasthi *et al*. 2004; Chatterjee *et al*. 2004). The level of polymorphism between the cultivars indicated that distinction should be possible with a small number of appropriate primers. In the present study, RAPD markers have been used to characterize the morphological variants obtained after crossing M-5 plants with S-13 irradiated pollen in order to find out the similarity to the parent plants and determine the absence of paternal markers, if any. A better understanding of the extent of genetic variation in the morphological variants is essential for their utilization in breeding programmes. In RAPD assay, the nature of the

fragments that are amplified is highly dependent on the primer sequence and on the genomic DNA sequence being assayed. A total of 19 primers were selected for the study based on their ability to distinguish between M-5 and S-13 cultivars. The primers selected for the study revealed considerable DNA polymorphism among the morphological variants, which ranged from 33.3-100%. Polymorphisms revealed through the RAPD technique may be caused by a number of events, such as a deletion eliminating the primer binding site, an insertion making a fragment too large for polymerization, nucleotide substitutions in the primer annealing sites leading to failure of polymerization, or a small addition or deletion leading to larger or smaller fragments, respectively (Shasany *et al*. 2005).

Of the 19 primers used in the study, only three primers, *viz*., OPY-03, OPY-06 and OPY-10 produced male parent specific fragments. Analysis of the morphological variants did not provide any evidence of the absence of male specific fragments when the bands produced by all the primers were considered together. It was observed that four primers *viz*., OPA-02, OPA-04, OPY-03 and OPY-05 produced unique bands in 10 morphological variants. Thus the study demonstrated the existence of large amount of genetic variation in the morphological variants, which could be brought about by genome rearrangements induced in the male genome by gamma irradiation. However, a detailed analysis using a large number of markers is essential to obtain comprehensive information regarding the nature of variation. Senthamizh *et al*. (2007) estimated the DNA polymorphisms in amla mutants induced by gamma rays using RAPD markers. Appearance and disappearance of new bands was explained as the result of DNA structural changes (breaks, transpositions, deletions etc.) due to gamma irradiation.

Several workers have shown that RAPD markers, which can quickly detect a large number of genetic polymorphisms have led to the creation of genetic maps in a number of woody fruit crops and detection of mutations in sunflower (Erdem and Oldacay, 2004) and grape (Khawale *et al*. 2007).

In the present study, the UPGMA dendrogram divided the morphological variants into three major groups and five outliers. Three morphological variants *viz*., Plant 33, 34 and 57 were placed in the sub-group of A along with M-5 cultivar depicting high similarity towards female parent. Majority of the morphological variants were placed away from the parents indicating the existence of high level of genetic variation. Bhagwat *et al*. (1997) characterized the radiation induced mutants of groundnut cv. Spanish improved showing distinct morphological differences with the parent for RAPD variability. The analysis revealed characteristic band differences among the 12 mutants and the parent.

RAPD analysis of the eighteen M_1 interspecific hybrids produced from crosses of M-5 cultivar with China White irradiated pollen was carried out to find out the similarity to the parents. A high level of polymorphism ranging from 56.3-100.0% was observed using 10 random primers. Out of the ten primers used, except for primer OPG-16, the remaining primers produced bands specific to China White cultivar. On the whole, 6 bands specific to M-5 cultivar and 25 bands specific to China White cultivar were produced using ten primers. The exclusive polymorphic RAPD bands are interesting, since they could be converted to STS (Sequence tagged sites) markers which could have

great value for detection of mixes between cultivars and for DNA fingerprinting as was reported in barley (Fernandez *et al*. 2002).

Luo *et al*. (2002) studied the inheritance of RAPD markers in an interspecific F_1 hybrid of grape between *Vitis quinquangularis* and *V. vinifer* and observed five non-parental markers. In the present study, comparison of the banding pattern of the morphological variants with the parents showed that presence of 3-6 bands specific to M-5 cultivar and 7-22 bands specific to China white cultivar. In addition, the morphological variants showed unique bands in the presence of seven primers. UPGMA analysis revealed that M_1 interspecific hybrids differed from the parents and among themselves in varying degrees. The high amount of genetic variation observed in the morphological variants could be due to the combination of genomes of different origin and also due to chromosomal structural rearrangements brought about by irradiation in the male genome. The promising variants obtained in the study have been proved to be due to variations at the DNA level. A detailed molecular and genetic analysis is however required to understand the nature of such variation. Shasany *et al*. (2005) demonstrated the use of RAPD and AFLP markers for identification of inter and intraspecific hybrids of *Mentha*.

AFLP technique is a powerful tool for identification of a greater number of polymorphisms than RAPD analysis. AFLP-based finger printing has been applied for characterization of genetic diversity in a germplasm collection of mulberry for the first time by Sharma *et al*. (2000). In the present study, AFLP markers were used to characterize 4 M_1 plants in comparison to parent cultivars and control hybrid. These variants produced male catkins and mixed catkins consisting of bisexual and female

florets. Eight primer combinations amplified an average of 140 AFLP markers across the eight plants analyzed. A high degree of polymorphism ranging from 77.7-97.6% was observed using all the primer combinations. The highest number of polymorphic markers was observed using the primer combination EACC/MCTC. The primer combinations used the present investigation were different from those used in the previous studies. All the primer combinations tested produced distinct banding pattern in the parents and the morphological variants and could be useful for fingerprinting purposes in mulberry. Recently, Botton *et al*. (2005) demonstrated the utility of AFLP technique in determining the origin of introduced mulberry (*Morus* spp.) accessions in Italy.

Comparison of the AFLP banding pattern of M_1 plants with the parent cultivars showed a high degree of genetic variation. It was interesting to note that the control hybrid had 22 M-5 specific bands and 26 S-13 specific bands whereas the variants lacked many S-13 specific bands (22-30) apart from the presence of many unique bands (23-135). Cluster analysis revealed that the morphological variants *viz*., Plant 40 and Plant 45 were distinct from the parent cultivars as well as China white cultivar. Thus the magnitude of variation observed in the M_1 plants was found to be higher than that observed between M-5 and China White cultivars which belonged to different species. Thus the study showed that changes observed in the morphology and sex expression in the M_1 plants were the result of variations at the DNA level. Further experiments are required in order to isolate and characterize the nucleotide sequence of the highly polymorphic markers identified in this work. The majority of polymorphic AFLP fragments are considered as the result from base substitutions within the restriction sites or within the nucleotides complementary to the selective nucleotides (Kuiper, 1998).

Polanco and Ruiz (2002) showed the usefulness of AFLP in identifying the specific genomic alterations associated with tissue culture variation in the regenerated plants of *Arabidopsis thaliana*. Scott *et al*. (2000) used AFLP markers for distinguishing an early mutant of Flame seedless grape. Two AFLP markers could be successfully used for differentiating the somatic mutant from its parental line. Wang *et al*. (2005) demonstrated extensive *de novo* genomic variation in rice induced by introgression from wild rice (*Zizania latifolia* Griseb) using AFLP analysis. These variations in Recombinant inbred lines (RILs) were found to represent base substitutions or small insertions/deletions.

The present experiments on RAPD and AFLP analysis of M_1 plants showed the superiority of AFLP over RAPD method as more number of bands was produced per assay with greater number of polymorphic markers. These results are in conformity with that reported by Vogel *et al*. (1994) and Sanchez *et al*. (1999) where AFLP analysis detected about 12 times the number of polymorphic loci per assay as compared to RAPD in soybean varieties.

In the present study, the two variants *viz.*, Plant 14 and Plant 15 were found to be clustered together when AFLP analysis was used but were placed away in RAPD analysis. The differences observed in clustering of AFLP and RAPD could be explained by the difference in the DNA segments targeted by the two methods. The variation observed between RAPD and AFLP might be due to the fact that the PCR amplified profiles in the two marker assays originated from different repetitive and non-repetitive regions of the genome. In a recent work, Gil-Vegas *et al*. (2006) also demonstrated the

existence of asexual genetic variability in *Agave tequilana* using AFLP, in contraction with their previous finding with RAPD, also supporting the fact that different marker systems, e.g. AFLP and RAPD, examine distinct regions in the genome. From these results, it is apparent that different methods individually reveal information about distinct and different regions of the genome.

SUMMARY

The present studies were initiated with an aim to induce haploids through anther, unpollinated ovary cultures and pollen irradiated method. The ploidy level of M_1 plants obtained after pollination with M-5 and China White pollen separately was determined using flow cytometry and cytology. The M_1 plants obtained from irradiated pollen experiments were characterized using molecular markers in order to find out the extent of similarity to parents and absence of paternal markers if any. The significant findings that emerged from the study are as follows:

- Anther culture studies led to induction of callus with a frequency of 6.8% from anthers of S-13 cultivar on MS medium with 8 mg/l 2,4-D, 0.1 mg/l NAA and 6% sucrose. Rhizogenesis was observed from the callus upon subculture on medium with 1 mg/l 2,4-D, 0.1 mg/l NAA and 3% sucrose.
- Studies on unpollinated ovary cultures of M-5 cultivar showed callus induction with a frequency of 4.5% on MS medium with 2 mg/l 2,4-D, 0.5 mg/l BAP and 3% sucrose and formation of nodular structures upon subculture on medium with 2 mg/l BAP and 3% sucrose.
- Pollen irradiation had a profound effect on pollen viability and decreased to 9.8% with the increase in the dose of gamma irradiation to 2000 Gy. The germination capacity of S-13 irradiated pollen decreased with the increase in the irradiation dose (50-1000 Gy) under *in vitro* and *in situ* conditions. The reduced pollen germination by gamma irradiation could be due to hampered protein synthesis.
- Irradiation of S-13 pollen up to 1000 Gy resulted in seed set whereas doses above 1000 Gy led to withering of the fruits.

- Interspecific hybridization between M-5 and China White cultivars was successful and seed set was observed with irradiated pollen from 50 to 1000 Gy.
- The germination capacity of the seeds obtained from crosses of M-5 with S-13 and China White irradiated pollen decreased with increase in irradiation dose and was lowest at 1000 Gy irradiation dose. The possible causes of germination reduction could be due to lack of completely developed or abnormal embryos within the seeds.
- Morphological variations were observed in M_1 plants obtained after pollination with S-13 irradiated pollen. Eight variants were found to be superior with respect to leaf size and one variant *viz*., Plant 40 exhibited vigorous growth along with large leaf size than parents.
- Interspecific hybridization of M-5 cultivar with China White irradiated pollen resulted in isolation of 18 morphological variants which displayed differences with respect to leaf size, shape and margin, plant height, stem thickness and internodal distance. While two M_1 interspecific hybrids exhibited larger leaf size than M-5 cultivar, eight variants exhibited slow growth with small leaves.
- Flow cytometric analysis of M_1 plants obtained from S-13 and China White irradiated pollen showed a peak corresponding to diploid as that of parents.
- Cytological studies using root tip squash method showed that the M_1 plants obtained after pollination with irradiated pollen were diploids (2n=28).
- Four M_1 plants obtained from crosses of M-5 with S-13 irradiated pollen produced male catkins and mixed catkins with female and bisexual florets. The

fruits of mixed catkins of Plant 40 were larger by 2 times as compared to that of the female parent.

- Analysis of leaf protein profiles of morphological variants using SDS-PAGE showed an increase in the content of 47 kDa and 18 kDa proteins in three variants obtained from S-13 irradiated pollen and six variants obtained from China White irradiated pollen in comparison to the parents indicating differential gene expression.
- RAPD analysis of the morphological variants obtained from S-13 irradiated pollen using nineteen primers revealed polymorphism ranging from 33.3-100%. The morphological variants showed 16-29 bands specific to M-5 cultivar and 1-3 bands specific to S-13 cultivar of a total of 35 M-5 specific bands and 3 S-13 specific bands identified with different primers.
- The dendrogram derived from UPGMA cluster analysis grouped the variants into three major groups and five outliers. Three morphological variants *viz*., 33, 34 and 57 were placed in the sub-group of A along with M-5 cultivar depicting high similarity towards the female parent while many morphological variants were placed away from the parents indicating the existence of high level of genetic variation.
- RAPD analysis of the morphological variants obtained from China White irradiated pollen using 10 random primers showed polymorphism ranging from 56.3-100%. The M_1 interspecific hybrids showed the presence of 3-6 bands specific to M-5, 7-22 bands specific to China White of a total of 6 M-5 specific bands and 25 China White specific bands identified with different primers. The

dendrogram generated by the UPGMA cluster analysis grouped the morphological variants into two major groups and an outlier revealing differences with parents and among themselves in varying degrees.

- AFLP analysis of four morphological variants that produced male catkins and mixed catkins with bisexual and female florets revealed a high degree of polymorphism from 77.0% to 97.6% with eight primer combinations. The variant Plant 40 was found to be highly distinct from parents and possessed 14 bands specific to M-5 cultivar, 3 bands specific to S-13 cultivar and 135 unique bands. The dendrogram showed that the two variants *viz*., Plant 40 and Plant 45 got entirely separated from the tree with low similarity to parents.

In conclusion, the present study revealed a low success rate of callus induction without plant regeneration from anther and unpollinated ovary cultures in mulberry. The pollen irradiation method was chosen as an alternative for production of haploids. Although no haploid could be recovered through this approach, a large number of variants were observed which could be used in varietal improvement programmes. Thus the study showed the usefulness of pollen irradiated method for recovering promising variants in mulberry including those altered in sex expression with induction of mixed catkins consisting of bisexual and female florets. Interspecific hybridization of M-5 cultivar with China White irradiated pollen resulted in recovery of two useful variants with larger leaf size than M-5 cultivar. Molecular characterization of the morphological variants obtained from intervarietal and interspecific crosses with irradiated pollen revealed a high level of genetic variation. A detailed genetic, cytogenetic and molecular

analysis employing large number of markers will throw light on the nature of genetic variation.

Future Scope of Work

- The efforts aimed at isolation of haploids through *in vitro* androgenesis, gynogenesis and irradiated pollen approach have resulted in promising variants which will be very useful for basic and applied research. These promising variants can be subjected to genetic and cytogenetic analysis for understanding the basis of inheritance.
- Four M_1 plants obtained after pollination with S-13 irradiated pollen produced male catkins and mixed catkins with female and bisexual florets. These variants offer scope for understanding the molecular basis of sex expression and identification of molecular markers linked to sex if any.
- The promising variants can be multiplied and analyzed for leaf yield and quality parameters for silkworm rearing for utilization in breeding programmes of mulberry.

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Fig. 1

Fig. 2

Fig. 3

Fig. 1: Differentiation of female catkin and shoot from the sprouted axillary bud of M-5 cultivar on MS medium with 0.3 mg.l 2,4-D

Fig. 2: Differentiation of male catkins from the sprouted axillary bud of V-1 cultivar on MS medium with 0.3 mg/l 2,4-D

Fig. 3: Differentiation of male catkins and shoot from the sprouted axillary bud of S-13 cultivar on MS medium with 0.3 mg/l 2,4-D

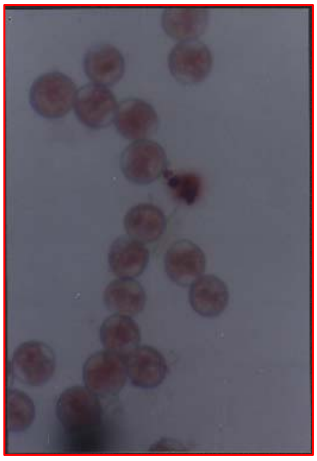


Fig. 4

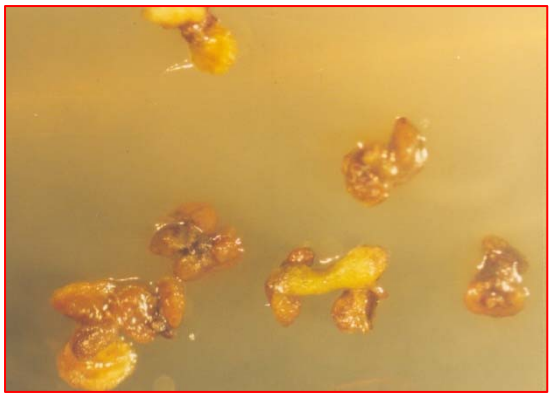


Fig. 5

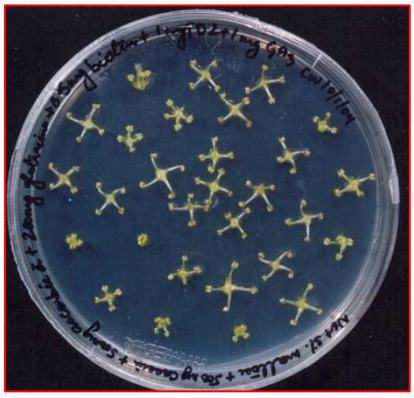


Fig. 6

Fig. 7

Fig. 4: Microspores of S-13 cultivar at uninucleate stage of development

Fig. 5: Browning of anthers of S-13 cultivar after culture on MS medium with 16 mg/l 2,4-D + 0.1 mg/l NAA + 6% sucrose

Fig. 6 & 7: Dehiscence of anthers from the cultured staminate flowers of S-13 cultivar after 12 days of culture

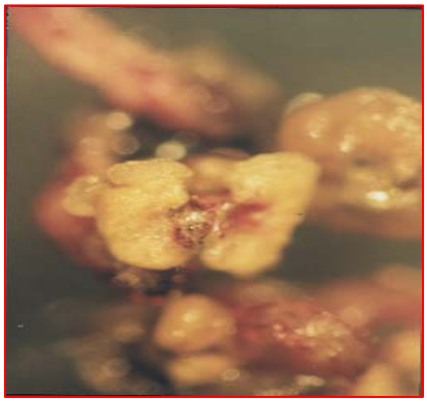


Fig. 8

Fig. 9

Fig. 8: Enlargement of the anther lobes of the cultured staminate flowers of S-13 cultivar

Fig. 9: Development of hard callus from the anthers of S-13 cultivar cultured on MS medium with 8 mg/l 2,4-D + 0.1 mg/l NAA + 6% sucrose

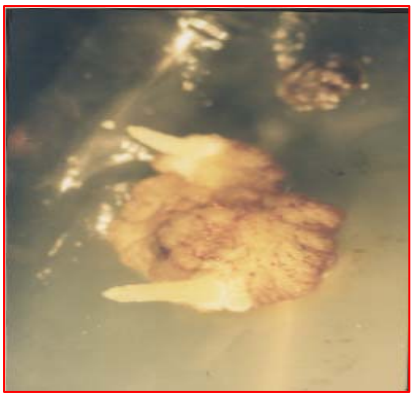


Fig. 10

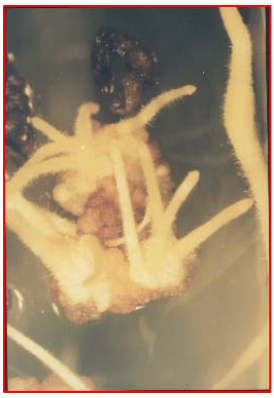


Fig. 11

Fig. 12

Fig. 10 &11: Induction of rhizogenesis from the callus derived from anthers of S-13 cultivar on MS medium with 1 mg/l 2,4-D + 0.1 mg/l NAA + 3% sucrose

Fig. 12: Callus induction from the anthers of V-1 cultivar on MS medium with 4 mg/l 2,4-D + 0.1 mg/l TDZ + 6 % sucrose

Fig. 13

Fig. 14

Fig. 13: Pistillate flowers of M-5 cultivar used for gynogenesis

Fig. 14: Enlargement of ovaries of the pistillate flowers of M-5 cultivar cultured on MS medium with 2 mg/l 2,4-D + 0.5 mg/l BAP + 3% sucrose

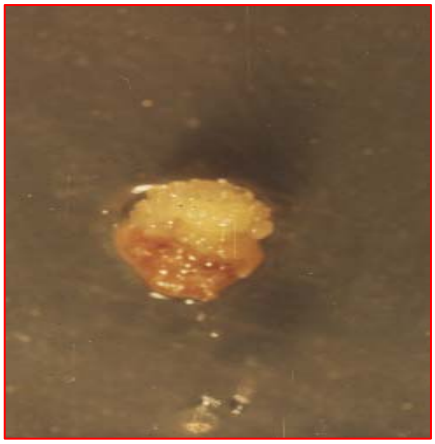


Fig. 15

Fig. 16

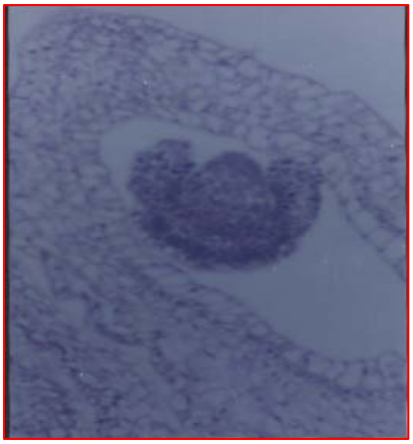


Fig. 17

Fig. 15: Induction of callus from the unpollinated ovaries of M-5 cultivar cultured on MS medium with 2 mg/l 2,4-D + 0.5 mg/l BAP + 3% sucrose

