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An overview of tissue culture techniques for crop improvement in mulberry

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Abstract
Mulberry is an economic plant grown for cultivating silk producing insect *Bombyx mori* worldwide. It is propagated through stem cuttings mainly but the success of stem cuttings is dependent on many factors *viz*., origin of genotype, age of stem, rooting ability of plant, physiological condition of plant and environmental conditions. The biotechnological approaches have been facilitated widely to improve mulberry crop production. Tissue culture techniques multiply and regenerate novel plants from genetically engineered cells in culture under aseptic conditions in a short period of time. Micropropagation, organogenesis and somatic embryogenesis are widely used tissue culture methods. Tissue culture methods are applicable in mulberry germplasm conservation, induction of tiploidy and tetraploidy, screening for stress tolerance, genetic transformation. Tissue culture techniques are convenient and cost-effective to improve the quality and quantity mulberry leaf production. This review presents a description of tissue culture techniques and their useful genetic transformation for the mulberry crop improvement to sustain silk industry.

Keywords: Genetic, micropropagation, stress tolerance, somatic embrogenesis, triploidy

Introduction
Mulberry (*Morus*) belongs to family Moraceae is one of the most economically important plant. It is a fast growing deciduous woody perennial tree with a deep root system and an invaluable tree of immense economic importance in silk industry for its foliage. It is the chief food plant for the silkworm, *Bombyx mori* L. (Das and Krishnaswami, 1965) [12]. It is widely grown in Asia either as a bush plant or tree for the main aim to sustain silk industry. The high leaf productivity is essential for sustaining profitability in sericulture. The improvement of productivity traits in mulberry plays a vital role in the progress of sericulture industry (Tewary *et al*., 1995; Dandin *et al*., 2003; Biasiolo *et al*., 2004) [42, 8, 11]. However, perennial nature of the plant coupled with prolonged juvenile period slows down the process of mulberry improvement (Kavyashree *et al*., 2001) [26]. Mulberry is propagated through both sexual and asexual modes of reproduction. However, the heterozygous nature of mulberry makes propagation of mulberry through seeds practically risky at commercial scale due high degree of variability. The dioecious nature, prolonged juvenile period and high inbreeding depression makes it uneasy to develop inbred lines (Vijayan *et al*., 2011) [49-50]. Mulberry can be vegetatively propagated through stem cuttings, grafting or budding. However, success of these methods depends on a number of factors such as genetic makeup of the plant, age and physiological conditions of the parental cutting, climatic conditions and cultural practices. Additionally, newly developed mulberry varieties cannot be immediately propagated through stem cuttings as at least 6-7 months of maturity is required before cuttings can be isolated from the parental plant (Kapur *et al*., 2001) [24]. The propagation of mulberry through stem cuttings is commercially followed method of planting mulberry crop. The success of stem cuttings is dependent on many factors *viz*., origin of genotype, age of stem, rooting ability of plant, physiological condition of plant and environmental conditions (Taha *et al*., 2020) [41]. The other methods of propagation include grafting, budding, layering also implicit some complexity at commercial scale. Vegetative propagation of mulberry through grafting is not economically viable (Attia *et al*., 2014) [4]. Mulberry tree improvement through conventional breeding is slow and also difficult due to its heterozygous nature.

However, the biotechnological techniques are always promising means when conventional methods are practically inapplicable at commercial scale (Guha *et al.*, 2010; Taha *et al*., 2020) [18, 41]. Plant tissue culture is an essential component of plant biotechnology which provides the means to multiply and regenerate novel plants from genetically engineered cells in culture under aseptic conditions (Hussain *et al*., 2012) [22]. It is widely exploited in rapid propagation of plants, obtaining disease free plants, somatic hybridization and obtaining androgenic and gynogenic haploids for breeding programmes.

Importance of tissue culture in mulberry

1. In a relatively short time and space a large number of plantlets can be produced starting from the single explant.
2. Taking an explant does not usually destroy the mother plant, so rare and endangered plants can be cloned safely.
3. It is easy to select desirable traits directly from the culture setup (*in vitro)* thereby decreasing the amount of space required, for field trials.
4. Once established, a plant tissue culture line can give a continuous supply of young plants throughout the year.
5. The time required is much shortened, no need to wait for the whole life cycle of seed development. For species that have long generation time, low level of seed production, or seeds that readily do not germinate, rapid propagation is possible.
6. *In vitro* growing plants usually free, from the bacterial and fungal diseases. Virus eradication and maintenance of plants in virus free state. This facilitates movement of plant across international boundaries.
7. Plant tissue banks can be frozen and then regenerated through tissue culture. It preserves the pollen and cell collections from which plants may be propagated.

