Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 1795-1808
Received: 15-03-2019
Accepted: 20-04-2019

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Polyploidy in agriculture: With special reference to mulberry

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Abstract
The mulberry is an economically important crop, cultivated for its foliage to rear the silkworm *Bombyx mori* L. Its chromosome number varies from 2n = 28 to 2n = 308 with ploidy level from x to 22x. Although there are good number of breeding techniques available in various agricultural crops; including mulberry, yet each of these methods suffers from one drawback or the other. Polyploidy has been intensively used in improving the mulberry crop, and there are good numbers of reports available which have indicated improvement in various commercial characters of the plant. However, the technique has not been popularized in mulberry due to many reasons. The suitability of breeding method usually depends upon the nature of crop. So, I am of the opinion that selection of the breeding method depends upon the kind and nature of crop which is under study.

Keywords: Mulberry (Morus spp.), Polyploid, Triploid, Breeding, Sericulture.

Introduction
Polyploidy is an intriguing phenomenon in plants that has provided an important pathway for evolution and speciation. Although the first polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy are still being elucidated (Bennett, 2004; Soltis *et al*., 2003). On a more practical level, there are many opportunities for utilizing polyploidy as a valuable tool in traditional plant breeding programs. Polyploids are organisms with multiple sets of chromosomes in excess of the diploid number (Acquaah, 2007; Chen, 2010; Comai, 2005; Ramsey and Schemske, 1998) [1, 14, 19, 66]. Polyploidy is common in nature and provides a major mechanism for adaptation and speciation. Approximately 50-70% of angiosperms, which include many crop plants, have undergone polyploidy during their evolutionary process (Chen *et al*., 2007) [13]. Flowering plants form polyploids at a significantly high frequency of 1 in every 100,000 plants (Comai, 2005) [19]. Many studies have been carried out to understand the nature of polyploidism.
To understand polyploidy, a few basic notations need be defined.

- **Genome**: refers to the complete genetic material present in an organism.
- **Haploid number (n)**: is the number of chromosomes in a gamete (n), half the number of total chromosomes present in the somatic cells. Two gametes form a diploid zygote with twice this number (2n). For humans, a diploid species, n=23. a typical human somatic cell contains 46 chromosomes: 2 complete haploid sets, which make up 23 homologous chromosome pairs (Acquaah, 2007; Otto and Whitton, 2000) [1, 59].
- **Monoploid number (x):** is the total number of chromosomes in a single complete set of chromosomes and this doesn't change whether we are taking about a somatic cell or a gametic cell. E.g. wheat is an hexaploid species, it has 6 sets of chromosomes, 2 sets from each of three different diploid species that are its distant ancestors. The somatic cells are thus hexaploid, with 6 sets of chromosomes, 2n=6x=42. The gametes are haploid for their own species, but triploid in respect to wheat. The Monoploid number x=7 and haploid number n= 21.

Ploidy
Ploidy is the number of sets of chromosomes in a cell. Usually a gamete (sperm or egg, which fuse into a single cell during the fertilization phase of sexual reproduction) carries a full set of chromosomes that includes a single copy of each chromosome, as aneuploidy generally leads to severe genetic disease in the offspring. The gametic or haploid number (*n*) is the number of chromosomes in a gamete. Two gametes form a diploid zygote with twice this number (2*n*, the zygotic or diploid number) i.e. two copies of autosomal chromosomes. For humans, a diploid species, *n* = 23. A typical human somatic cell contains 46 chromosomes: 2 complete haploid sets, which make up 23 homologous chromosome pairs. It is also possible on rare occasions

for the ploidy to increase in the germline, which can result in polyploid offspring and ultimately polyploid *species*. This is an important evolutionary mechanism in both plants and animals. As a result, it becomes desirable to distinguish between the ploidy of a species or variety as it presently breeds and that of an ancestor.

As the chromosome number is generally reduced only by the specialized process of meiosis, the somatic cells of the body inherit and maintain the chromosome number of the zygote. However, in many situations somatic cells double their copy number by means of end reduplication as an aspect of cellular differentiation. For example, the hearts of two-year-old children contain 85% diploid and 15% tetraploid nuclei, but by 12 years of age the proportions become approximately equal, and adults examined contained 27% diploid, 71% tetraploid and 2% octaploid nuclei (John,1979) [36].