Fig. 16: Induction of green, nodular structures from the callus derived from unpollinated ovaries of M-5 cultivar cultured on MS medium with 2 mg/l BAP + 3 % sucrose

Fig. 17: Development of an embryo within 30 day-old unpollinated ovule of M-5 cultivar cultured on MS medium with 2 mg/l 2,4-D + 0.5 mg/l BAP + 3% sucrose

Fig. 18: M-5 plants of mulberry bagged for pollination experiments

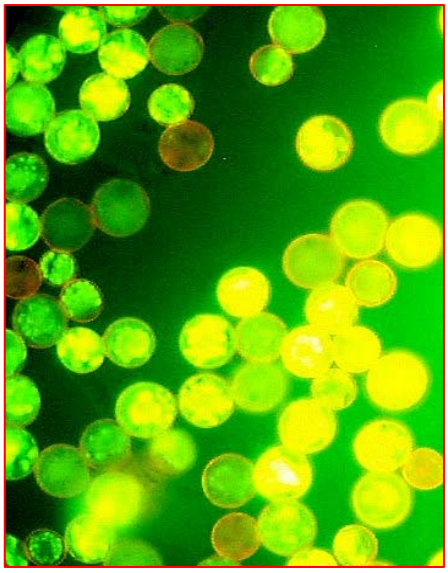


Fig. 19

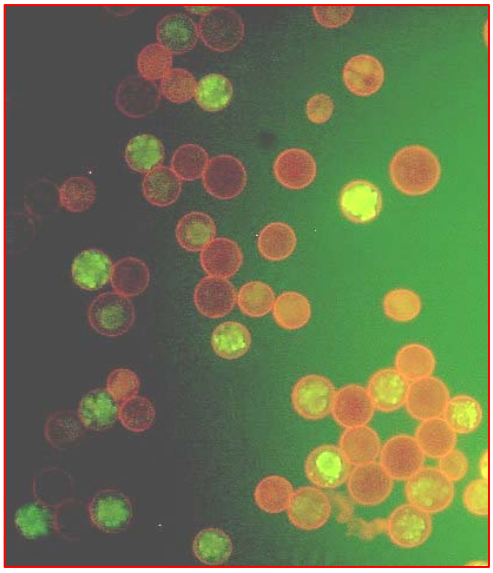
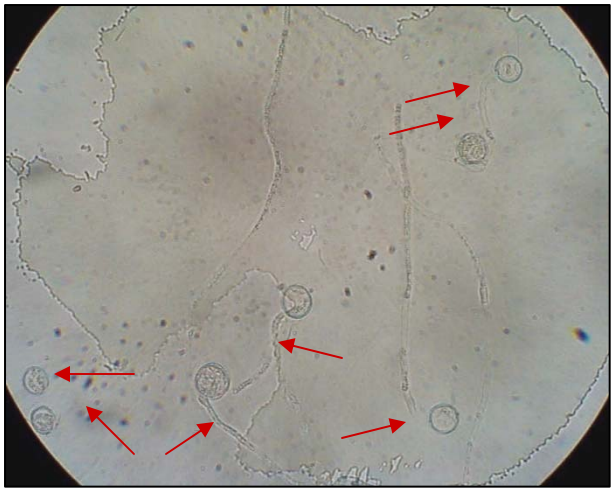


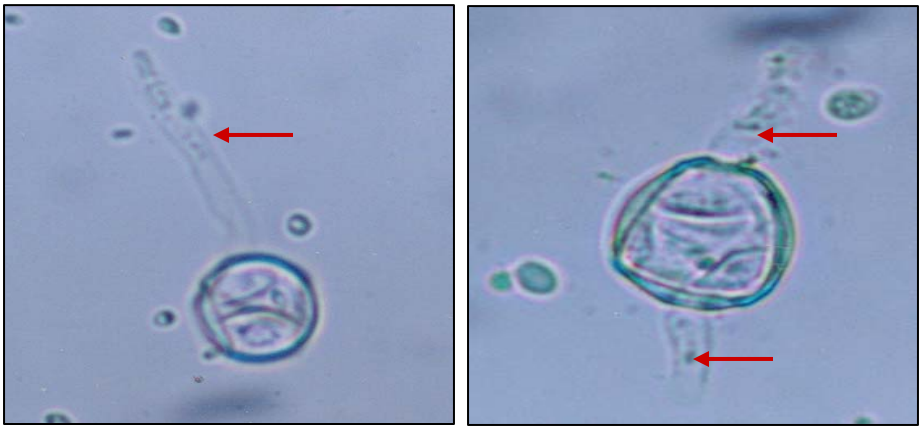
Fig. 20

Fig. 19: Viability of control pollen after staining with FDA and PI (Green fluorescence emitted by viable pollen and red fluorescence by non-viable pollen)

Fig. 20: Viability of irradiated pollen (1000 Gy) after staining with FDA and PI



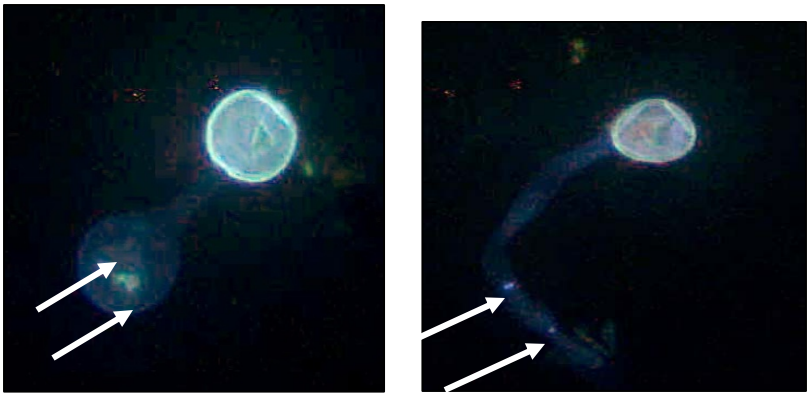
(a)



(b) (c)

Fig. 21 a-c: *In vitro* germination of S-13 pollen

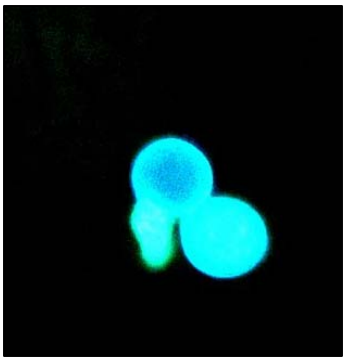
(a) (b)



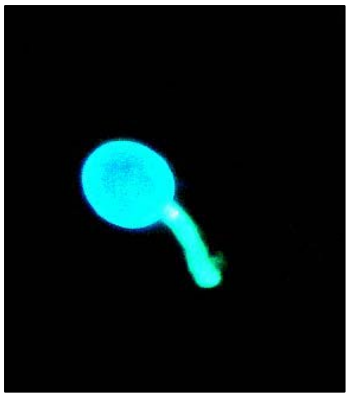
(c) (d)

Fig. 22 a-d: Fluorescence micrographs of different stages of germination in control pollen of S-13 cultivar

(a)

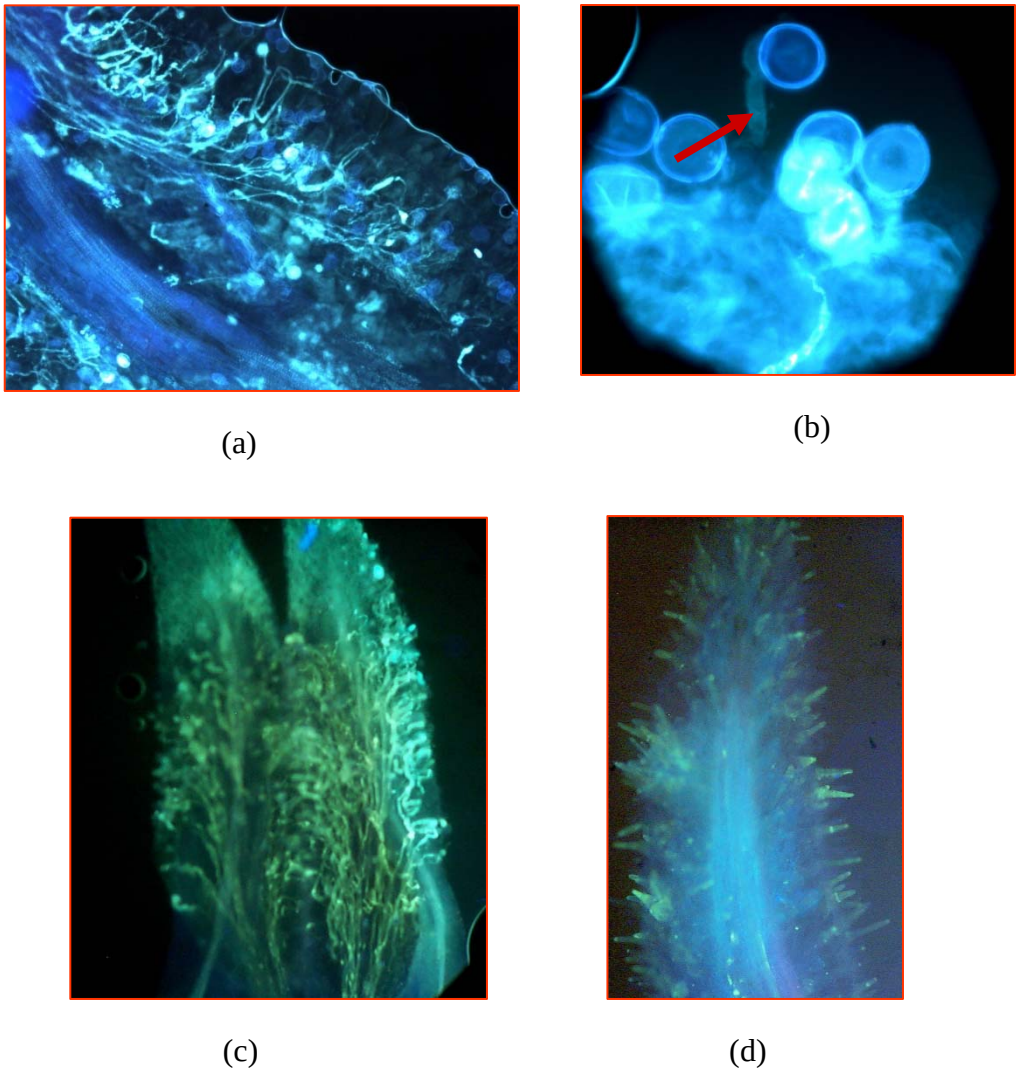


(b)



(c)

Fig. 23 a-c: Fluorescence micrographs of different stages of germination in irradiated (1000 Gy) pollen of S-13 cultivar



(a) (b)

(c) (d)

Fig. 24 a-d: Aniline blue staining of control and irradiated S-13 pollen during *in situ* germination

(a) Control pollen germinating on the stigma of M-5 plant

(b) Enlarged view of a single pollen tube entering the stigmatic surface

(c) Control pollen showing germination after 24 hrs of placing on stigma of M-5 plant

(d) 2000 Gy irradiated pollen showing no germination

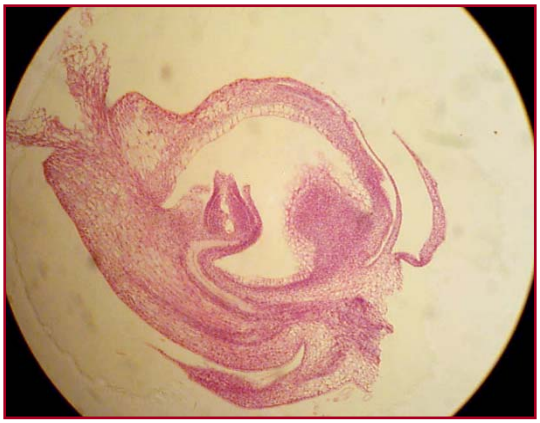


Fig. 25

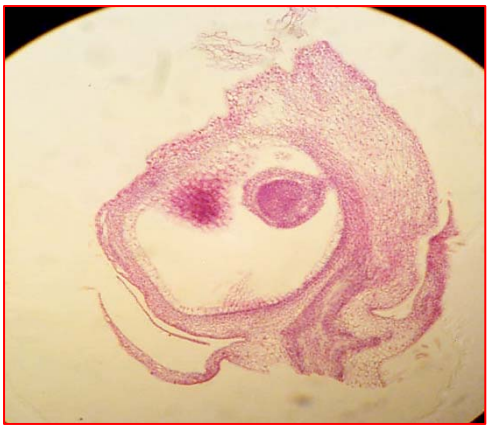


Fig. 26

Fig. 25: Anatomical section of unpollinated ovule of M-5 cultivar

Fig. 26: Embryo and endosperm development in ovules pollinated with control pollen

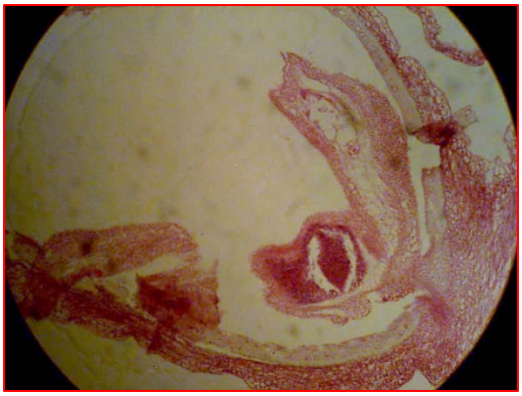


Fig. 27

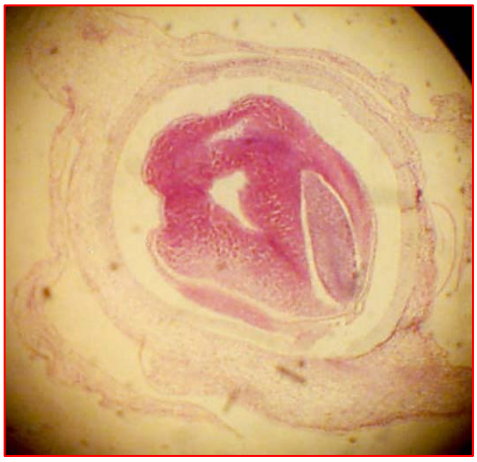


Fig. 28

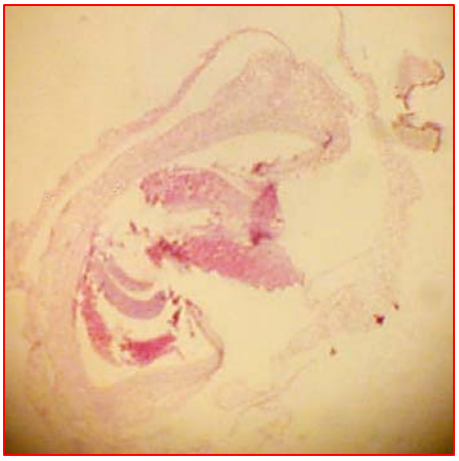


Fig. 29

Fig. 27: Development of twin embryos in the embryo sac of M-5 cultivar after pollination with 50 Gy irradiated pollen

Fig. 28: Embryo and endosperm development in the embryo sac of M-5 cultivar after 10 days of pollination with S-13 irradiated (80 Gy) pollen

Fig. 29: Abnormal embryo development and endosperm disintegration in the embryo sac of M-5 cultivar after 10 days of pollination with S-13 irradiated (100 Gy) pollen

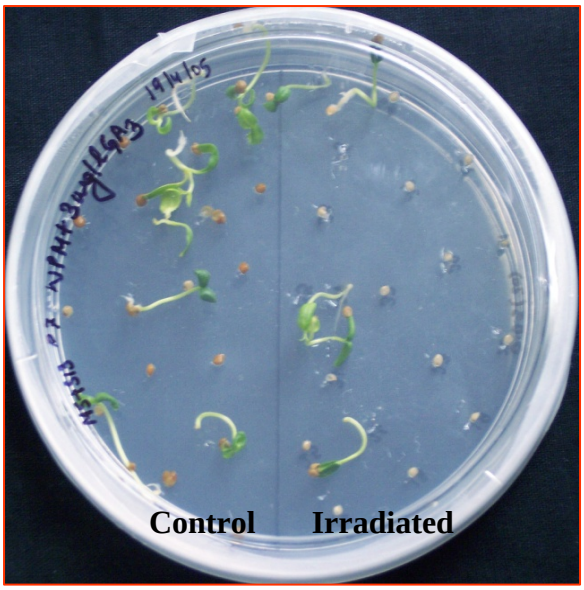
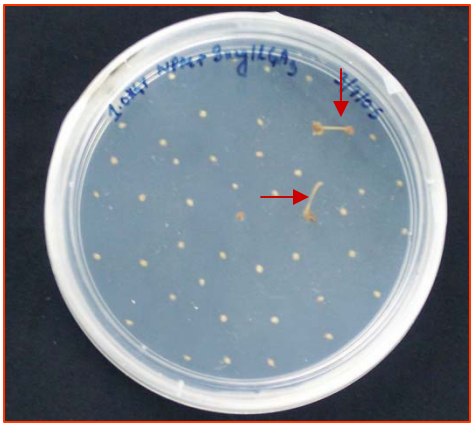


Fig. 30: Germination response from seeds obtained from control and 1000 Gy irradiated pollen of S-13 cultivar

(a)

(b)



(c)

(d)

Fig. 31: Germination response of seeds obtained after crossing M-5 with control and irradiated pollen of S-13.