General tissue culture techniques in plants

The tissue culture employs plant cells, tissues, organs, embryos, meristems, somatic cells, protoplast, cell suspension, anther or pollen accordingly named as cell culture, tissue culture, meristem culture, somatic cell culture and so on as depicted in figure 1 (Desai *et al*., 2022) [14]. Callus culture refers to production and maintenance of an unorganized mass of proliferative cell from isolated plant cell, tissue or organ by growing them on artificial nutrient medium in glass vials under controlled aseptic conditions (Neumann *et al*., 2020) [33]. The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordial or whole or part of an organ in way and function whereas single cell culture is a method of growing isolated single cell aseptically on nutrient medium under controlled condition (Uysal *et al.,* 2018) [45]. Suspension cultures are used in induction of somatic embryos and Shoot, production of secondary metabolites, *in vitro* mutagenesis, selection of mutants and genetic transformation studies (Kong *et al*., 2020) [27]. Embryo culture refers to aseptic isolation of embryo (of different developmental stages) from the bulk of maternal tissue of mature seed or capsule and *in vitro* culture under aseptic and controlled condition in nutrient semisolid or liquid medium to grow directly into plantlet. Androgenesis is the *in vitro* development of haploid plants originating from potent pollen grains through a series of cell division and differentiation whereas Pollen culture is the *in vitro* technique by which the pollengrains (preferably at the microscope stages) are squeezed from the intact anther and then cultured on nutrient medium where the microspores without producing male gametes (Horner, 1977) [21]. The isolated protoplast or naked plant cells are surrounded by plasma membrane which is potentially capable of cell wall regeneration, cell division, growth and plant regeneration on suitable medium under aseptic condition (Davey *et al*., 2010; Kumari, 2019) [13, 29]. The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro* producing clumps of shoots from either axillary or adventitious buds (Kane, 2018) [23]. This method can be used for clonal propagation. The explants of plants *viz*., root, stem and leaves possess meristematic and parenchyma is the most versatile of all types of tissues (Haque *et al*., 2022) [20]. They are capable of division and growth.

