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.

1
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 F₁ 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 M₁ 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 M₁ plants obtained after pollination with irradiated pollen of S-13 and China White.
- Flow cytometric and cytological analysis of M₁ 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.