(a) Germination response from seeds obtained after pollination with control pollen of S-13 cultivar on Woody Plant medium containing 3 mg/l GA_3

(b) Enlarged view of a seedling obtained after pollination with S-13 pollen

(c) Germination response from seeds obtained after pollination with 1000 Gy irradiated pollen of S-13 cultivar

(d) Enlarged view of a seedling obtained after pollination with S-13 irradiated pollen that died immediately after emergence

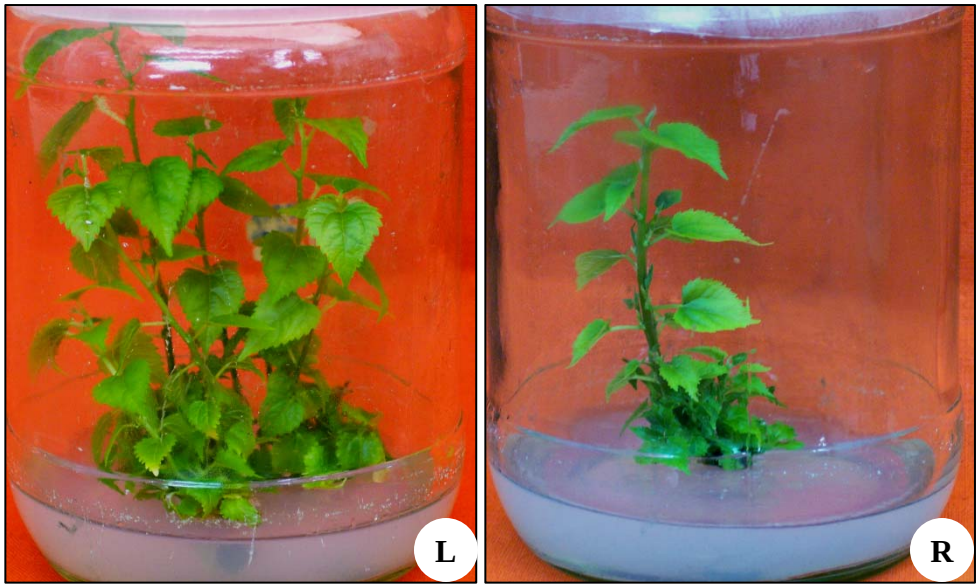
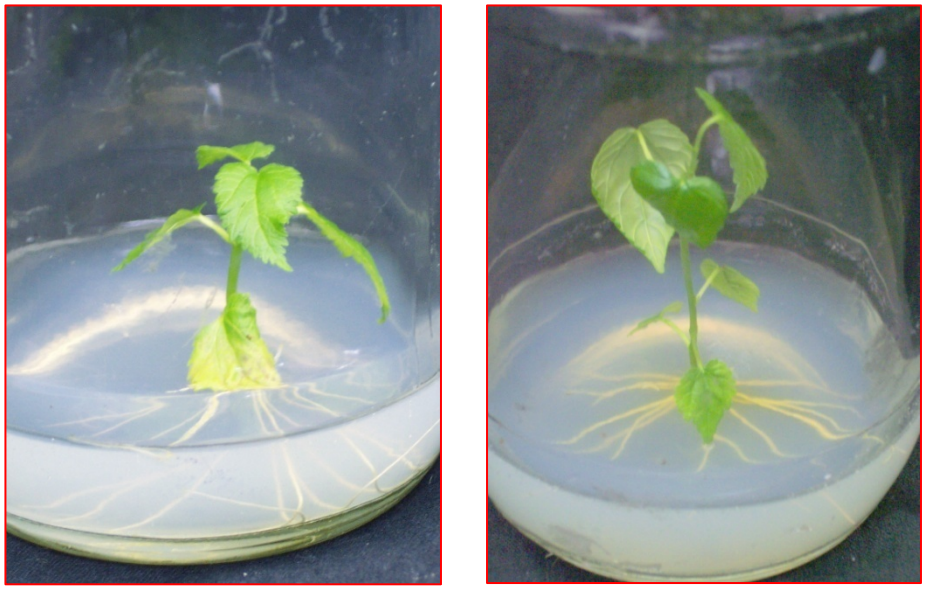


Fig. 32: Response of shoot multiplication from the plants obtained after pollination with control and irradiated pollen on MS medium with 0.5 mg/l BAP

Left: Two-month-old culture of control plant

Right: Two-month-old culture of Plant 36 obtained after pollination with 50 Gy irradiated pollen



(a) (b)

Fig. 33: Comparison of root induction from shoots of control and M_1 plant obtained after pollination with S-13 irradiated pollen

(a) Root induction from shoot of control plant after one month of culture on MS medium with 0.1 mg/l IBA

(b) Root induction from shoot of Plant 16 after one month of culture on MS medium with 0.1 mg/l IBA

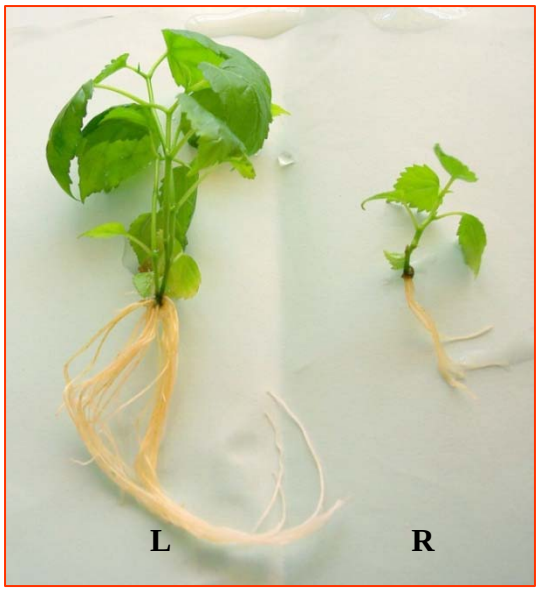


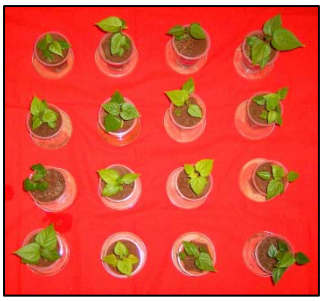
Fig. 34: Rooting response from shoots of control and M_1 plant obtained after pollination with S-13 irradiated pollen

Left: Roots developed in control shoot after one month of culture on MS medium with 0.1 mg/l IBA

Right: Roots developed in Plant 23 after one month of culture on MS medium with 0.1 mg/l IBA

(a)

(b)



(c)

(d)

(e) (f)

Fig. 35: a-f: Acclimatization and field establishment of *in vitro* raised plants obtained after pollination with control and irradiated pollen along with parents

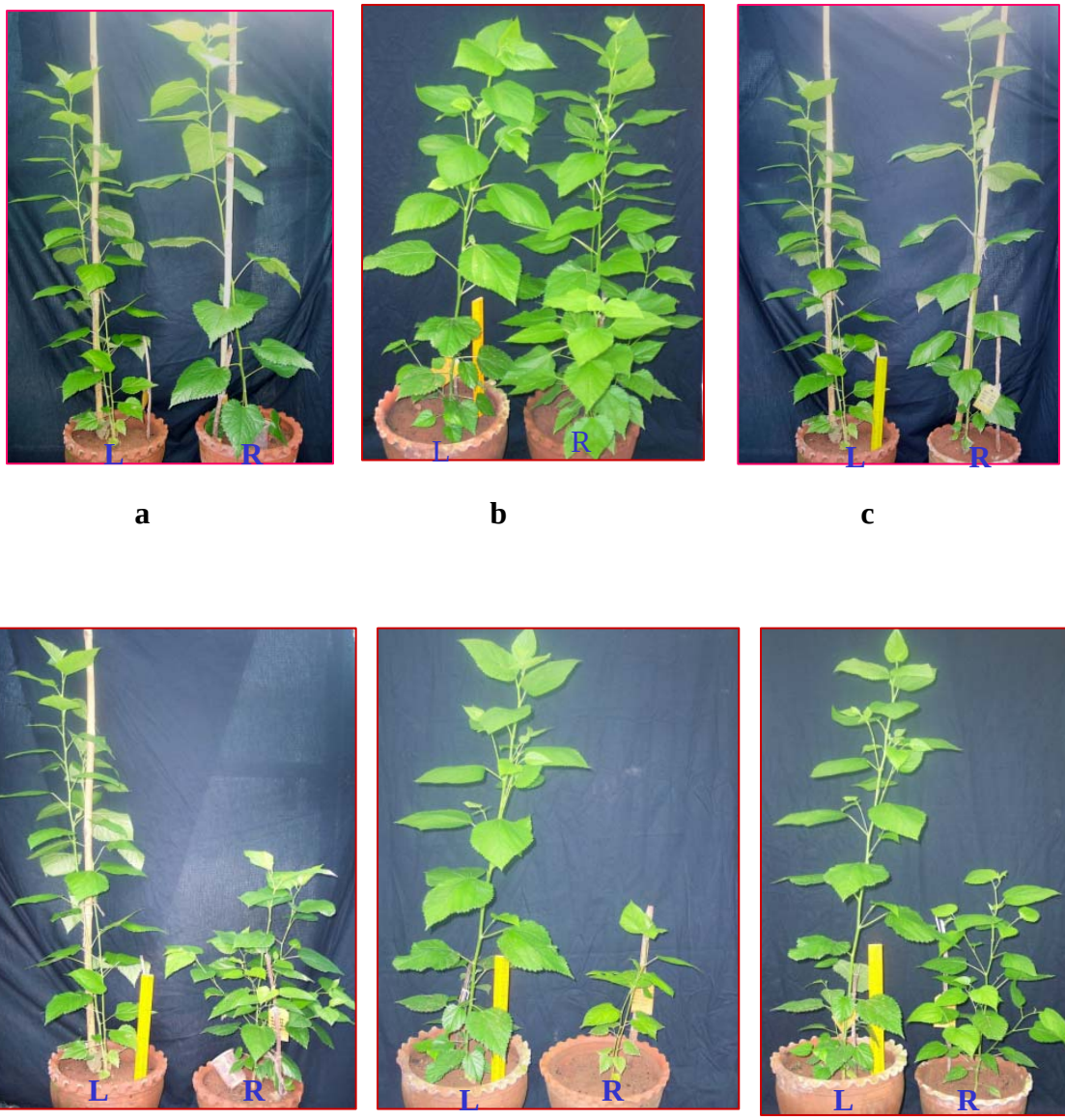


Fig. 36 a-f: Comparison of 3-month-old plants obtained after pollination with control and S-13 irradiated pollen

(a) Left: Control plant Right: Plant 40 (80 Gy)

(b) Left: Control plant Right: Plant 15 (50 Gy)

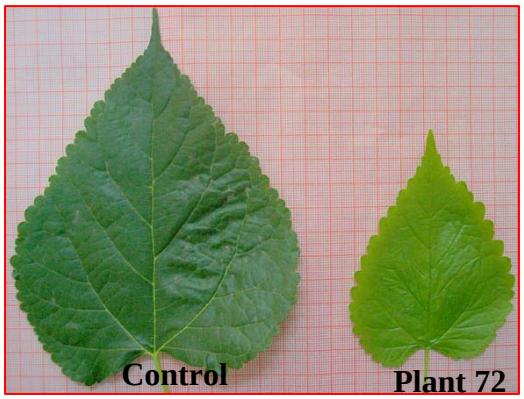
(c) Left: Control plant Right: Plant 33 (80 Gy)

(d) Left: Control plant Right: Plant 32 (100 Gy)

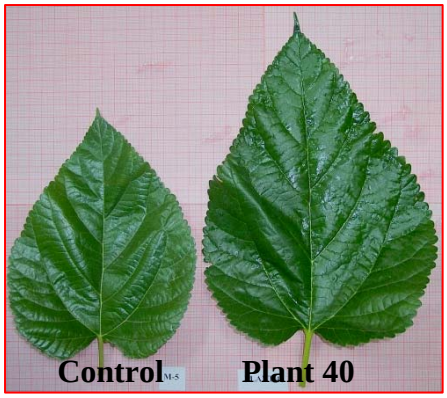
(e) Left: Control plant Right: Plant 72 (50 Gy)

(f) Left: Control plant Right: Plant 52 (80 Gy)

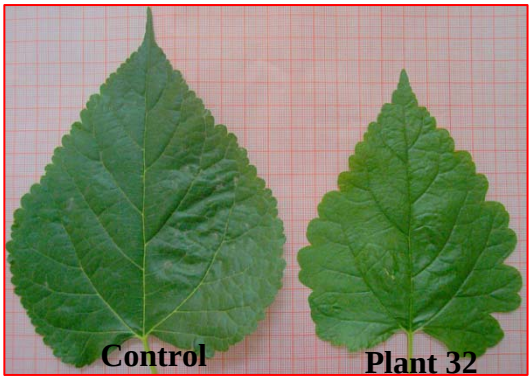
a



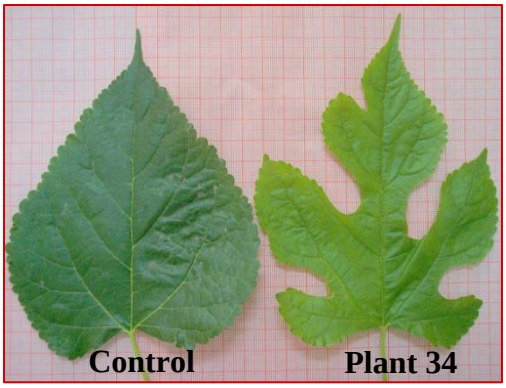
b



c



d



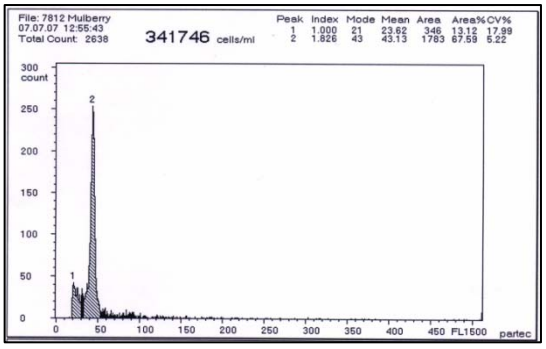
e

Fig. 37 a-e: Comparison of leaves of morphological variants with control hybrid and parents

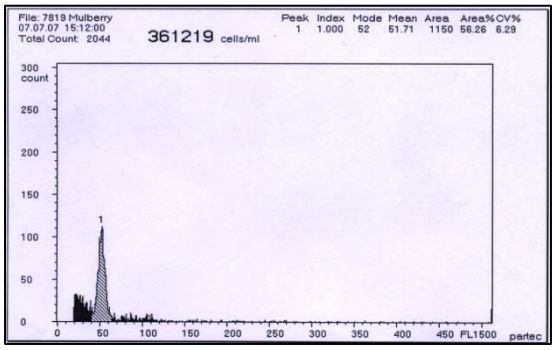
a) Leaves of M-5 and S-13 cultivars b) Leaves of Plant 72 (Small leaf) with control

c) Leaves of Plant 40 (Large leaf) with control

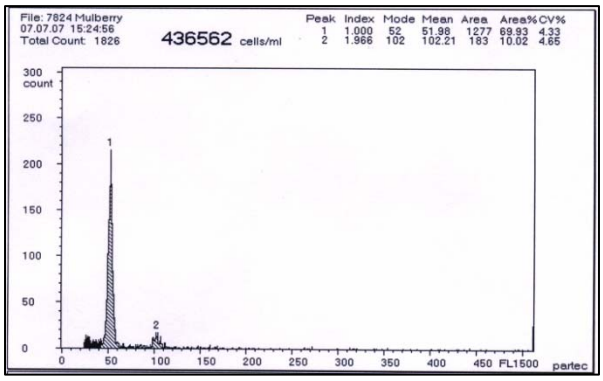
d) Leaves of Plant 32 (Bicrenate leaf) with control f) Leaves of Plant 34 (Five-lobed leaf) with control



a



b



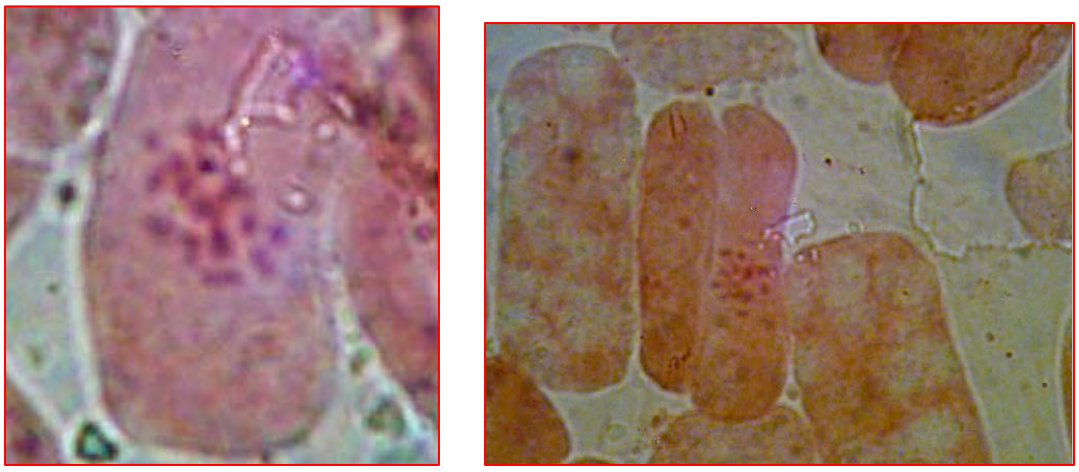
c

Fig. 38 a-c: Flow Cytometric analysis of leaf tissues of plants obtained after pollination with control and irradiated pollen

(a) Control plant

(b) Plant 14

(c) Plant 40

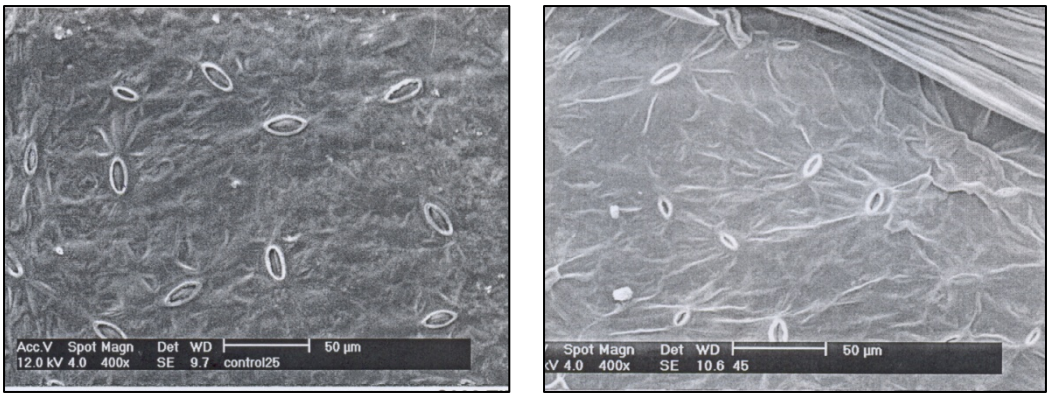


a b

Fig. 39 a-b: Metaphase plate of root tip cell of control plant and morphological variant

(a) Control plant (2n = 28)

(b) Plant 63 (2n = 28)

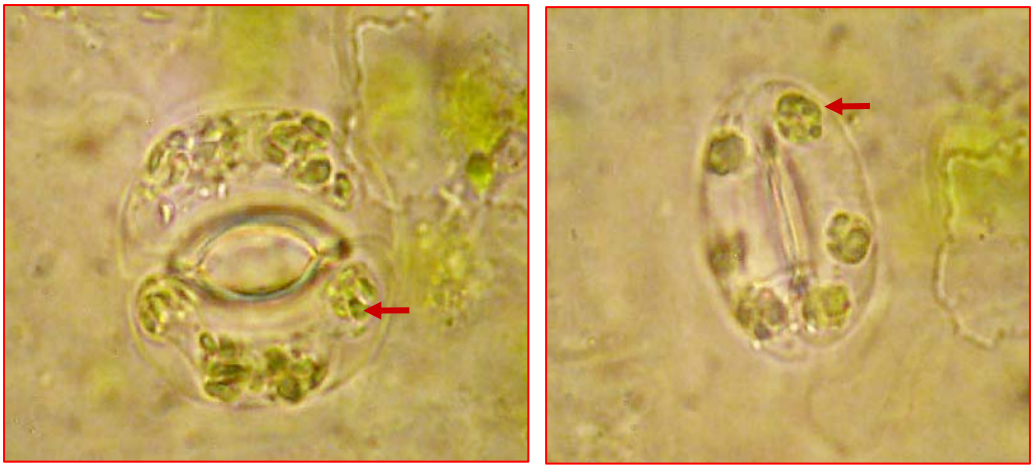


a b

Fig. 40 a-b: Scanning electron micrographs of leaf surface of plants obtained after pollination with control and S-13 irradiated pollen

(a) Control Plant

(b) Plant 40



a b

Fig. 41a-b: Comparison of stomatal chloroplast of plants obtained after pollination with control and irradiated pollen

(a) Control plant

(b) Plant 40 (80 Gy)

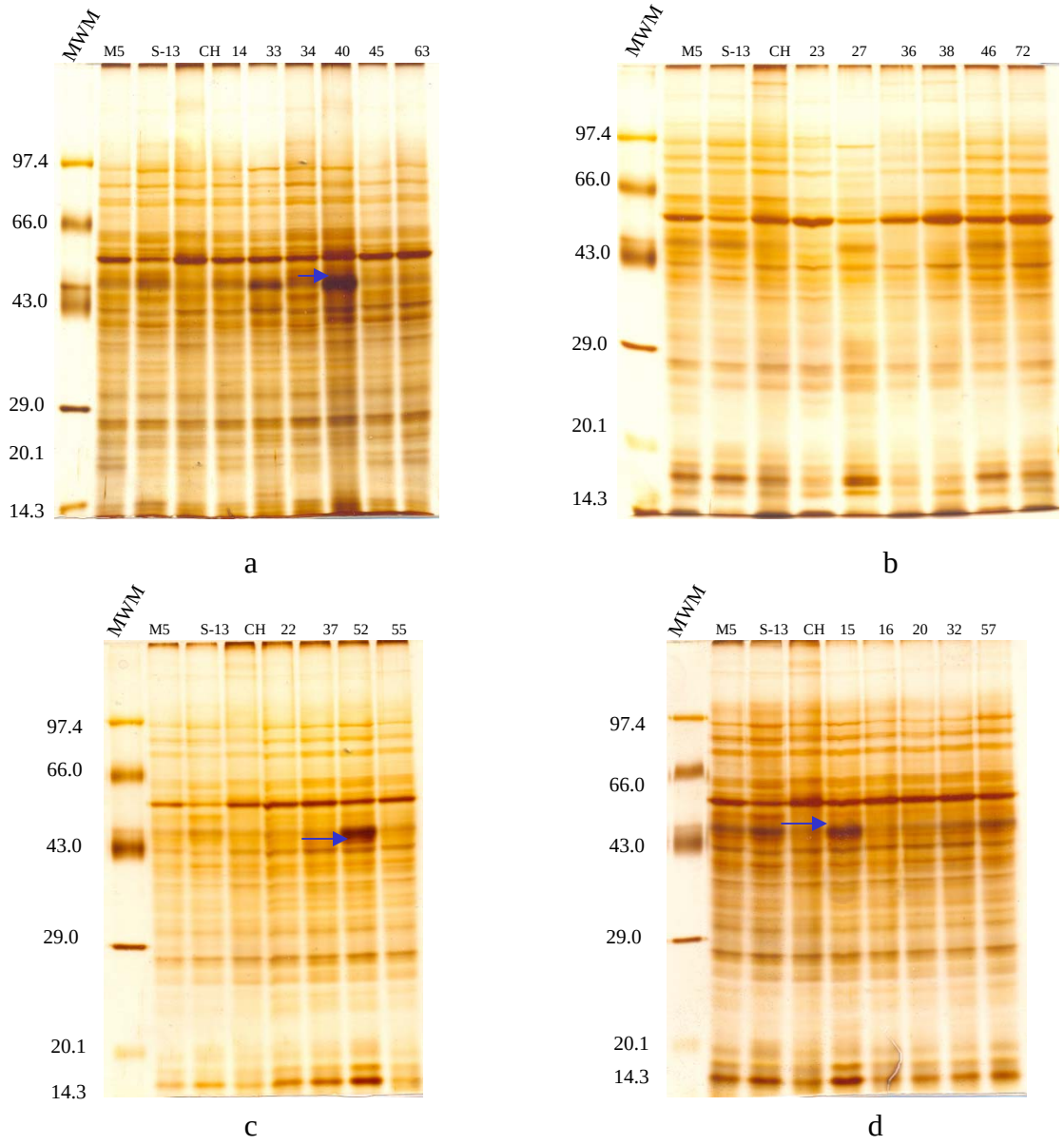


Fig. 42 a-d: Protein profiles in leaves of morphological variants obtained after pollination with S-13 irradiated pollen as analyzed on SDS-PAGE (Lane 1 in Figs. a-d represent Molecular Weight Marker (MWM) in kDa.