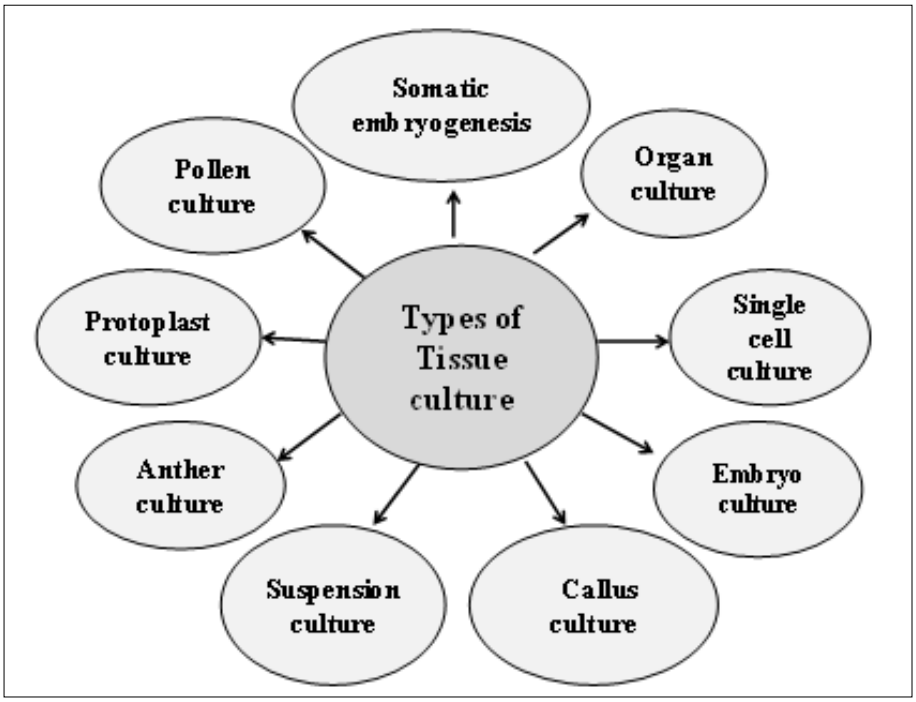

Fig 1: Types of tissue culture techniques in mulberry

Tissue culture technique in mulberry has developed and ramified into different areas such as micropropagation, callus culture, organogenesis, and screening of genotypes for stress tolerance, induction of polyploids, cryopreservation, and transgenesis. There are three methods widely used in tissue culture which includes micro propagation, organogenesis and somatic embryogenesis. Micropropagation provides such an alternative tool for the rapid and cost-effective multiplication of mulberry, as a large number of clones can be produced in a relatively short time and space.

Micropropagation

Micropropagation allows multiplication of the plant in a short period under the controlled conditions (Cardoso *et al*., 2018) [9]. Further, in conventional method of propagation through stem cuttings, each stem cutting produces only one plant, whereas in micropropagation thousands of plants can be produced from a single plant piece (explants) (Kumar and Reddy, 2018). Micropropagation can provide plantlets throughout the year irrespective of seasonal variations (Abdalla *et al*., 2022) [1]. The procedure for multiplication of mulberry platelets through micro propagation is depicted in figure 2. Thus, micropropagation is an efficient and cost effective method for rapid multiplication of mulberry in a relatively short time and limited space. It facilitates production of virus free plants from the apical meristematic tissues (Mallick and Sengupta, 2022) [31]. mulberry micropropagation was initiated by regenerating whole plants from axillary buds of *M. alba* (Ohyama, 1970) [34]. Shoot tips and dormant axillary buds were found suitable for mulberry micropropagation. It is used to develop high-quality clonal plants (Kumar and Reddy, 2011) [28]. The main advantages attributed to the potential of rapid, large scale propagation of new genotypes and the use of small amount of original germplasm (Smith, 1990) [44]. These methods have been used for numerous mulberry species, including *Morus nigra*, *Morus cathayana* Hemsl., *Morus serrate* Roxb., *Morus latifolia* Poilet, *Morus laevigata* Wall., *Morus multicaulis* Perr., *Morus alba*, and *Morus alba* var. V1 (Vijayan *et al*., 2011) [49-50]. The composition of media is one of the factors that significantly affect micropropagation in mulberry. Among the different media compositions and hormones tested, MS (Murashige and Skoog, 1962) [32] medium containing 2 mg L-1 6-benzylamino purine (BAP) is the best for shoot multiplication (Lalitha *et al*., 2013) [30]. However, it is also important to note that concentrations of 6-benzyladenine (BA) above 2 mg L-1 is inhibitory for shoot initiation and multiplication (Bhau and Wakhlu, 2003) [6]. Among the different sugars such as sucrose, glucose and fructose tested, sucrose is the best (Bhau and Wakhlu, 2003) [6]. It was found that 3% glucose is better for shoot formation from leaf explants (Vijayan, 2003) [46-48]. Among different pH levels tested, 5.6-5.8 is optimum for shoot multiplication (Enomoto, 1987) [16]. Agar in the culture medium also plays an important role in the success of micropropagation. Among various concentrations tested, 0.8% agar is the best whereas above 1% reduced shoot growth (Pattnaik and Chand, 1997) [36]. Among the plant hormones, 0.5 mg L-1 NAA is the best for *M. alba*, *M. indica*, *M. multicaulis* and *M. latifolia* resulting in 95% rooting (Vijayan *et al*., 2003) [46-48], though indole-3- butyric acid (IBA) is the best auxin for *M. nigra* (Rao and Bapat, 1993) [38]. It was observed that higher concentrations of most of the auxins (>1.0 mg L-1) were inhibitory for root formation in mulberry and there was a strong interactive relationships among genotype, hormone type and concentration (Vijayan *et al*., 2014) [47].