(a)Lanes 2, 3, 4, 5, 6, 7, 8, 9 & 10 represent proteins extracted from leaves of M-5, S-13, control hybrid (CH), Plant 14, Plant 33, Plant 34, Plant 40, Plant 45 and Plant 63, respectively.

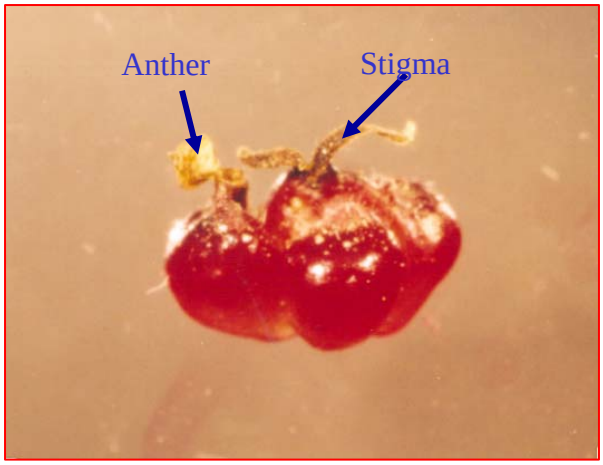
(b) Lanes 2, 3, 4, 5, 6, 7, 8, 9 & 10 represent proteins extracted from leaves of M-5, S-13, control hybrid (CH), Plant 23, Plant 27, Plant 36, Plant 38, Plant 46 and Plant 72, respectively.

(c) Lanes 2, 3, 4, 5, 6, 7 & 8 represent proteins extracted from leaves of M-5, S-13, control hybrid (CH), Plant 22, Plant 37, Plant 52 and Plant 55, respectively.

(d) Lanes 2, 3, 4, 5, 6, 7, 8 & 9 represent proteins extracted from leaves of M-5, S-13, control hybrid (CH), Plant 15, Plant 16, Plant 20, Plant 32 and Plant 57, respectively.

a

b



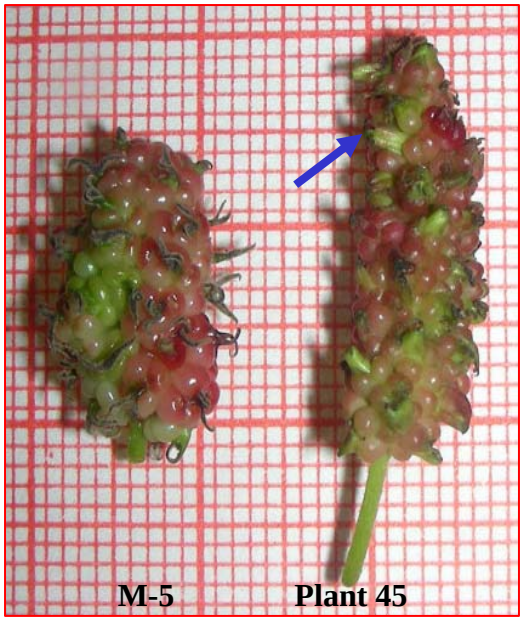
c

Fig. 43 a -c: Induction of male and mixed catkins in Plant 40 obtained after pollination with S-13 irradiated pollen

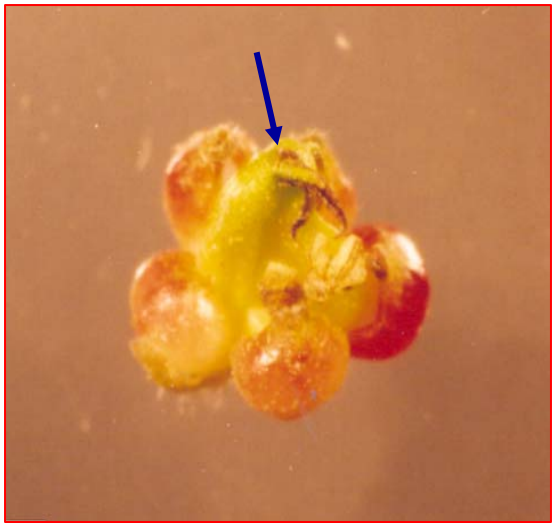
(a) Male catkins

(b) Mixed catkin with bisexual and female florets

(c) Magnified view of a single bisexual floret seen in stereomicroscope



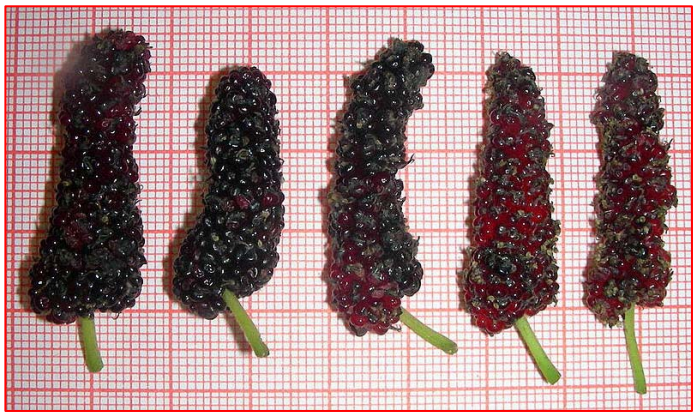
a



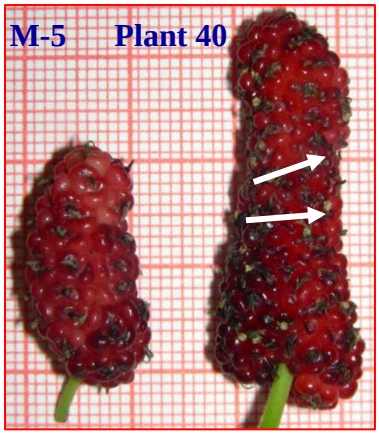
b

Fig. 44 a-b : Catkin of Plant 45 with elongated style
(a) Catkin of Plant 45 with elongated styles in comparison to M-5 catkin
(b) Magnified view of a single bisexual floret with elongated style

a



b



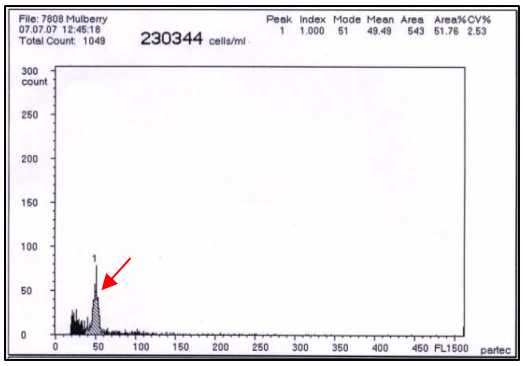
c

Fig. 45 a-c: Comparison of fruits of M-5 cultivar with fruits of Plant 40

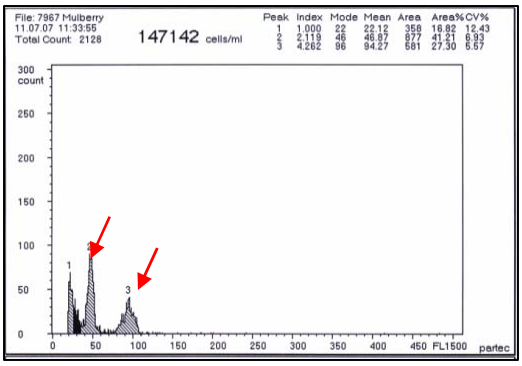
(a) Fruits of M-5 cultivar

(b) Fruits of Plant 40 developed from mixed catkins

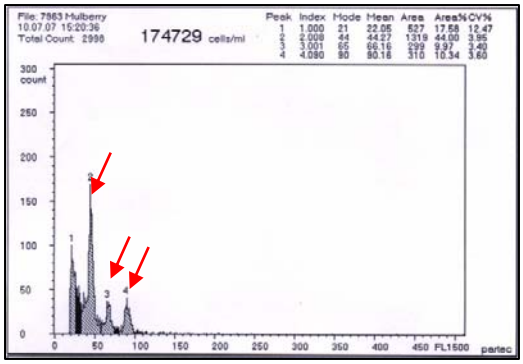
(c) Fruit of Plant 40 along with M-5 fruit (The dehisced anthers from the bisexual florets could be seen on fruits)



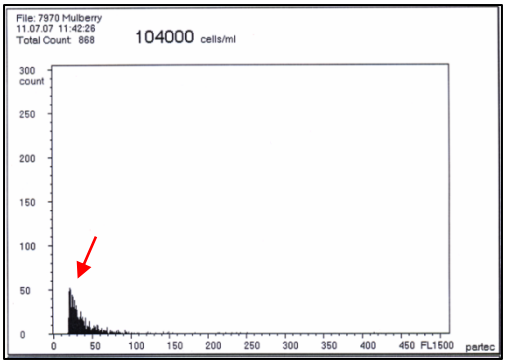
a



b



c



d

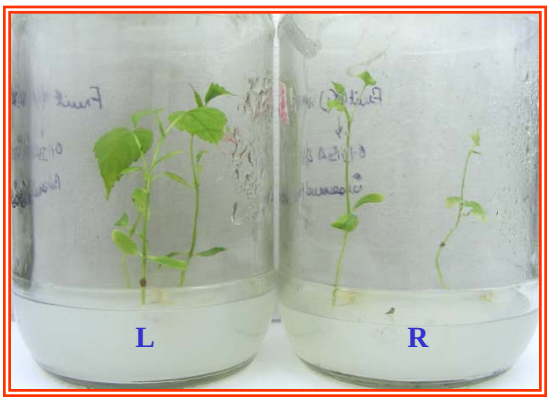
Fig. 46 a-d: Flow Cytometric analysis of seeds of M-5 cultivar and Plant 40

(a) Seed of M-5 cultivar showing single peak at 2C

(b) Bisexual seed obtained from Plant 40 showing two peaks at 2C and 4C

(c) Bisexual seed obtained from Plant 40 showing three peaks at 2C,3C and 4C

(d) Non-viable bisexual seed of Plant 40



a

b

c

Fig. 47 a-c: Comparison of plants raised from seeds of female and bisexual florets of Plant 40

(a) Plant development from seeds of female and bisexual florets of Plant 40 (Left: Female Right : Bisexual)

(b) Establishment of plants obtained from seeds of female florets of Plant 40 in pots

(c) Establishment of plants obtained from seeds of bisexual florets of Plant 40 in pots

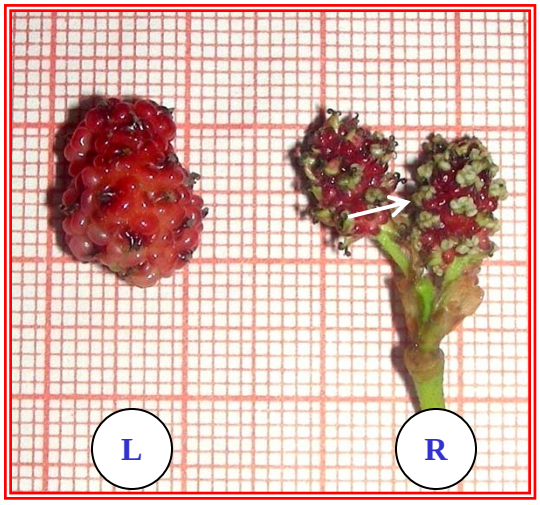


Fig. 48: Fruits of M_2 plants from bisexual seed of Plant 40 in comparison to M-5 cultivar

Left: Fruit of M-5 cultivar

Right: Fruit of M_2 plant raised from bisexual seed of Plant 40 (The dehisced anthers on the fruits are shown by arrow)

a b

Fig. 49

Fig. 49 a-b: Comparison of plant development from the seeds obtained after pollination with control and irradiated pollen of China White cultivar

a: Shoot and root development observed in control on MS medium with 0.1 mg/l IBA

b: Seedling obtained after pollination with1000 Gy irradiated pollen that died after transfer to MS medium with 0.1 mg/l IBA

a

b

c

d

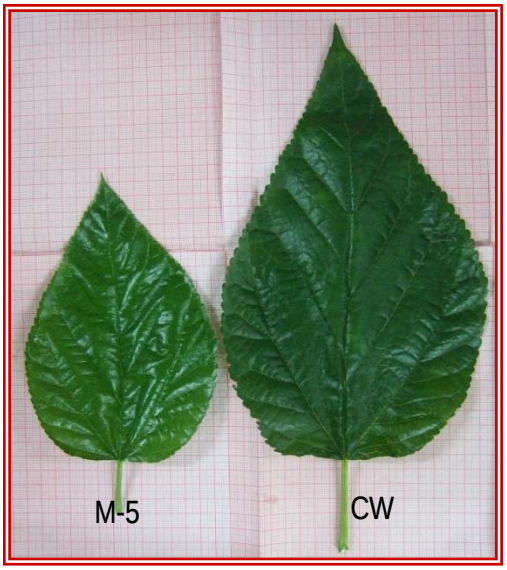
Fig. 50 a-d: Comparison of 3-month-old plants obtained after pollination with control and irradiated pollen of China White cultivar

(a) Left: Control plant Right: Plant C32 (500 Gy)

(b) Left: Control plant Right: Plant C34 (80 Gy)

(c) Left: Control plant Right: Plant C7 (1000 Gy)

(d) Left: Control plant Right: Plant C51 (1000 Gy)



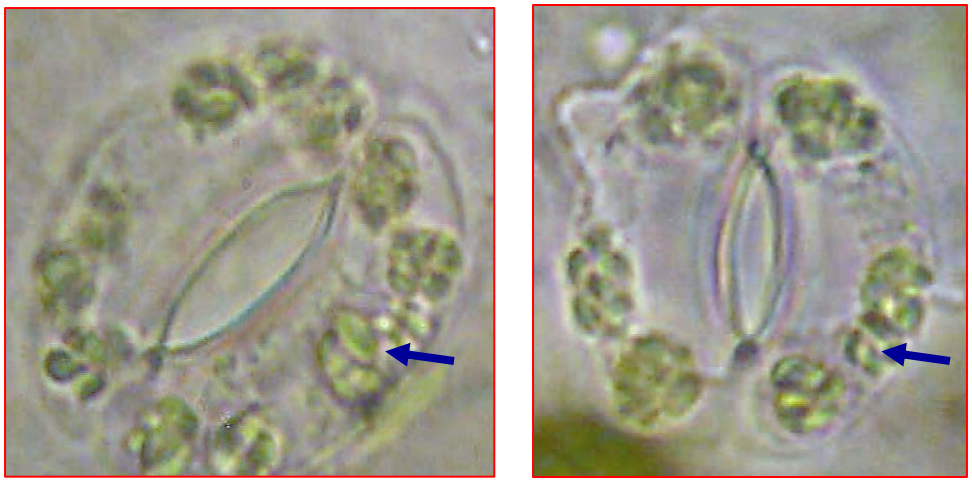
a

b

c

d

Fig. 51 a-d: Comparison of leaves of M_1 interspecific hybrids obtained after pollination with China White irradiated pollen with parents
a) Leaves of M-5 and China White cultivars
b) Leaves of Plant C17 with acuminate apex
c) Leaves of Plant C7 with cordate leaf base
d) Leaves of Plant C4 with dentate leaf margin

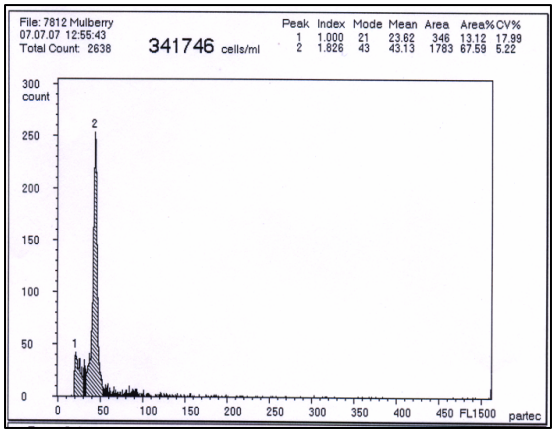


a b

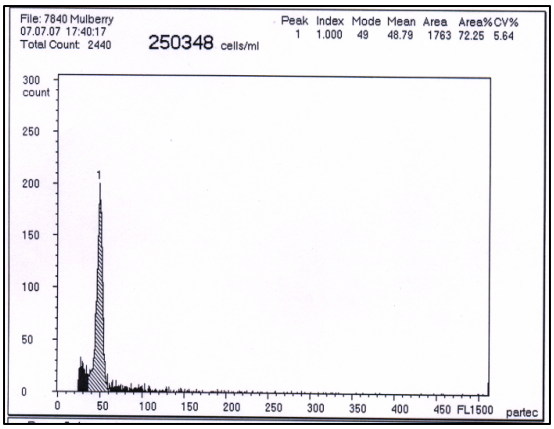
Fig. 52 a-b: Stomatal chloroplast in leaves of plants obtained after pollination with control and irradiated pollen of China White cultivar

(a) Control plant

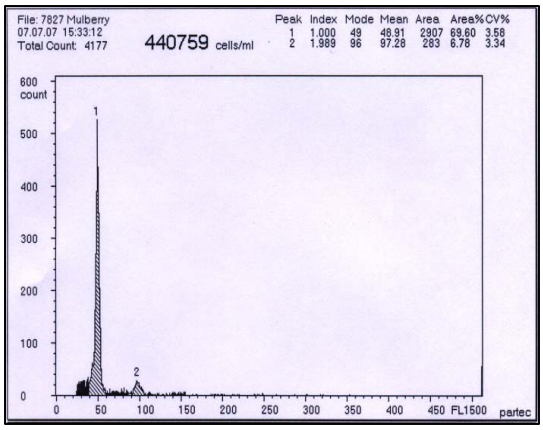
(b) Plant C7 (1000 Gy)



a



b



c

Fig. 53 a-c: Flow Cytometric analysis of leaf tissues of plants obtained after pollination with control and irradiated pollen of China White cultivar

(a) Control plant

(b) Plant C2

(c) Plant C7

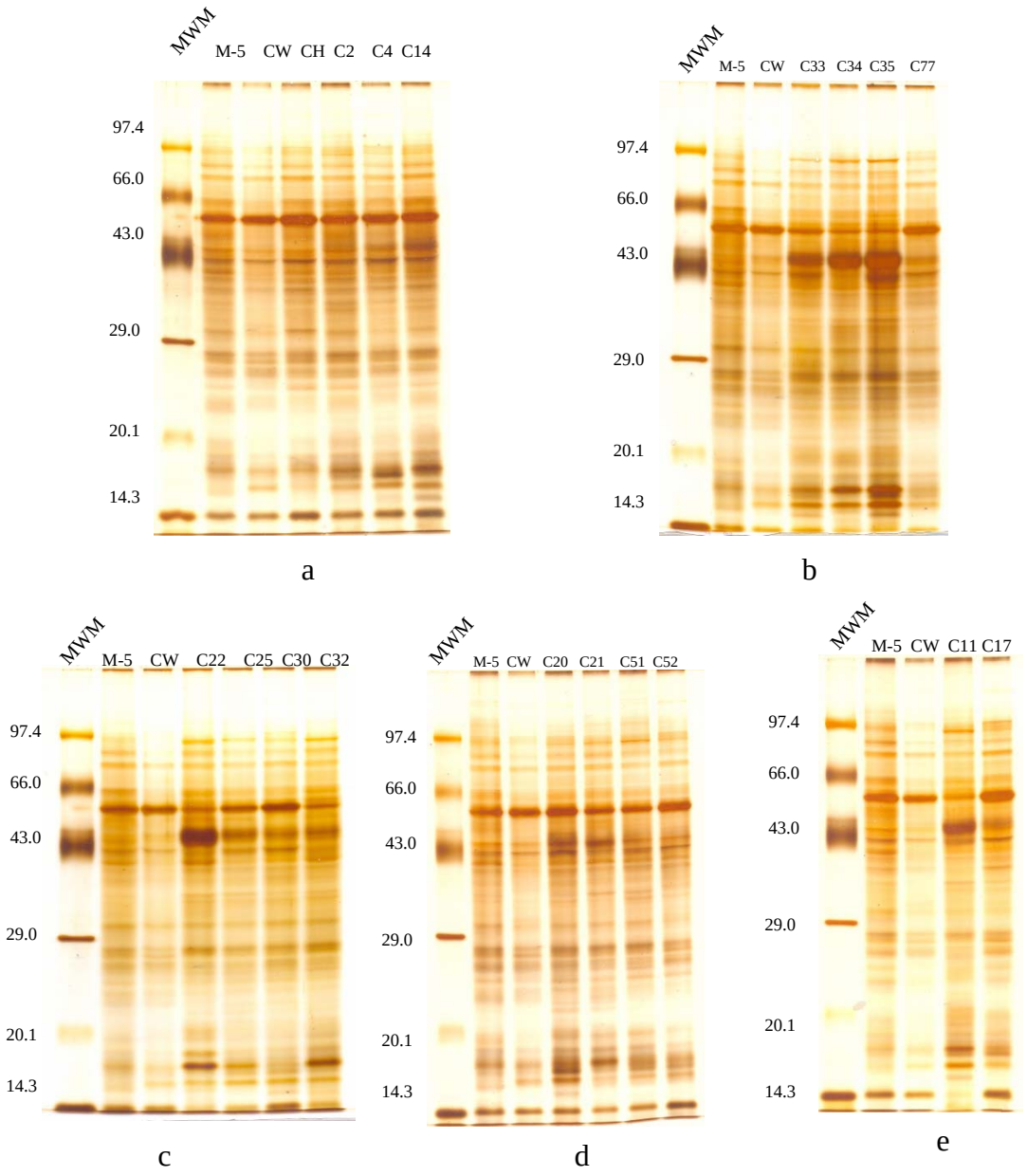


Fig. 54 a-e: Protein profiles in leaves of morphological variants obtained after pollination with China White irradiated pollen as analyzed on SDS-PAGE (Lane 1 in Figs. a-e represent Molecular Weight Marker (MWM) in kDa.

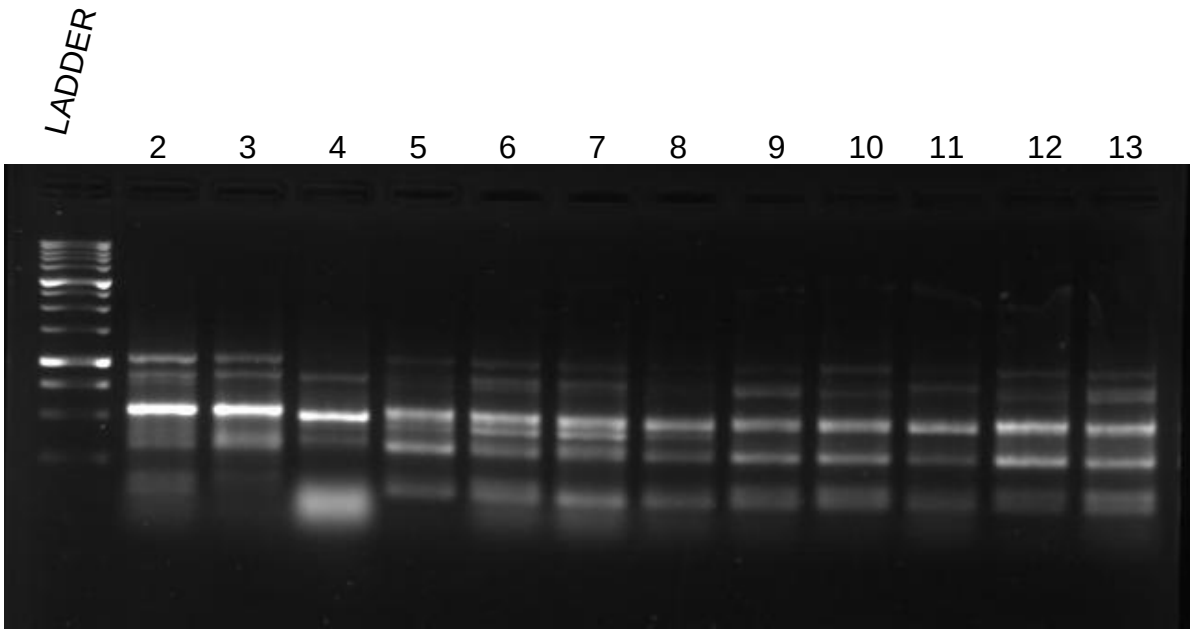
(a)Lanes 2, 3, 4, 5, 6 & 7 represent proteins extracted from leaves of M-5, China White, control hybrid (CH), Plant C2, Plant C4 and Plant C14, respectively.

(b) Lanes 2, 3, 4, 5, 6 & 7 represent proteins extracted from leaves of M-5, China White, Plant C 33, Plant C34, Plant C35 and Plant C77, respectively.

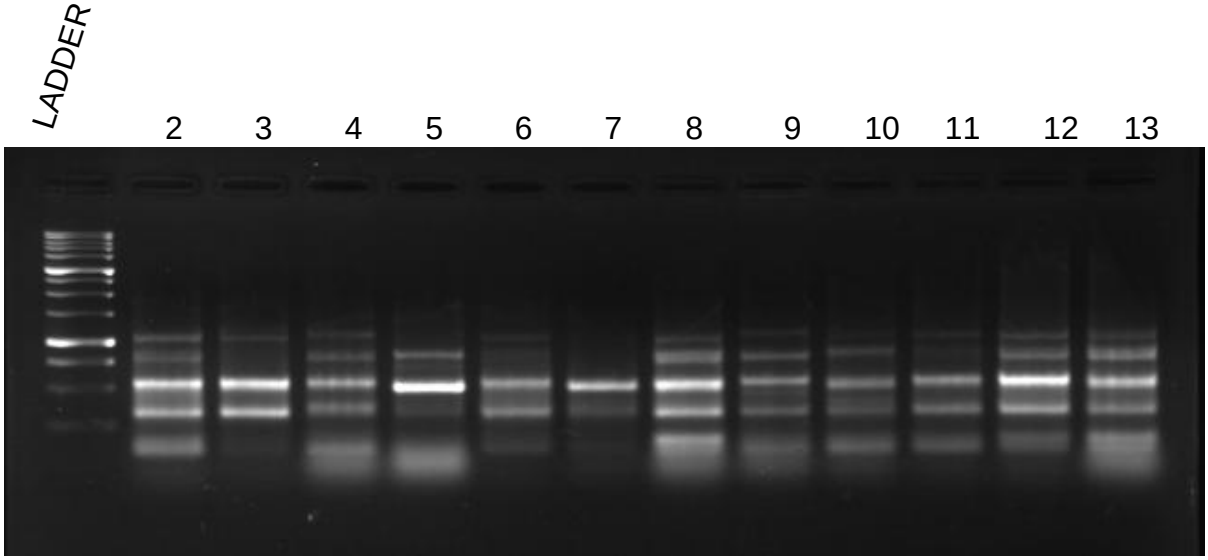
(c) Lanes 2, 3, 4, 5, 6 & 7 represent proteins extracted from leaves of M-5, China White, Plant C22, Plant C25, Plant C30 and Plant C32, respectively.

(d) Lanes 2, 3, 4, 5, 6 & 7 represent proteins extracted from leaves of M-5, China White, Plant C20, Plant C21, Plant C51 and Plant C52, respectively.

(e) Lanes 2, 3, 4 & 5 represent proteins extracted from leaves of M-5, China White, Plant C11 and Plant C17, respectively.



a



b

Fig. 55 a-b: RAPD profiles of the parent plants and morphological variants obtained after pollination with S-13 irradiated pollen with primer OPY-10. Lane 1 in Figs. a & b represent DNA ladder (0.5 kb to 10 kb)

a)Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 & 13 represent M-5, control hybrid, S-13, Plant 14, Plant 33, Plant 34, Plant 40, Plant 45, Plant 63, Plant 23, Plant 27 and Plant 38, respectively

b) Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 & 13 represent Plant 46, Plant 72, Plant 22, Plant 36, Plant 37, Plant 52, Plant 55, Plant 15, Plant 16, Plant 20, Plant 32 and Plant 57, respectively

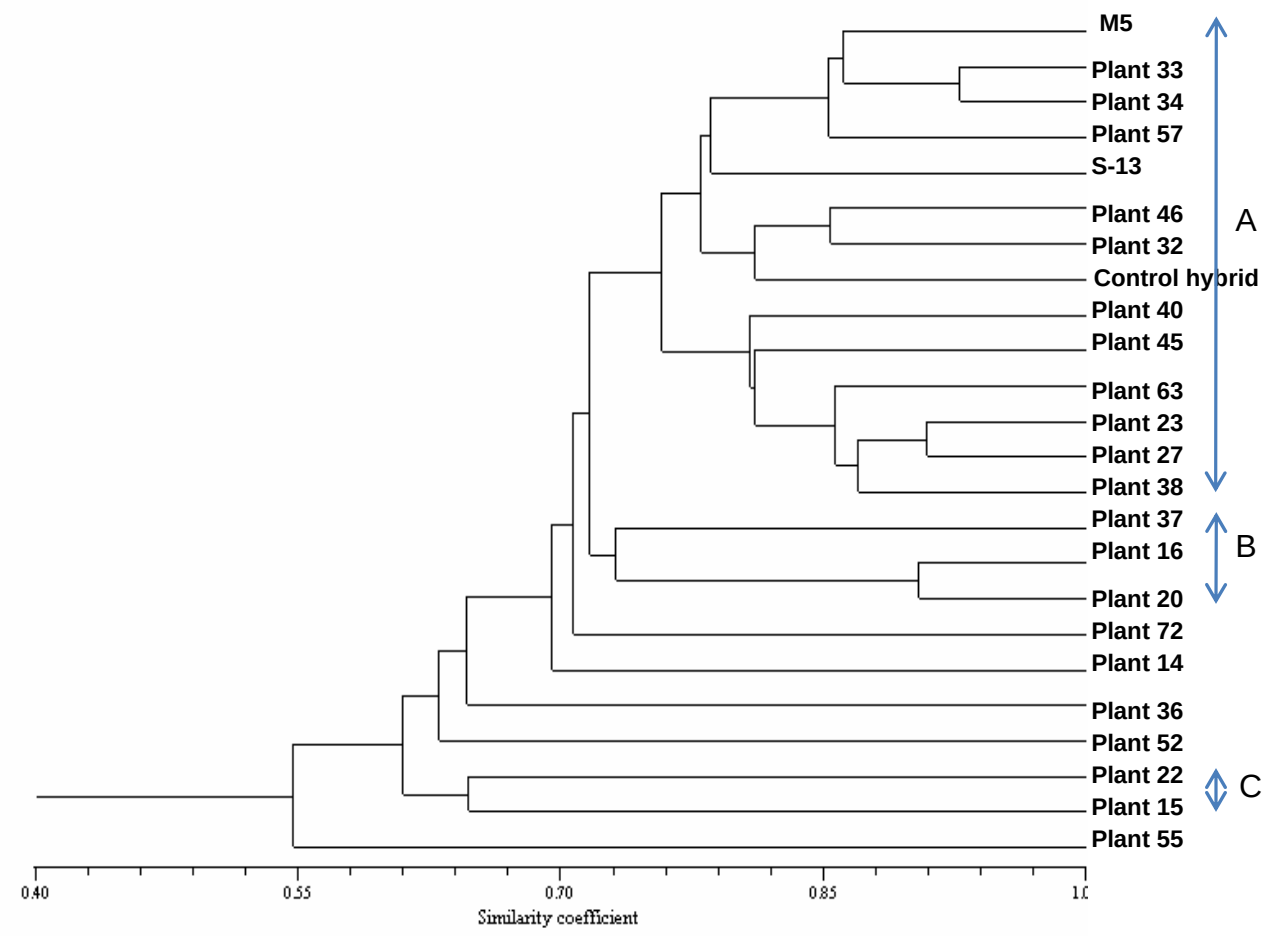
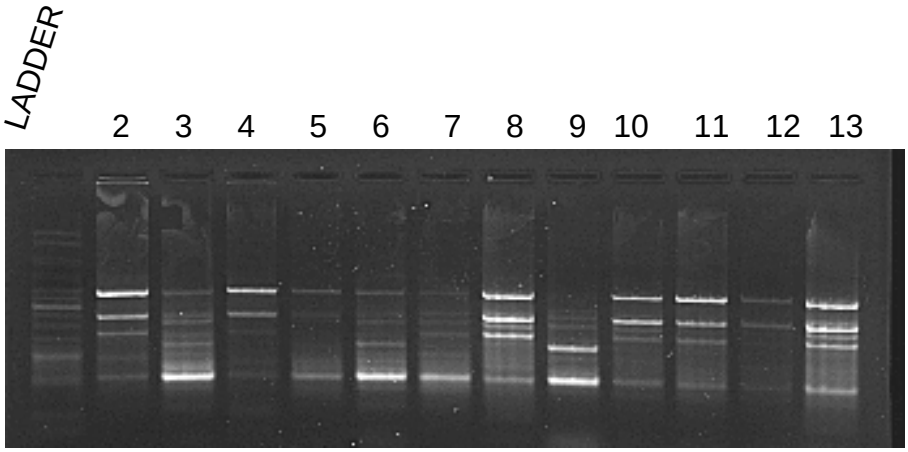
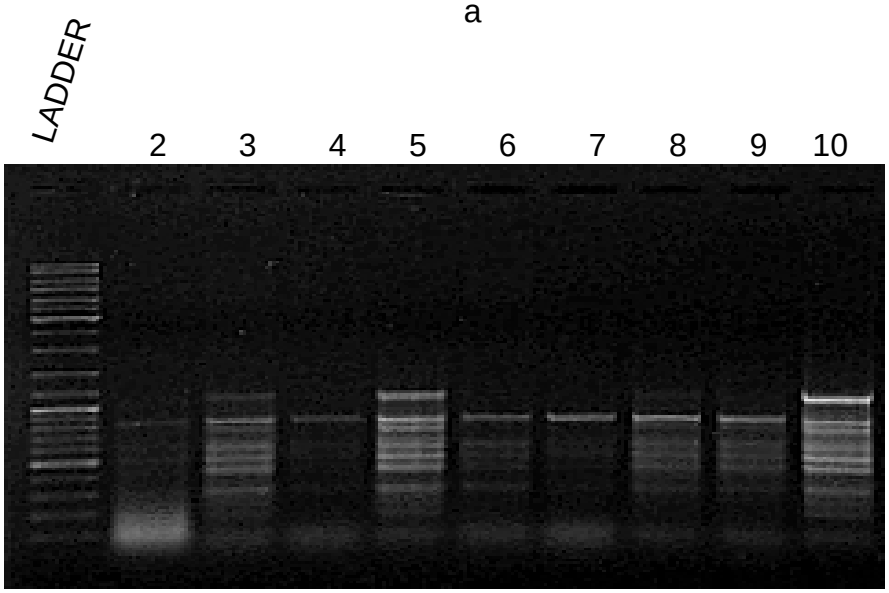


Fig. 56: Dendrogram of morphological variants obtained after pollination with S-13 irradiated pollen generated by RAPD data using UPGMA method



a



b

Fig. 57 a-b: RAPD profiles of the parent plants and morphological variants obtained after pollination with China White irradiated pollen with primer OPW-04 Lane 1 in Figs. a & b represent DNA ladder (0.5 kb to 10 kb)

a) Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 & 13 represent M-5, China White, control hybrid, Plant C22, Plant C25, Plant C30, Plant C32, Plant C33, Plant C34, Plant C35, Plant C77 and Plant C7, respectively

b) Lanes 2, 3, 4, 5, 6, 7, 8, 9 & 10 represent Plant C2, Plant C4, Plant C14, Plant C20, Plant C21, Plant C51, Plant C52, Plant C17, Plant C11, respectively

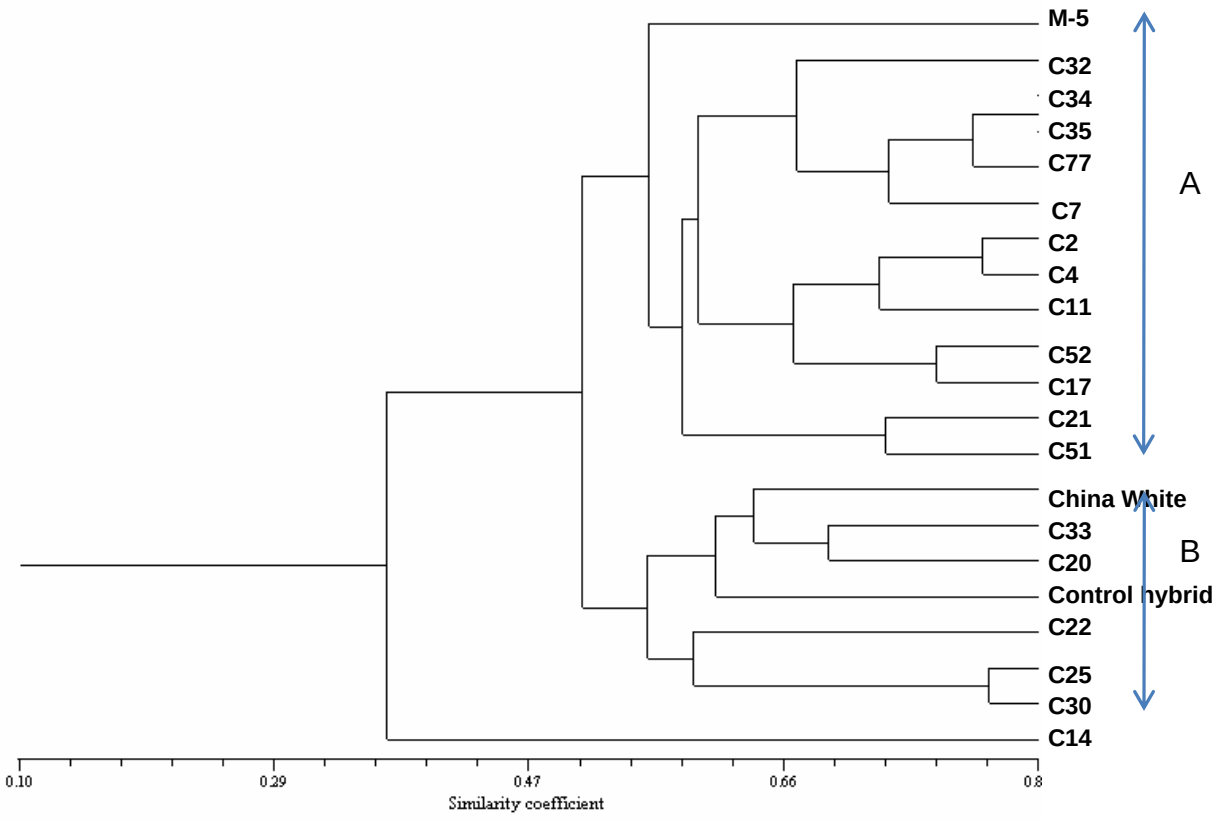


Fig. 58: Dendrogram of morphological variants obtained after pollination with China White irradiated pollen generated by RAPD data using UPGMA method