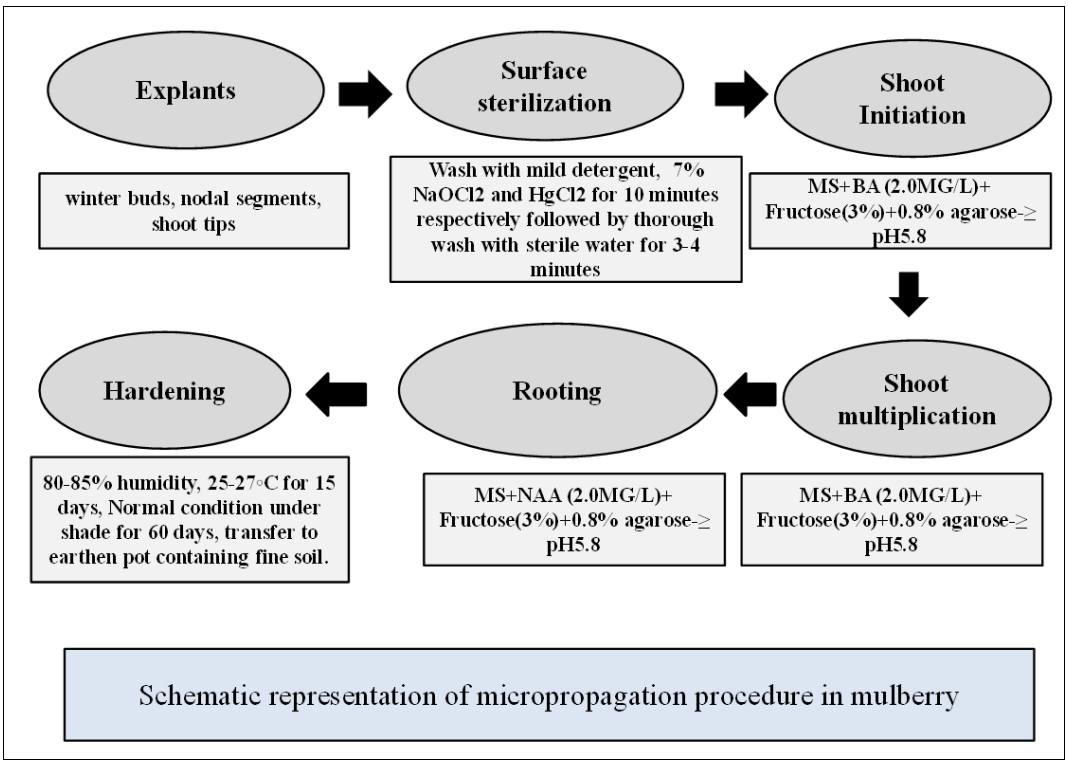

Fig 2: Micropropagation procedure followed in mulberry

Organogenesis

Plant regeneration from somatic cells has a high impact in woody tree improvement as it facilitates the development of transgenic plants, isolation of somaclonal variants, and development of stress-tolerant plants through cell line selection and multiplication of desirable genotypes (Bajaj 1986; Gupta 1988) [5, 19]. Therefore, attempts have been made in mulberry to develop protocols for direct plant regeneration from explants

like leaves, cotyledons, and embryos and also for indirect plant development via callus culture. Direct organogenesis from explants has the advantage of inducing the least genetic variation among regenerated plants, i.e. clonal propagation whereas organogenesis from callus has the advantage of higher plant productivity (Desai *et al*., 2022) [14]. Plants developed from callus serve as a significant source of variation variation for isolation of somaclonal variants with desirable traits.

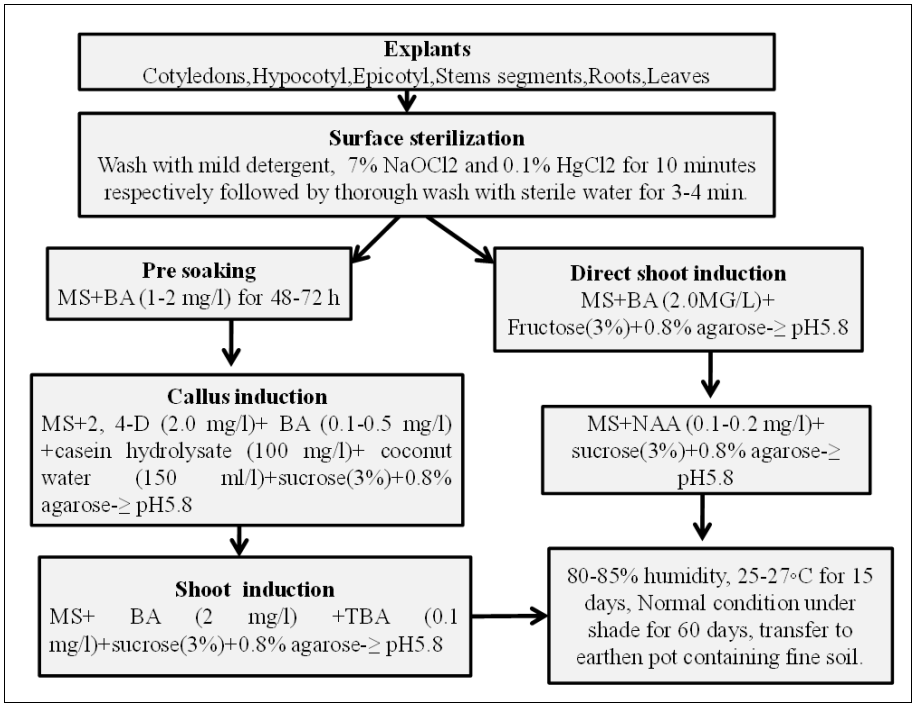

Fig 3: Schematic representation of organogenesis procedure in mulberry.

Somatic Embryogenesis

Somatic embryogenesis is the process of a single or group of cells initiating the development pathway that leads to reproducible regeneration of non zygotic embryos capable of germinating to form complete plants. Somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of commercial crop species. Several investigating groups attempted induction of somatic embryos in mulberry but the rate of success is less. Shajahan *et al.* (1995) [39] obtained heart shaped embryos from *M. alba* hypocotyl segments cultured on MS medium supplemented with 2,4 D (0.45-4.52 µM) and BAP (2.2 µM). Primary and secondary somatic embryoids were obtained by culturing zygotic embryos on MS medium containing 0.05 mg L-1 2,4-D + 0.1 mg L-1 BAP and 6% sucrose (Agarwal, 2002) [2]. However, due to the difficulty in hormonally controlling the formation of adventitious shoots and roots in mulberry, somatic embryogenesis has not been developed as it is in many other crop plants (Tiku *et al*., 2021) [44]. Thus, concerted efforts are needed to make somatic embryogenesis successful in mulberry.

Application of tissue culture technique for induction of tetraploidy

In general the mulberry is propagated through vegetative means. Hence, sterile high yielding

Varieties/cultivars do not pose any problems for their true to type multiplication. Triploidy in mulberry is considered as the optimum level of ploidy because triploids show several advantages over plants of other ploids. Triploids are superior in leaf yield, stress resistance and chemical components of the leaf (Vijayan *et al*., 2014) [47]. Considering these advantages, tetraploids are developed from diploids by colchicine treatment of the growing shoots. In this method, small cotton pads soaked with 1.0-2.0% colchicine solution is applied over the growing buds for 2-3 consecutive days. Though this method is easier to apply, it suffers from quick drying of the cotton pad, excessive loss of colchicines and difficulty in maintaining the uniform concentration of the colchicines solution. Application of colchicine *in vitro* solves most of these problems and also makes the system more economic. The cultured apical buds of field grown mulberry on MS medium supplemented with BAP (2 mg L-1) and four concentrations of colchicine (0.0, 0.05, 0.1 and 0.2%) revealed that 0.1% produced 39.4 ± 4.8% tetraploids (Chakraborty *et al*., 1998) [10]. Higher concentrations of colchicines reduced the survivability of the buds and low concentration reduced the percentage of tetraploid formation. Additionally, *in vitro* application of colchicine is more cost effective as the same medium can be used for at least 4 repeated treatments without reducing the efficiency of the conchicine to induce tetraploidy. Another method of getting triploidy in mulberry is to culture the endosperm because in angiosperm, endosperm is a triploid tissue formed via double fertilization (Bhojwani and Razdan, 1996) [7]. In mulberry, for the first time, successfully developed triploids from endosperm of the variety S36 (Thomas *et al.*, 2000) [43]. It clearly indicates the efficiency of the tissue culture protocol for developing more triploids in mulberry.