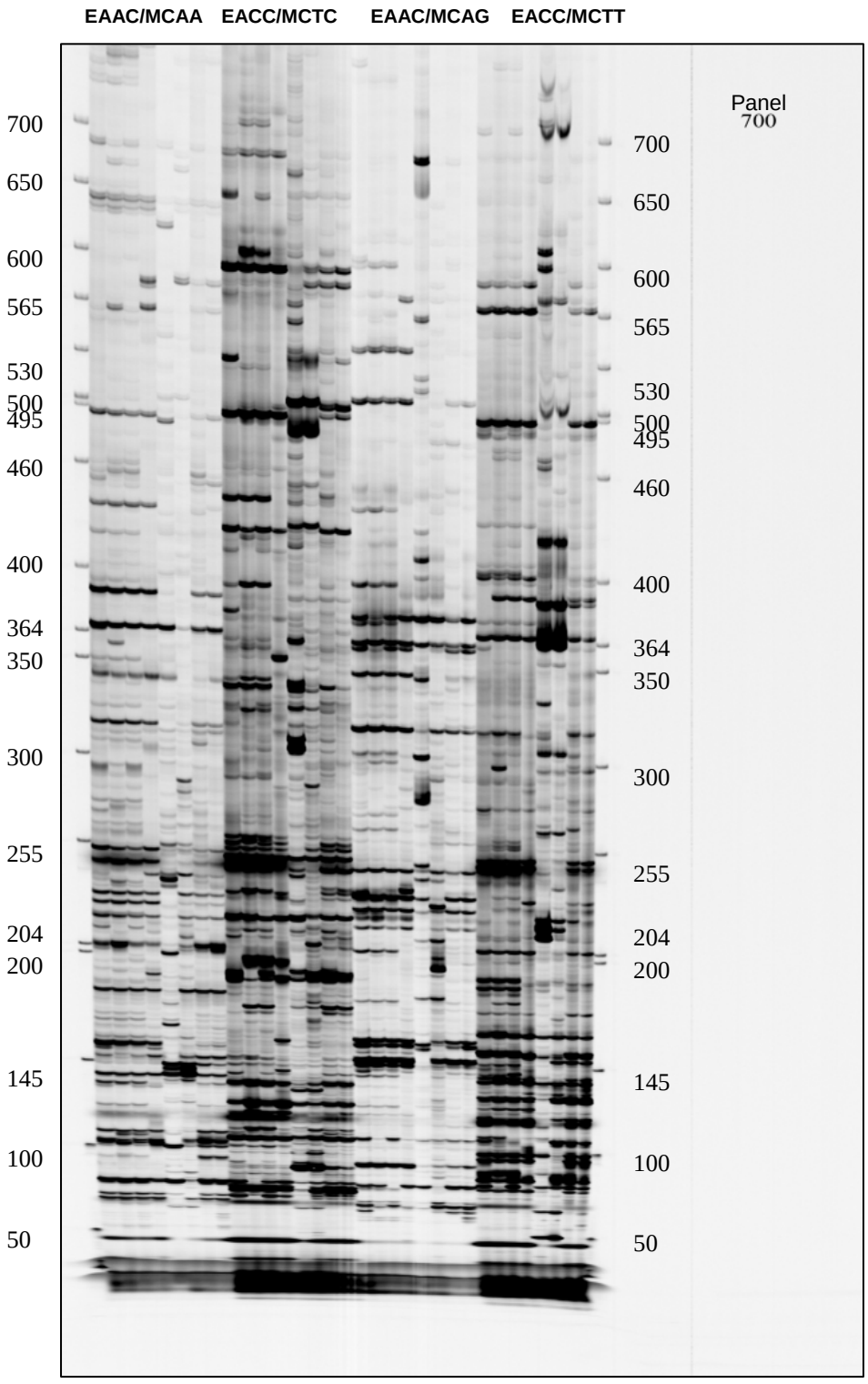


Fig. 59a: Banding pattern of morphological variants obtained after pollination with S-13 irradiated pollen as compared with M-5, S-13, control hybrid, China White detected by AFLP analysis with primer combinations EAAC/MCAA. EACC/MCTC. EAAC/MCAG and EACC/MCTT

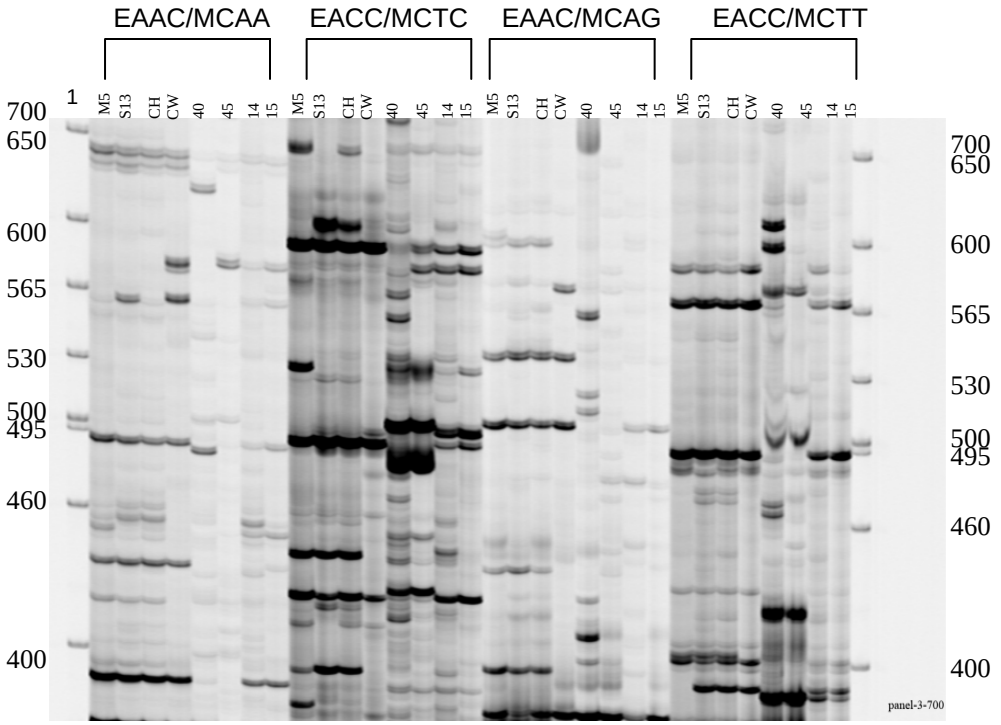


Fig. 59b: A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1 - DNA ladder (50 bp to 700 bp) **, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAA

Lanes 10, 11, 12,13, 14 15, 16 & 17 - AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACC/MCTC

Lanes 18, 19, 20, 21, 22, 23, 24 & 25 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAG

Lanes 26, 27, 28, 29, 30, 31, 32 & 33 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACC/MCTT

Lane 33 - DNA ladder (50 bp to 700 bp)

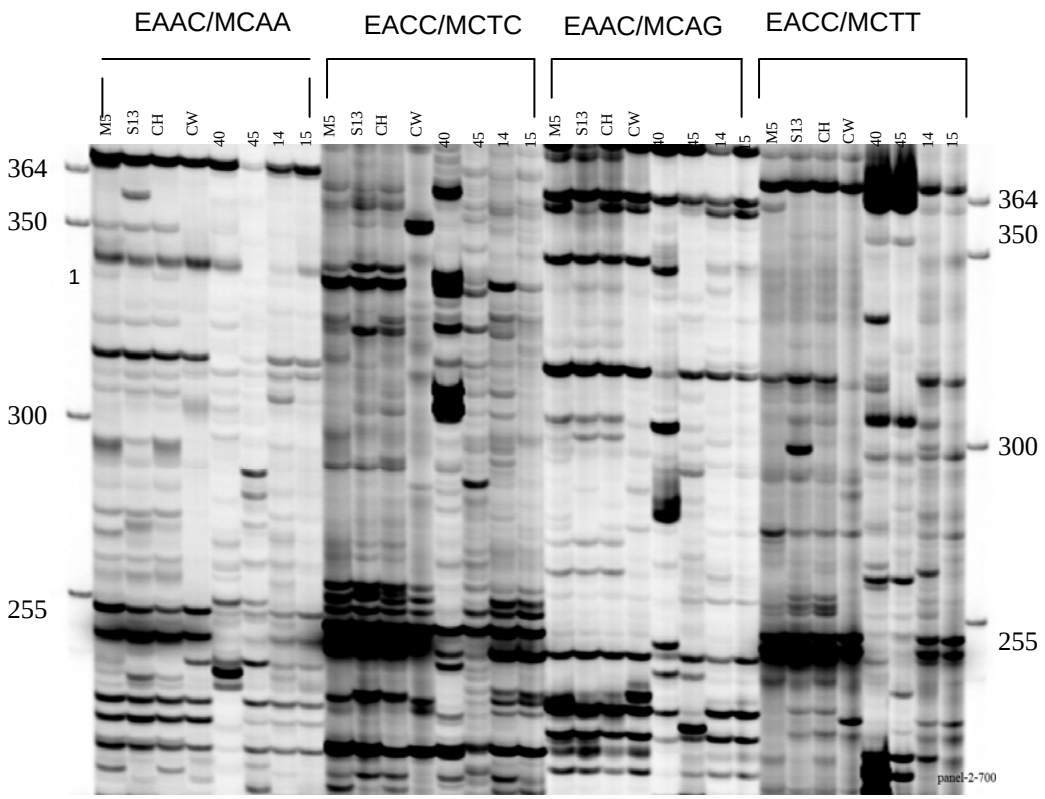


Fig. 59c.: A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1- DNA ladder (50 bp to 700 bp)**, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAA

Lanes 10, 11, 12, 13, 14 15, 16 & 17- AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACC/MCTC

Lanes 18, 19, 20, 21, 22, 23, 24 & 25– AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAG

Lanes 26, 27, 28, 29, 30, 31, 32 & 33 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACC/MCTT

Lane 34 - DNA ladder (50 bp to 700 bp)

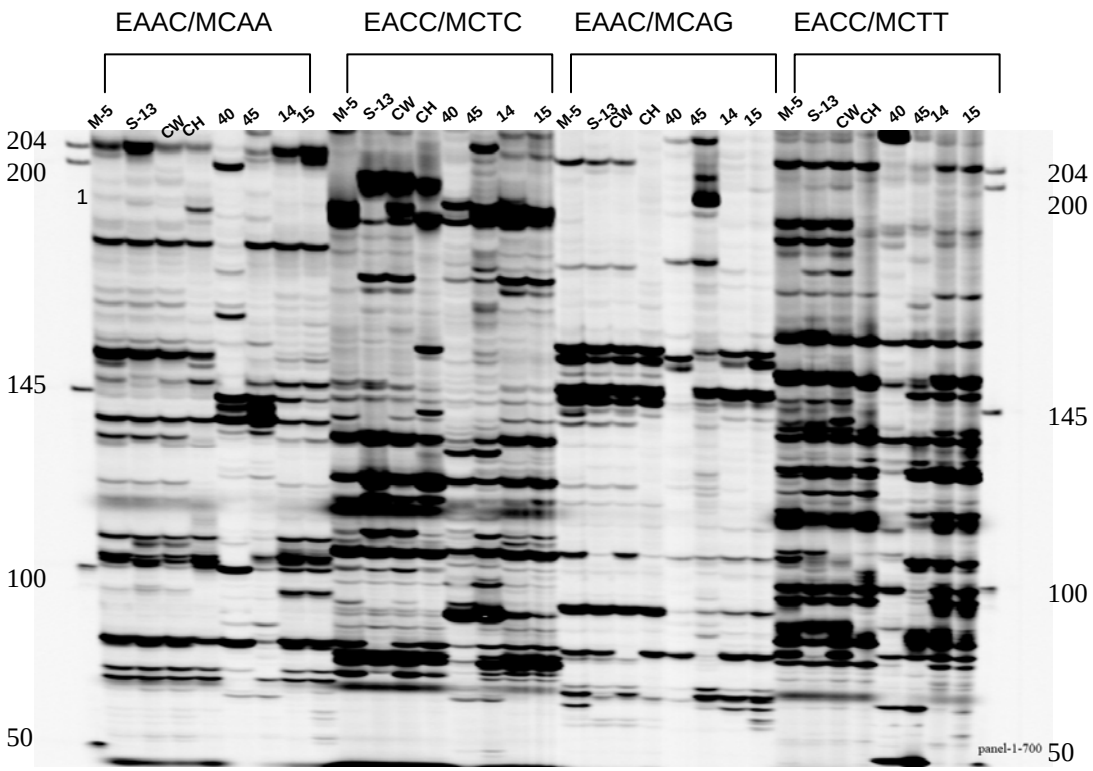


Fig. 59d. A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1 - DNA ladder (50 bp to 700 bp)**, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAA,

Lanes 10, 11, 12, 13, 14 15, 16 & 17- AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACC/MCTC,

Lanes 18, 19, 20, 21, 22, 23, 24 & 25– AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EAAC/MCAG,

Lanes 26, 27, 28, 29, 30, 31, 32 & 33 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACC//MCTT

Lane 34 - DNA ladder (50 bp to 700 bp)

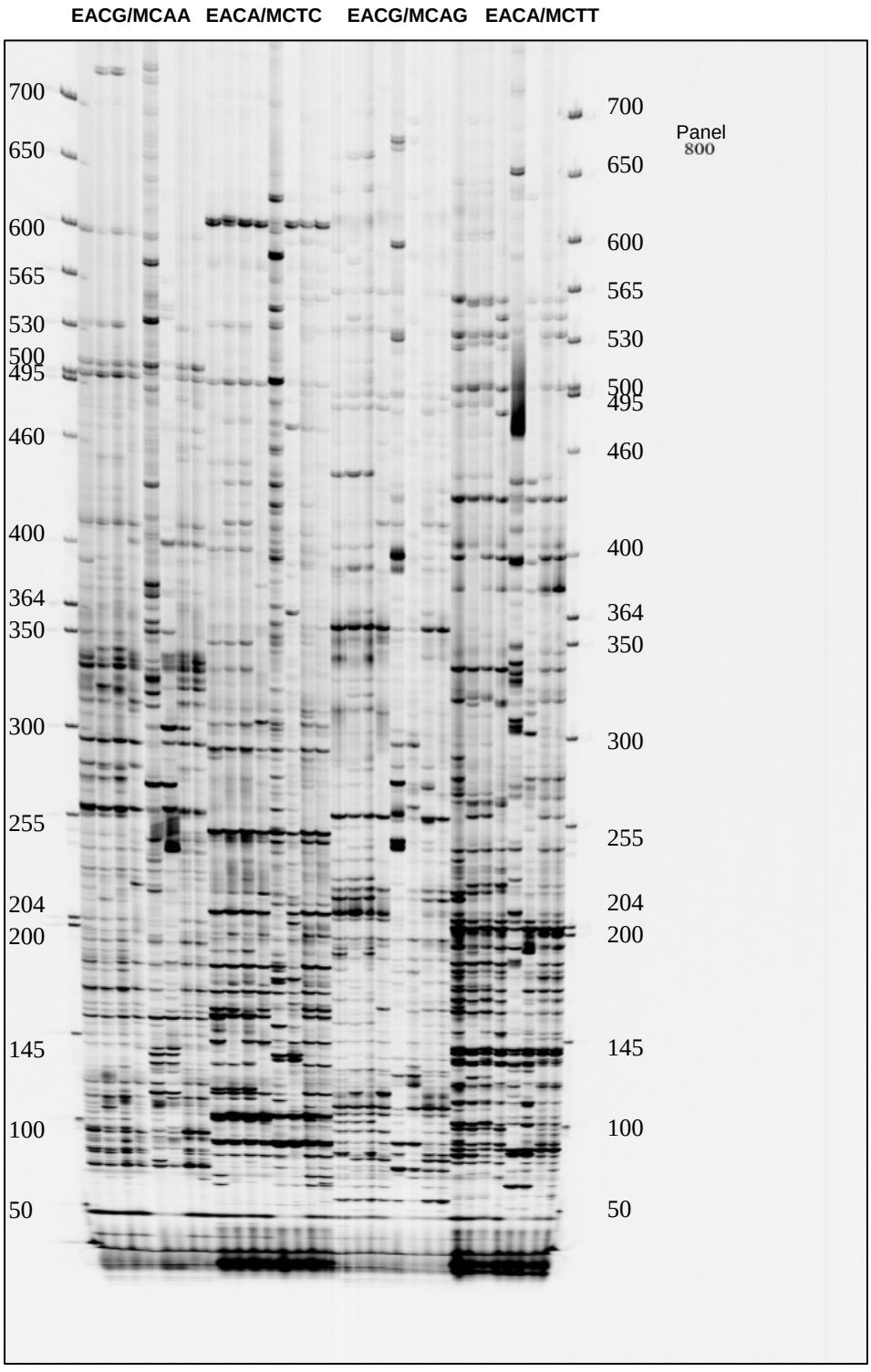


Fig. 60a: Banding pattern of morphological variants obtained after pollination with S-13 irradiated pollen as compared with M-5, S-13, control hybrid, China White detected by AFLP analysis using primer combinations EACG/MCAA, EACA/MCTC, EACG/MCAG and EACA/MCTT

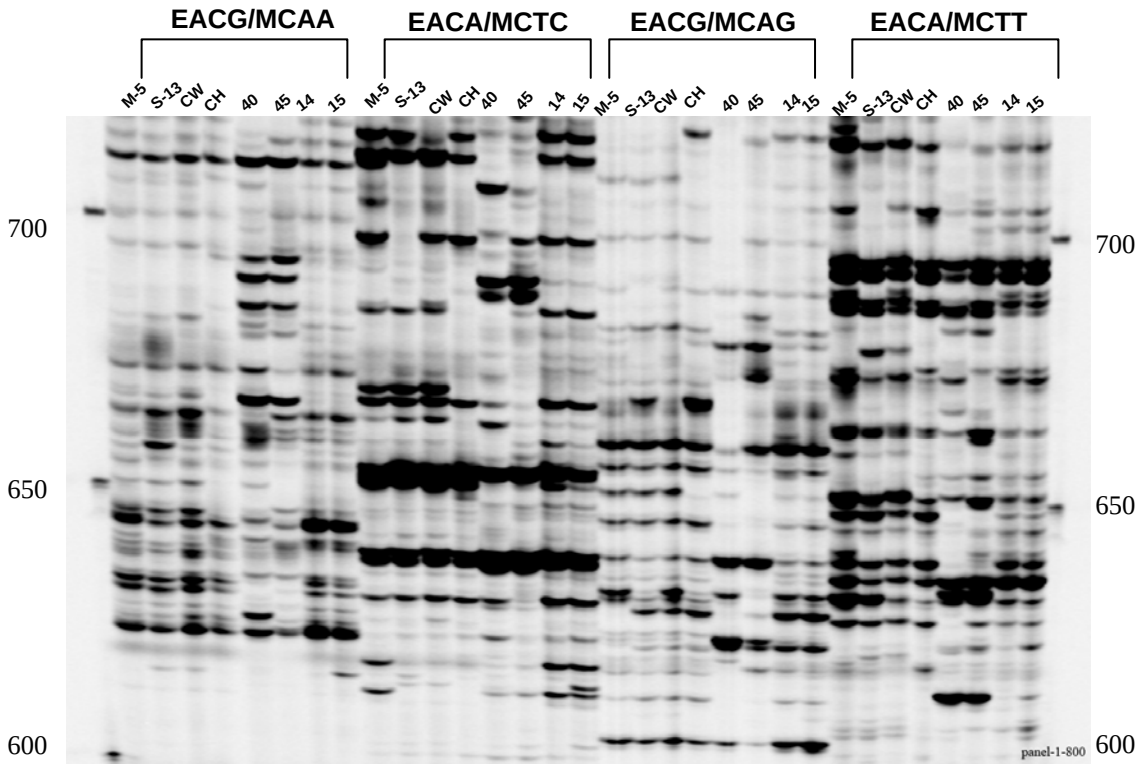


Fig. 60b: A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1 - DNA ladder (50 bp to 500 bp)**, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MCAA

Lanes 10, 11, 12, 13, 14 15, 16 & 17 - AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACA/MCTC,

Lanes 18, 19, 20, 21, 22, 23, 24 & 25 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MC AG,

Lanes 26, 27, 28, 29, 30, 31, 32 & 33 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACA/MCTT

Lane 34 - DNA ladder (50 bp to 700 bp)

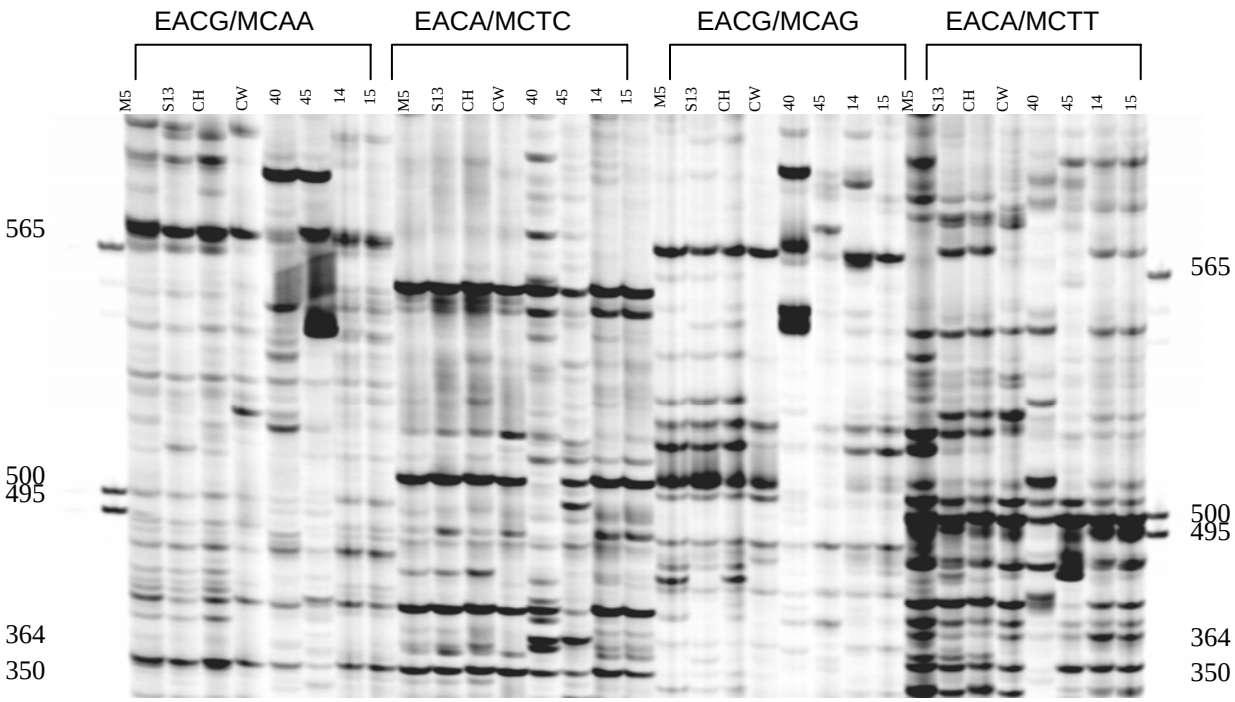


Fig. 60c. A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1 - DNA ladder (50 bp to 700 bp)**, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MCAA

Lanes 10, 11, 12, 13, 14 15, 16 & 17 - AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACA/MCTC,

Lanes 18, 19, 20, 21, 22, 23, 24 & 25– AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MCAG,

Lanes 26, 27, 28, 29, 30, 31, 32 & 33– AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACA/MCTT

Lane 34 - DNA ladder (50 bp to 700 bp)

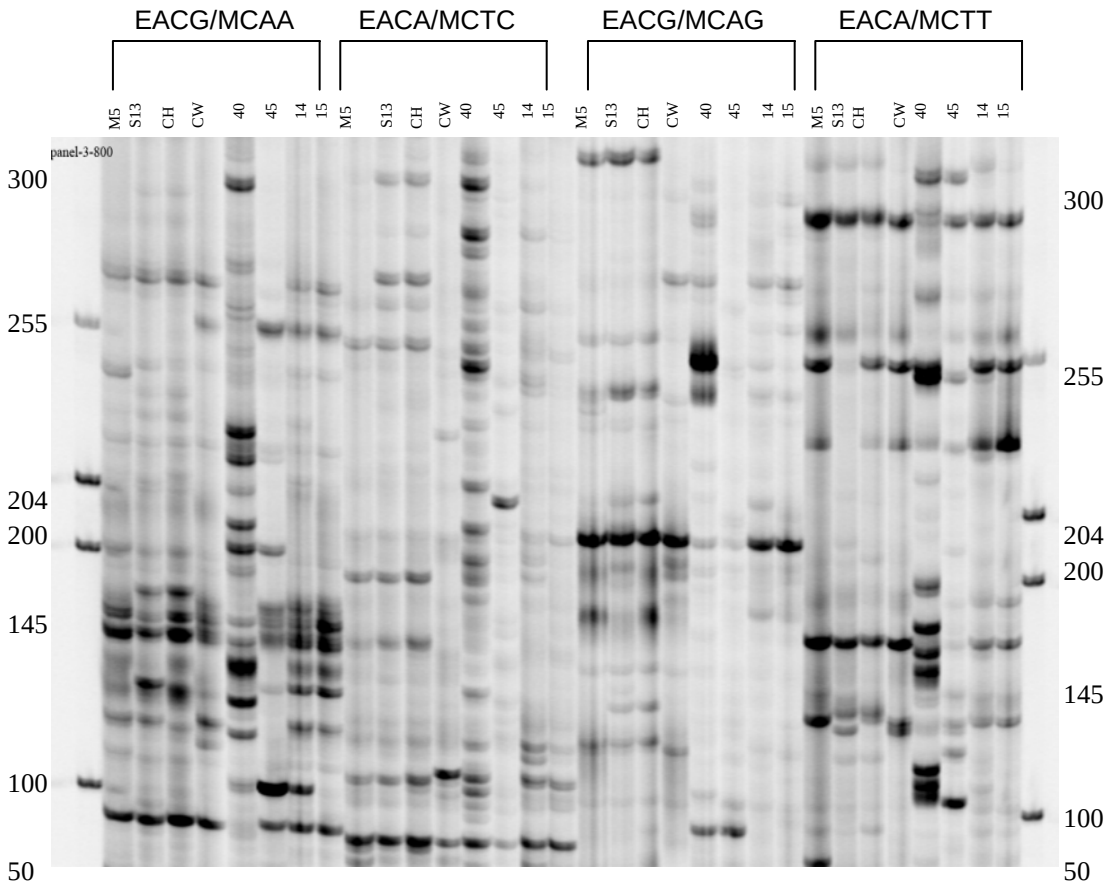


Fig. 60d: A section of AFLP fingerprint of the morphological variants along with M-5, S-13, China White and control hybrid using four primer combinations

Lane 1 - DNA ladder (50 bp to 700 bp)**, Lanes 2, 3, 4, 5, 6, 7, 8 & 9** AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MCAA

Lanes 10, 11, 12, 13, 14 15, 16 & 17 - AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACA/MCTC,

Lanes 18, 19, 20, 21, 22, 23, 24 & 25 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14 and Plant 15, respectively, with primer combination EACG/MCAG,

Lanes 26, 27, 28, 29, 30, 31, 32 & 33 – AFLP fingerprint of M-5, S-13, control hybrid, China White, Plant 40, Plant 45, Plant 14, Plant 15, respectively with primer combination EACA/MCTT

Lane 34 - DNA ladder (50 bp to 700 bp)

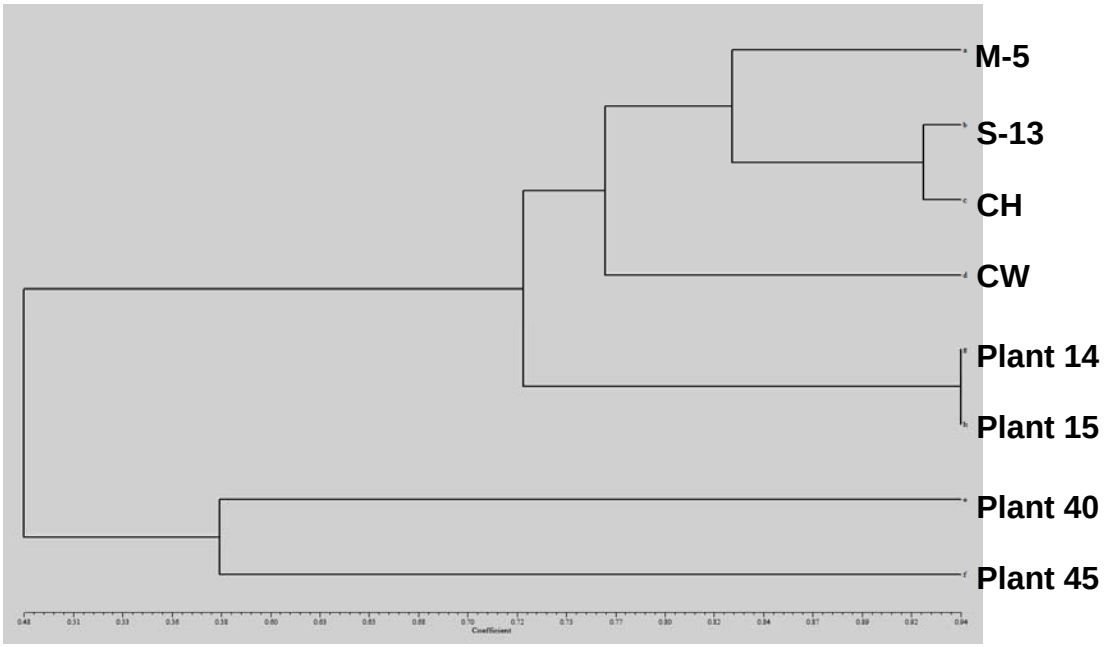


Fig. 61: Dendrogram of morphological variants obtained after pollination with S-13 irradiated pollen along with M-5, S-13, China White (CW) and control hybrid (CH) generated by AFLP data using UPGMA method

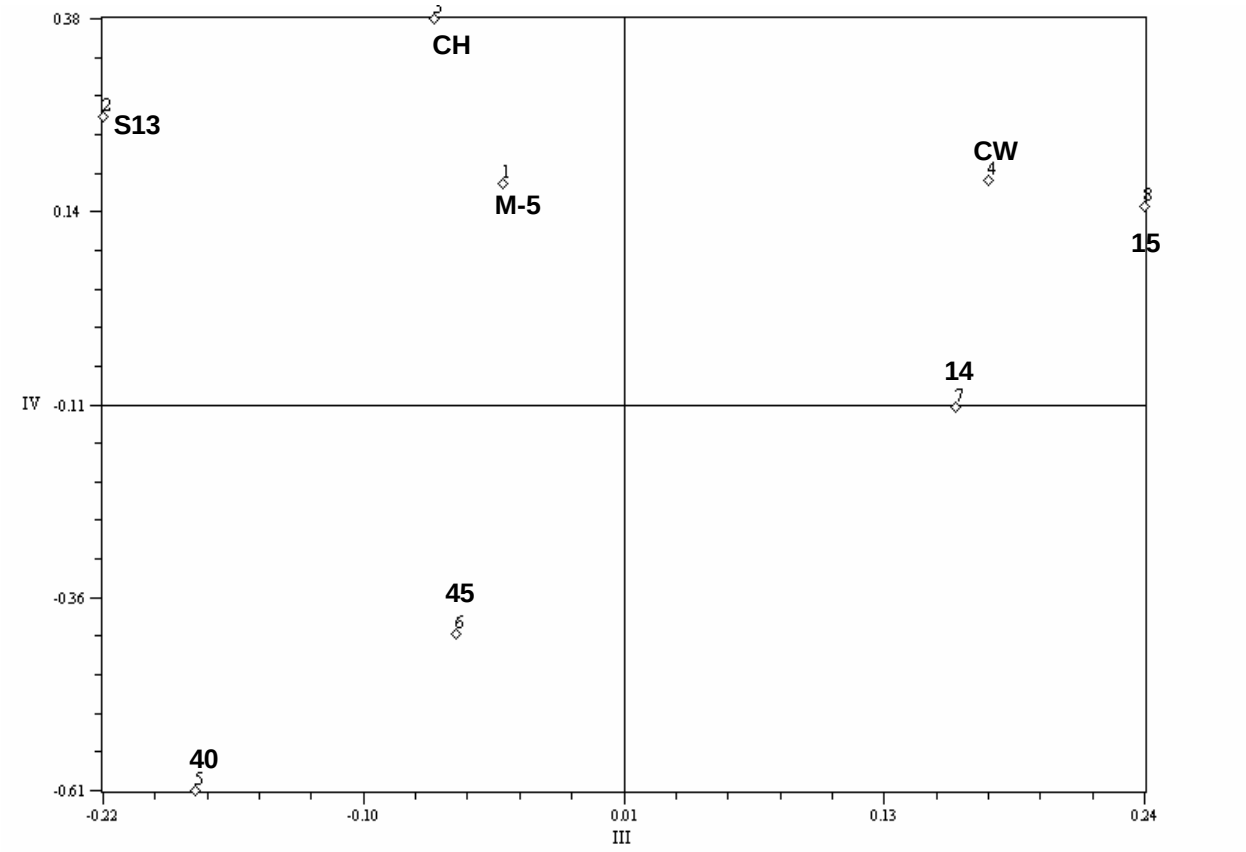


Fig. 62: Principal coordinate analysis illustrating divergence in morphological variants obtained after pollination with S-13 irradiated pollen in relation to M-5, S-13, control hybrid and China white

Table 1: Characteristics of different mulberry cultivars used in the study

Cultivar	Species	Sex	Ploidy
M-5	*Morus indica* L.	Female	2n = 28 Diploid
S-36	*Morus indica* L.	Female	2n = 28 Diploid
S-13	*Morus indica* L.	Male	2n = 28 Diploid
V-1	*Morus indica* L.	Male	2n = 28 Diploid
China White	*Morus alba* L.	Monoecious	2n = 28 Diploid

Table 2: RAPD primers used to detect polymorphism in M_1 plants obtained after pollination with S-13 irradiated pollen

Primer name	Sequence 5′←→3′
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-04	AATCGGGCTG
OPA-11	CAATCGCCGT
OPA-13	CAGCACCCAC
OPG-05	CTGAGACGGA
OPG-16	AGCGTCCTCC
OPW-02	ACCCCGCCAA
OPW-03	GTCCGGAGTG
OPW-04	CAGAAGCGGA
OPY-02	CATCGCCGCA
OPY-03	ACAGCCTGCT
OPY-04	GGCTGCAATG
OPY-05	GGCTGCGACA
OPY-06	AAG GCTCACC
OPY-09	AGCAGCGCAC
OPY-10	CAAACGTGGG
OPY-13	GGGTCTCGGT
OPY-16	GGGCCAATGT

Table 3: RAPD primers used to detect polymorphism in M_1 interspecific hybrids obtained after pollination with China White irradiated pollen

Primer name	Sequence 5′←→3′
OPG-05	CTGAGACGGA
OPG-16	AGCGTCCTCC
OPW-01	CTCAGTGTCC
OPW-02	ACCCCGCCAA
OPW-03	GTCCGGAGTG
OPW-04	CAGAAGCGGA
OPY-11	AGACGATGGG
OPY-13	GGGTCTCGGT
OPY-15	AGTCGCCCTT
OPY-16	GGGCCAATGT

Table 4: Pollen viability of *in vitro* and *in vivo* induced catkins

Cultivar	Pollen viability percentage (%)	
	In vitro	*In vivo*
S-13	81.4 ± 1.3	88.2 ± 2.2
V-1	81.8 ± 1.3	90.2 ± 0.4

Table 5: Anther culture response in mulberry cultivars on different media

Cultivar	Media and growth regulators	Duration of cold pretreatment (days)	Frequency of callus induction (%)	Response after subculture
S-13	MS + 8 mg/l 2,4-D + 0.1 mg/l NAA + 6% sucrose	4	6.8%	Rhizogenesis
S-13	MS + 10 mg/l 2,4-D + 1 mg/l KN + 6% sucrose	4	3.3%	Browning of callus
V-1	MS + 4 mg/l 2,4-D + 0.1 mg/l TDZ + 6% sucrose	6	3.8%	Browning of callus

Table 6: Effect of gamma irradiation on the viability of S-13 pollen

Dose of irradiation (Gy)	Per cent viability
Control (Non-irradiated)	96.2 ± 1.2e
50	60.4 ± 1.9 d
80	57.6 ± 1.7 d
100	40.5 ± 1.2 c
200	37.4 ± 1.2 c
500	28.1 ± 2.9 b
1000	13.7 ± 1.2 a
2000	9.8 ± 0.6 a

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 7: Germination response of S-13 pollen on different media

S. No.	Composition of the media and culture conditions	Per cent pollen germination after 48 hours
1.	10 mg/l EDTA + 100 mg/l H_3BO_3 + 400 mg/l $Ca(NO_{3)2}$ + 10% sucrose, pH 5.3, 19^oC	59.6 $\pm$ 2.2 b
2.	10,000 mg/l Ca $(NO_3)_2$+ 300 mg/l $MgSO_4$ + 100 mg/l KNO_3 + 100 mg/l H_3BO_3, pH 7.0, 26^oC	15.1 $\pm$ 1.1 a
3.	100 mg/l H_3BO_3 + 300 mg/l $Ca(NO_3)_2$ + 200 mg/l $MgSO_4$ + 100 mg/l KNO_3, 10 % sucrose, pH 7.0, 36^oC	14.4 $\pm$ 1.5 a
4.	150 mg/l H_3BO_3 + 500 mg/l $Ca(NO_3)_2$ +10% sucrose , pH 5.6, 19^oC	9.7 $\pm$ 1.1 a

Values are means $\pm$ standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 8: Effect of gamma irradiation on *in vitro* pollen germination of S-13 cultivar

Dose of irradiation (Gy)	Pollen germination (%)
Control (Non-irradiated)	59.6 ± 2.2 d
50	27.7 ± 2.7 c
80	13.9 ± 1.7 b
100	13.1 ± 1.7 b
200	12.6 ± 1.6 b
500	9.6 ± 2.3 ab
1000	4.9 ± 1.3 a

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 9: Effect of gamma irradiation on *in situ* pollen germination of S-13 cultivar

Dose of irradiation (Gy)	Pollen germination (%)
Control (Non-irradiated)	81.9 ± 2.6 a
50	50.5 ± 1.5 b
80	39.8 ± 2.6 c
100	29.4 ± 2.9 d
200	25.8 ± 2.6 d
500	20.1 ± 1.0 e
1000	12.3 ± 0.6 f
2000	--

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 10: Effect of S-13 pollen irradiation on fruit and seed formation in M-5 cultivar in 2004

Dose of irradiation (Gy)	Fruit size (cm)	Fruit weight (mg)	No. of seeds per fruit	Single seed weight (mg)
Control	1.4 ± 0.1 a	368.0 ± 15.2 a	32.6 ± 1.4 a	1.1 ± 0.0 a
50	1.4 ± 0.1 a	358.4 ± 24.2 b	31.6 ± 1.4 a	1.1 ± 0.0 a
80	1.4 ± 0.1 a	341.1 ± 20.0 c	27.8 ± 1.4 a	1.0 ± 0.0 a
100	1.4 ± 0.2 a	345.6 ± 29.8 c	29.0 ± 1.3 a	0.9 ± 0.0 a
200	1.4 ± 0.1 a	313.0 ± 32.2 d	28.2 ± 1.3 a	0.9 ± 0.0 a
500	1.3 ± 0.1 a	240.2 ± 32.8 e	2.4 ± 0.7 d	--

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 11**:** Effect of S-13 pollen irradiation on fruit and seed formation in M-5 cultivar in 2005

Dose of irradiation (Gy)	Fruit size (cm)	Fruit weight (mg)	No. of seeds per fruit	Single seed weight (mg)
Control	1.1 ± 0.1 a	397.1 ± 22.4 a	29.7 ± 2.4 a	0.9 ± 0.0 c
50	1.0 ± 0.1 a	354.5 ± 24.0 a	20.9 ± 1.1 a	0.6 ± 0.0 b
80	1.0 ± 0.2 a	392.1 ± 31.7 a	26.4 ± 1.4 a	0.6 ± 0.0 b
100	1.1 ± 0.2 a	349.0 ± 22.6 a	22.6 ± 1.5 a	0.5 ± 0.0 b
500	1.0 ± 0.2 a	289.7 ± 36.0 a	18.3 ± 2.0 a	0.4 ± 0.0 a
1000	1.0 ± 0.1 a	443.0 ± 31.8 a	25.8 ± 1.4 a	0.6 + 0.0 b

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 12: Germination response from M-5 seed on different media

Media and growth regulators	Germination percentage	Shoot length (cm)	Root length (cm)
MS basal	68.0 ± 1.4 a	2.2 ± 0.1 a	3.3 ± 0.2 a
WPM basal	75.4 ± 1.3 b	2.3 ± 0.1 a	3.5 ± 0.2 a
MS + 3 mg/l GA_3	83.0 ± 1.4 c	3.2 ± 0.1 b	3.9 ± 0.1 b
WPM+ 3 mg/l GA_3	91.5 ± 1.1 d	3.2 ± 0.1 b	4.1 ± 0.0 b

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 13: Germination response from M_1 seed obtained after pollination with S-13 irradiated pollen in 2004

Dose of irradiation (Gy)	Germination percentage	Shoot length (cm)	Root length (cm)
Control	91.5 ± 1.1 a	3.2 ± 0.1 a	4.0 ± 0.1 a
50	88.9 ± 1.2 a	3.1 ± 0.2 a	3.9 ± 0.1 a
80	59.2 ± 1.2 b	3.1 ± 0.2 a	3.6 ± 0.2 a
100	58.3 ± 2.5 b	2.3 ± 0.2 b	1.3 ± 0.2 b
200	46.7 ± 4.5 c	1.6 ± 0.1 c	0.9 ± 0.1 c

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 14: Germination percentage from M_1 seeds obtained after pollination with S-13 irradiated pollen in 2005

Dose of irradiation (Gy)	Germination percentage
Control	91.3 ± 3.3 e
50	71.8 ± 3.1 d
80	63.9 ± 3.8 d
100	41.6 ± 6.2 c
500	24.9 ± 4.0 b
1000	9.4 ± 1.2 a

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 15: Response of shoot multiplication in M_1 plants obtained after pollination with S-13 irradiated pollen