Application of tissue culture technique in screening for stress tolerance in mulberry

Salt tolerance in plants is a complex phenomenon involving morphological, physiological and biochemical processes, screening of genotypes for salt tolerance need to be done in such conditions where the influence of external factors is minimal (Vijayan *et al*., 2011) [49-50]. Maintenance of uniformity of salinity across the plants under *in vitro* is considered as an ideal option, where most of the environmental conditions can be controlled. The axillary buds of 63 mulberry germplasm

accessions maintained at the Central Sericultural Research and Training Institute, Berhampore, West Bengal, India isolated salt tolerant genotypes by surface sterilizing the nodal explants and culturing on MS medium (Murashige and Skoog, 1962) [32] supplemented with 2 mg L-1 BAP, 30 mg L-1 sucrose and 0.0% to 1.0% NaCl. Genotypes showing early sprouting and better growth rate in 1.0% NaCl were selected as salt tolerant. Out of the 63 accessions tested, 16 sprouted in 1.0% NaCl, and 13 of them survived till 30 days and these genotypes were considered as salt tolerant.The study while confirming the higher salt tolerance of the selected genotypes also demonstrated the efficacy of *in vitro* screening to isolate salt tolerant genotypes in mulberry (Vijayan *et al*., 2003) [46-48]. This technique was also used to investigate the effect of NaHCO3 on various characters of mulberry (Ahmad *et al.*, 2007) [3]. This method is more economical, efficient and less time consuming for screening large number of mulberry accessions for salt tolerance.

Application of tissue culture technique in cryopreservation of mulberry germplasm

The high heterozygosity hinders conservation of mulberry germplasm through seeds as the progenies from such seeds are heterogenous in nature and getting true to the parental type is difficult. Thus, mulberry germplasm is conserved as *ex situ* germplasms, which is laborious, needs huge resources, and is in a risk of destruction by natural calamities, pests and diseases (Vijayan *et al*., 2011) [49-50]. Thus, safe alternative methods with economically viability need to be explored. Cryopreservation is one such alternative wherein plant materials are stored at ultra-low temperatures (-196 °C) in liquid nitrogen. At this temperature all the metabolic activities of the cell including divisions remain arrested; hence, the material remains unaltered for long period. Two different cryopreservation techniques in vogue are the classical one and the modern vitrification (Engelmann, 2000) [15]. In classical cryopreservation technique, the plant material is cool down slowly at a controlled rate of 0.1-4 °C/min to about -40 °C and rapidly immersed in liquid nitrogen. In vitrification, plant material is physically or osmotically dehydrated and is subsequently subjected to ultra rapid freezing resulting in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without occurrence of ice crystals (Kaviani, 2011) [25]. Although different plant materials are used for cryopreservation, the most appropriate material for cryopreservation of mulberry is winter buds. Genetic constitution of the plant material also influences significantly the success and duration of the preservation. Cryopreservation techniques have been standardized for *M. indica*, *M. alba*, *latfolia*, *M. cathayana*, *M. laevigata*, *M. nigra*, *M. australis*, *M. bombycis*, *M. sinensis*, *M multicaulis* and *M. rotundiloba* species (Rao *et al*., 2007) [37]. Encapsulation of winter-hardened shoot tips with calcium alginate coating was also tested successfully (Padro *et al*., 2012) [35]. It is concluded from different experiments that dormant buds of mulberry can be cryopreserved for 11 years without reducing the viability of the buds (Fukui *et al*., 2011) [17].

Conclusion

Tissue culture have been explored for the improvement of crop plants by micropropgation techniques, isolation of somaclonal variants, organogenesis, induction of polyploidy, germplasm conservation, abiotic stress tolerance, production of synthetic seeds, cryopreservation, transgenesis and other traits associated with economic plants. However, exploitation of other biotechnological approaches such as molecular markers, with full potential can facilitate development of more and faster improvement of economic crops. The transfer of technology at commercial is yet to achieve. The desirable gene transfer approaches, genetic maps, quantitative triat loci (QTL), androgenesis, gynogenesis and haploid production protocols for easy and rapid development of mulberry crop improvement could be facilitated.

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