Plant No.	Dose of irradiation (Gy)	Average No. of shoots per culture	Average length of shoots (cm)	Average leaf length (cm)
Control	--	11.4 ± 0.5 a	9.6 ± 0.3 a	2.4 ± 0.0 a
Plant 23	80	5.2 ± 0.4 b	3.4 ± 0.2 b	0.9 ± 0.0 b
Plant 36	100	8.8 ± 0.2 c	2.3 ± 0.1 c	0.6 ± 0.0 c

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 16: Morphological variations in four-month-old M_1 plants of mulberry obtained after pollination with S-13 irradiated pollen in the year 2004

Plant Number	Dose of irradiation (Gy)	Height of the plant (cm)	Leaf length (cm)	Leaf width (cm)	Thickness of the main stem (cm)	Internodal distance (cm)	Remarks
M-5*	--	142.3	15.4	12.1	3.5	4.5-5.0	Ovate leaves
S-13*	--	132.7	16.5	13.2	3.1	4.5-5.0	Elongated leaves
Control hybrid*	--	135.8	15.3	11.4	2.8	4.0-5.0	Ovate leaves
Plant 14	80	145.2	20.5	14.0	3.0	5.0-5.5	Large leaves
Plant 15	50	152.0	15.5	10.5	2.4	4.5-6.5	Pale green leaves
Plant 16	100	100.0	17.0	12.0	2.5	5.0-5.5	Pale green leaves
Plant 20	100	110.0	20.0	16.0	2.8	4.5-5.0	Pale green leaves
Plant 22	80	69.0	14.0	10.0	2.0	4.0-4.5	Short height
Plant 23	100	95.0	11.0	9.4	2.0	3.5-4.0	Small leaves
Plant 27	80	90.0	9.5	8.4	2.0	4.0-5.0	Small leaves
Plant 32	100	88.0	16.5	12.5	2.8	4.0-4.5	Bicrenate leaf lamina
Plant 33	80	155.0	19.0	14.0	3.0	4.0-5.0	Large leaves
Plant 34	80	148.0	22.0	17.0	3.2	5.0-5.5	Five lobed leaves
Plant 36	80	82.0	9.0	8.4	1.8	4.0-5.0	Small leaves
Plant 37	80	74.0	13.0	9.5	2.5	4.0-5.0	Short height
Plant 38	80	100.0	11.0	9.2	4.0	5.0-6.0	Small leaves
Plant 40	80	170.0	22.0	14.0	3.5	5.0-6.0	Large leaves
Plant 45	80	150.0	19.5	14.0	3.0	3.0-4.0	Large leaves
Plant 46	50	54.0	11.0	9.0	1.5	4.0-4.5	Small leaves
Plant 52	80	70.0	9.3	8.0	1.8	3.5-4.0	Short height
Plant 55	80	86.0	14.5	12.5	2.5	4.5-5.0	Short height
Plant 57	80	90.0	15.0	10.0	2.3	4.0-4.5	Elliptical leaves
Plant 63	100	145.0	17.5	15.0	3.5	4.0-4.5	Large leaves
Plant 72	50	78.0	9.5	8.0	1.5	4.5-5.0	Short height

*Data represents an average of ten plants

Table 17: Morphological variations in four-month-old M_1 plants of mulberry obtained after pollination with S-13 irradiated pollen in 2005

Plant Number	Dose of irradiation (Gy)	Height of the plant (cm)	Leaf length (cm)	Leaf width (cm)	Thickness of the main stem (cm)	Internodal distance (cm)	Remarks
M-5*	--	145.3	13.4	12.2	3.5	4.0-5.0	Ovate leaves
S-13*	--	125.6	15.5	13.5	3.2	4.5-5.5	Ovate leaves
Control hybrid*	--	138.3	15.5	12.4	3.2	4.2-5.0	Ovate leaves
Plant N8	500	132.0	13.0	8.0	2.9	4.5-5.0	Five lobed leaves
Plant N15	100	145.0	19.0	14.0	3.2	4.8-5.3	Large leaves
Plant N17	1000	97.0	12.4	8.0	2.5	3.2-4.0	Cordate leaf base
Plant N18	500	87.0	11.1	7.0	2.0	3.0-4.2	Short height
Plant N20	1000	100.0	12.5	8.0	2.4	4.2-4.8	Acute leaf apex
Plant N21	100	148.0	21.0	15.0	3.1	4.7-5.5	Large leaves
Plant N34	500	87.0	5.2	7.2	1.7	2.9-3.7	Small curly leaves
Plant N53	100	128.0	19.2	14.5	3.0	4.5-5.0	Large leaves
Plant N57	80	125.0	12.4	9.0	3.0	4.5-5.2	Biserrate leaf margin
Plant N60	1000	76.0	6.0	5.0	1.5	3.2-3.5	Small leaves

*Data represents an average of ten plants

Table 18: Comparison of stomatal length and chloroplast number per stomata in M_1 plants of mulberry obtained after pollination with S-13 irradiated pollen

Plant Number	Dose of pollen irradiation (Gy)	Stomatal length (μm)	Chloroplast number per stomata
M-5	--	32.3 ± 1.2	10.4 ± 0.5
S-13	--	28.5 ± 0.8	9.8 ± 0.5
Control hybrid	-	31.8 ± 0.8	9.4 ± 0.5
Plant 14	80	20.1 ± 0.8	9.0 ± 0.4
Plant 15	50	20.5 ± 0.7	9.0 ± 0.3
Plant 16	100	20.4 ± 1.2	7.0 ± 0.3
Plant 20	100	22.3 ± 0.8	8.4 ± 0.3
Plant 22	80	25.0 ± 0.7	9.2 ± 0.3
Plant 23	100	21.8 ± 0.6	8.9 ± 0.3
Plant 27	80	25.4 ± 0.6	9.0 ± 0.3
Plant 32	100	22.7 ± 1.1	9.4 ± 0.5
Plant 33	80	21.2 ± 0.9	7.2 ± 0.4
Plant 34	80	23.7 ± 1.0	8.2 ± 0.3
Plant 36	80	22.9 ± 1.3	8.0 ± 0.5
Plant 37	80	25.0 ± 0.9	9.6 ± 0.5
Plant 38	80	20.5 ± 0.7	7.7 ± 0.3
Plant 40	80	17.6 ± 0.6	5.8 ± 0.5
Plant 45	80	23.7 ± 1.0	7.2 ± 0.3
Plant 46	50	20.2 ± 0.8	8.3 ± 0.3
Plant 52	80	23.7 ± 0.7	8.2 ± 0.3
Plant 55	80	23.4 ± 1.1	9.2 ± 0.3
Plant 57	80	24.6 ± 1.0	9.4 ± 0.3
Plant 63	100	25.2 ± 1.1	9.0 ± 0.3
Plant 72	50	21.8 ± 1.5	7.2 ± 0.3

Table 19: Pattern of sex expression in M_1 plants of mulberry obtained after pollination with S-13 irradiated pollen

Plant	Female catkins (%)	Male catkins (%)	Mixed catkins (%)	Catkins forming fruits (%)
M-5 Cultivar	100	--	--	99.0
S-13 Cultivar	--	100.0	--	--
Plant 14	--	78.0	22.0	82.3
Plant 15	--	84.0	16.0	82.5
Plant 40	--	80.0	20.0	85.3
Plant 45	--	76.0	24.0	81.2

Table 20: Characteristics of fruits formed on M_1 plants of mulberry obtained after pollination with S-13 irradiated pollen

Plant No.	Fruit size (cm)	Fruit weight (mg)	Fruit thickness (cm)	Peduncle length (cm)	Average number of seeds per fruit
M-5 cultivar	1.4 ± 0.1	579.3 ± 62.4	1.8 ± 0.1	0.3 ± 0.0	41.4 ± 1.2
Plant 14	1.6 ± 0.1	517.8 ± 59.6	1.6 ± 0.1	0.3 ± 0.0	37.7 ± 1.3
Plant 15	1.4 ± 0.1	492.4 ± 46.5	1.5 ± 0.0	0.2 ± 0.0	33.6 ± 1.2
Plant 40	3.1 ± 0.1	931.6 ± 66.2	2.8 ± 0.1	0.9 ± 0.0	51.9 ± 4.8
Plant 45	1.5 ± 0.1	504.5 ± 45.7	1.5 ± 0.0	0.3 ± 0.0	33.8 ± 1.1

Table 21: Seed development in the mixed catkins of M_1 plants obtained after pollination with S-13 irradiated pollen

Plant No.	Number of florets per catkin		Number of seeds in female florets per catkin	Number of seeds in bisexual florets per catkin	Single seed weight of female florets	Single seed weight of bisexual florets
	Female	Bisexual				
M-5 cultivar	54.1 ± 1.5	--	40.0 ± 1.6	--	1.0 ± 0.0	--
Plant 14	23.2 ± 1.9	32.8 ± 1.6	14.0 ± 1.4	23.0 ± 1.5	0.9 ± 0.1	0.7 ± 0.1
Plant 15	34.6 ± 2.1	22.8 ± 2.3	20.4 ± 1.9	9.6 ± 1.0	0.9 ± 0.0	0.6 ± 0.0
Plant 40	25.0 ± 3.5	60.4 ± 4.7	13.7 ± 3.3	40.3 ± 2.9	1.1 ± 0.1	0.6 ± 0.0
Plant 45	39.6 ± 2.0	19.0 ± 1.4	25.6 ± 2.4	6.6 ± 0.5	0.9 ± 0.0	0.5 ± 0.0

Table 22: Germination percentage from seeds of bisexual and female florets of M_1 plants

Plant No.	Germination percentage of seeds of female florets (%)	Germination percentage of seeds of bisexual florets (%)
M-5 cultivar	96.0 $\pm$ 2.5 a	--
Plant 14	81.4 $\pm$ 1.9 c	26.0 $\pm$ 1.1 a
Plant 15	83.5 $\pm$ 2.6 c	22.2 $\pm$ 1.1 a
Plant 40	95.4 $\pm$ 3.2 a	27.5 $\pm$ 1.2 a
Plant 45	86.0 $\pm$ 3.1 b	16.7 $\pm$ 1.1 b

Values are means $\pm$ standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 23: Effect of China White irradiated pollen on fruit and seed formation in M-5 cultivar

Dose of irradiation (Gy)	Fruit size (cm)	Fruit weight (mg)	No. of seeds per fruit	Single seed weight (mg)
Control	1.4 ± 0.1 a	631.1 ± 45.8 b	22.0 ± 2.7c	1.9 ± 0.1 a
50	0.9 ± 0.1 a	287.5 ± 38.3 a	5.6 ± 1.6 a	1.8 ± 0.2 a
80	1.1 ± 0.1 a	424.6 ± 54.7 ab	5.8 ± 0.8 a	1.6 ± 0.1 a
100	1.0 ± 0.1 a	276.2 ± 75.0 a	7.5 ± 1.3b	1.7 ± 0.1 a
500	1.2 ± 0.1 a	387.2 ± 47.3 ab	8.6 ± 0.5 b	1.8 ± 0.2 a
1000	1.3 ± 0.1 a	633.2 ± 69.0 b	13.8 ± 3.1 b	2.1 ± 0.5 a

Values are means ± standard error. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 24: Germination percentage of M_1 seed obtained after pollination with China White irradiated pollen

Dose of irradiation (Gy)	Germination percentage
Control	82.2 ± 1.1 c
50	63.9 ± 5.7 c
80	53.3 ± 3.3 b
100	46.3 ± 1.9 b
500	39.4 ± 1.6 a
1000	31.6 ± 3.1 a

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Table 25: Morphological variations in four-month-old M_1 interspecific hybrid plants obtained after pollination with China White irradiated pollen

Plant Number	Dose of irradiation (Gy)	Height of the plant (cm)	Leaf length (cm)	Leaf width (cm)	Thickness of the main stem (cm)	Internodal distance (cm)	Remarks
M-5*	--	135.3	15.4	12.4	3.2	4.0-5.0	Ovate leaves
China White*	--	112.7	28.5	16.5	3.5	4.2-5.0	Large leaves
Control Hybrid*	--	130.0	15.8	11.3	2.5	5.0-5.3	Ovate leaves
Plant C2	1000	63.0	7.0	4.5	1.7	2.5-3.2	Small leaves
Plant C4	1000	57.0	8.5	5.0	2.3	3.2-4.3	Dentate leaf margin
Plant C7	1000	92.0	12.7	10.5	2.7	4.8-5.0	Cordate leaf base
Plant C11	100	125.0	12.0	9.5	2.8	4.5-5.0	Dentate leaf margin
Plant C14	80	68.0	7.0	4.5	1.5	3.2-4.5	Small leaves
Plant C17	1000	115.0	14.5	9.5	2.3	3.5-3.8	Acuminate leaf apex
Plant C20	100	88.0	8.0	5.0	1.6	4.5-4.8	Small leaves
Plant C21	100	76.0	7.0	5.0	1.6	4.8-5.0	Small leaves
Plant C22	100	136.0	15.2	12.0	3.0	4.5-5.0	Dentate leaf margin
Plant C25	500	142.0	16.5	12.0	3.2	4.0-5.2	Cordate leaf base
Plant C30	80	136.0	16.5	12.0	2.5	3.5-4.2	Caudate leaf apex
Plant C32	500	140.0	20.6	14.0	3.3	4.5-5.0	Large leaves
Plant C33	80	133.0	16.0	12	3.2	4.0-5.5	Cordate leaf base
Plant C34	80	142.0	15.5	12.5	3.0	5.0-5.5	Dentate leaf margin
Plant C35	80	128.0	14.5	13	3.0	4.9-5.3	Cordate leaf base
Plant C51	1000	90.0	8.5	5.5	1.5	4.2-5.0	Small leaves
Plant C52	1000	92.0	7.8	4.9	1.7	3.4-4.5	Small leaves
Plant C77	80	148.0	21.5	14.5	3.0	4.5-4.8	Large leaves

*Data represents an average of ten plants

Table 26: Comparison of stomatal length and number of chloroplasts per stomata in M_1 interspecific hybrid plants obtained after pollination with China White irradiated pollen

Plant Number	Dose of irradiation (Gy)	Stomata length (μm)	Chloroplast number per stomata
M-5	--	32.1 ± 0.8	10.4 ± 0.2
CW	--	28.6 ± 0.8	11.4 ± 0.3
Control hybrid	--	29.7± 0.9	11.3 ± 0.2
Plant C2	1000	21.4 ± 0.6	7.9 ± 0.1
Plant C4	1000	22.7 ± 0.6	6.9 ± 0.3
Plant C7	1000	22.3 ± 0.7	6.5 ± 0.2
Plant C11	100	27.5 ± 0.6	8.5 ± 0.3
Plant C14	80	20.6 ± 0.5	6.5 ± 0.3
Plant C17	1000	23.8 ± 0.7	8.7 ± 0.3
Plant C20	100	22.8 ± 0.7	7.8 ± 0.1
Plant C21	100	20.0 ± 0.6	6.6 ± 0.3
Plant C22	100	26.3 ± 0.6	8.7 ± 0.3
Plant C25	500	26.9 ± 0.8	9.1 ± 0.3
Plant C30	80	23.4 ± 0.7	7.8 ± 0.1
Plant C32	500	27.6 ± 0.6	9.3 ± 0.3
Plant C33	80	24.2 ± 0.5	7.9 ± 0.2
Plant C34	80	25.7 ± 0.6	9.0 ± 0.4
Plant C35	80	25.4 ± 0.6	8.2 ± 0.2
Plant C51	1000	24.8 ± 0.6	8.5 ± 0.3
Plant C52	1000	22.7 ± 0.7	7.3 ± 0.4
Plant C77	80	26.6 ± 0.8	9.3 ± 0.3

Table 27: Polymorphism revealed by RAPD primers in the M_1 plants obtained after pollination with S-13 irradiated pollen

Primer	Total no. of bands per primer across all the plants	No. of monomorphic bands	No. of polymorphic bands	Polymorphism percentage
OPA1	78	24	54	69.2
OPA2	75	24	51	68.0
OPA4	58	0	58	100.0
OPA-11	70	0	70	100.0
OPA-13	74	0	74	100.0
OPG-05	51	0	51	100.0
OPG-16	98	24	74	75.5
OPW-02	79	24	55	69.6
OPW-03	67	0	67	100.0
OPW-04	75	24	51	68.0
OPY-02	76	24	52	68.4
OPY-03	104	0	104	100.0
OPY-04	66	24	42	63.6
OPY-05	67	24	43	64.2
OPY-06	72	48	24	33.3
OPY-09	70	24	46	65.7
OPY-10	85	24	61	71.8
OPY-13	80	24	56	70.0
OPY-16	53	24	29	54.7

Table 28: RAPD primers showing polymorphism in M_1 interspecific hybrids obtained after pollination with China White irradiated pollen

Primer	Total no. of bands per primer for all the plants	No. of monomorphic bands	No. of polymorphic bands	Polymorphism percentage
OPG-05	58	0	58	100.0
OPG-16	64	0	64	100.0
OPY-11	65	21	44	67.7
OPY-13	79	21	58	73.4
OPY-15	48	21	27	56.3
OPY-16	78	0	78	100.0
OPW-01	85	0	85	100.0
OPW-02	80	21	59	73.8
OPW-03	56	0	56	100.0
OPW-04	76	0	76	100.0

Table 29: Polymorphism revealed by AFLP primers in the morphological variants with mixed catkins obtained after pollination with S-13 irradiated pollen

Primer	Total number of fragments amplified per primer across all the plants	No. of monomorphic fragments	No. of polymorphic fragments	Polymorphism percentage
EAAC/MCAA	329	8	321	97.6
EACC/MCTC	381	48	333	87.4
EAAC/MCAG	235	32	203	86.4
EACC/MCTT	313	32	281	89.8
EACG/MCAA	320	32	288	90.0
EACA/MCTC	233	32	201	86.3
EACG/MCAG	272	8	264	97.1
EACA/MCTT	313	72	241	77.0

Table 30a: Banding pattern of M_1 plants and parents with different AFLP primer combinations

Primer	No. of amplified fragments specific to M-5 cultivar	No. of amplified fragments specific to S-13 cultivar	No. of amplified fragments specific to CW cultivar	Plants showing M-5 specific bands	Plants showing S-13 specific bands	Plants showing unique bands
EAAC/MCAA	6	5	4	Control hybrid (3) Plant 45 (1) Plant 14 (3) Plant 15 (3)	Plant 45 (1) Control hybrid (3)	Plant 40 (13) Plant 45 (15) Plant 14 (2) Plant 15 (2)
EACC/MCTC	8	7	4	Control hybrid (6) Plant 40 (5) Plant 45 (6) Plant 14 (4) Plant 15 (5)	Control hybrid (6) Plant 40 (2) Plant 45 (2) Plant 14 (2) Plant 15 (2)	Plant 40 (25) Plant 45 (22) Plant 14 (6) Plant 15 (2)
EAAC/MCAG	1	1	2	Plant 45 (1) Plant 14 (1) Plant 15 (1)	Control hybrid (1) Plant 14 (1) Plant 15 (1)	Plant 40 (13) Plant 45 (9)
EACC/MCTT	3	8	2	Control hybrid (2) Plant 40 (3) Plant 45 (3) Plant 14 (3) Plant 15 (3)	Control hybrid (7) Plant 40 (1) Plant 45 (1) Plant 14 (4) Plant 15 (3)	Plant 40 (21) Plant 45 (16) Plant 14 (8) Plant 15 (2)

Table 30b: Banding pattern of M_1 plants and parents with different AFLP primer combinations

Primer	No. of amplified fragments specific to M-5 cultivar	No. of amplified fragments specific to S-13 cultivar	No. of amplified fragments specific to CW cultivar	Plants showing M-5 specific bands	Plants showing S-13 specific bands	Plants showing unique bands
EACG/MCAA	0	4	1	--	Control hybrid (2) Plant 14 (1) Plant 15 (1)	Plant 40 (20) Plant 45 (9) Plant 14 (2) Plant 15 (1)
EACA/MCTC	4	1	1	Control hybrid (2) Plant 40 (1) Plant 45 (2) Plant 14 (3) Plant 15 (3)	Control hybrid (1)	Plant 40 (21) Plant 45 (6) Plant 14 (3) Plant 15 (6)
EACG/MCAG	3	2	3	Control hybrid (3) Plant 40 (1) Plant 14 (1) Plant 15 (1)	Control hybrid (1) Plant 14 (1) Plant 15 (1)	Plant 40 (8) Plant 45 (6) Plant 14 (2) Plant 15 (2)
EACA/MCTT	14	5	3	Control hybrid (6) Plant 40 (4) Plant 45 (6) Plant 14 (8) Plant 15 (8)	Control hybrid (5) Plant 14 (2) Plant 15 (2)	Plant 40 (14) Plant 45 (6)