Cells are described according to the number of sets present (the ploidy level): monoploid (1 set), diploid (2 sets), triploid (3 sets), tetraploid (4 sets), pentaploid (5 sets), hexaploid (6 sets), heptaploid (Murthy *et al*., 1973) [71] or septaploid (Tateoka, 1975) (7 sets), etc. The generic term polyploid is frequently used to describe cells with three or more sets of chromosomes (triploid or higher ploidy). Ploidy can also differ with life cycle (Parfrey *et al*., 2008; Qiu *et al.*, 2012) [61, 63]. In some insects it differs by caste. In humans, only the gametes are haploid, but in the Australian bulldog ant, *Myrmecia pilosula*, a haplodiploid species, haploid individuals of this species have a single chromosome, and diploid individuals have two chromosomes (Crossland and Croziea, 1986). In *Entamoeba,* the ploidy level varies from 4*n* to 40*n* in a single population (https://bcrc.bio.umass.edu/). Alternation of generations occurs in many plants.

There are mainly two main types of ploidys' present in the living system, they are:

1. Aneuploidy
2. Euploidy

Aneuploidy

Aneuploidy are polyploids that contain either an addition or subtraction of one or more specific chromosome(s) to the total number of chromosomes that usually make up the ploidy of a species (Acquaah, 2007; Ramsey and Schemske, 1998) [1, 66]. Aneuploids result from the formation of univalents and multivalents during meiosis of euploids (Acquaah, 2007) [1]. For example, several studies have found that 30-40% of progeny derived from autotetraploid maize are aneuploids (Comai, 2005) [19]. With no mechanism of dividing univalents equally among daughter cells during anaphase I, some cells inherit more genetic material than others (Ramsey and Schemske, 1998) [66]. Similarly, multivalents such as homologous chromosomes may fail to separate during meiosis leading to unequal migration of chromosomes to opposite poles. This mechanism is called non-disjunction (Acquaah, 2007) [1]. These meiotic aberrances result in plants with reduced vigor. Aneuploids are classified according to the number of chromosomes gained or lost as shown in Table 1.

Table 1: Classification of aneuploids

Term	Chromosome number
Monosomy	2n-1
Nullisomy	2n-2
Trisomy	2n+1
Tetrasomy	2n+2
Polysomy	>2n+1

Euploidy

Is the state of having variation in chromosome number that is an exact multiple of the characteristic haploid number. Leads to the formation of new species. Mostly in plants and rarely in animals. It can also be described as a state in which a cell or organism is having the same number of each homologous chromosome, possibly excluding the sex-determining chromosomes. For example, most human cells have 2 of each of the 23 homologous monoploid chromosomes, for a total of 46 chromosomes. A human cell with an abnormal number of 3 of each would also be considered as euploid. (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/P/Polyploidy.html).

Types of Euploidy

a) Basic Euploidy: it involves the basic states of haploidy and diploidy.

b) Abberant Euploidy: it involves the higher ploidy levels like triploidy, Tetraploidy, polyploidy etc.

Polyploidy

It is the heritable condition in which a normally diploid cell or organism acquires one or more additional sets of chromosomes. It is a kind of numerical change in a whole set of chromosomes. This phenomenon is present mainly in plants than in animals. A few animals exhibiting polyploidy are: fishes, lizards, amphibians and some insects. Polyploid types are labeled according to the number of chromosome sets in the nucleus.

Triploids (3x): organisms having three sets of chromosomes in their nucleus e.g. seedless watermelon, banana, citrus.

Tetraploid (4x): organisms having four sets of chromosomes in their nucleus e.g. Durum wheat, Gossypium hirsutum.

Hexaploid (6x): organisms having six sets of chromosomes in their nucleus e.g. Bread wheat, oat etc.

Polyploidy is most commonly observed in the plant kingdom. Thousands of years of selective cultivation and plant breeding have resulted in vigorous food plants that are commonly tetraploid and hexaploid. If you compare diploid and tetraploid varieties of the same type of plant, very often the tetraploid plants grow larger and more vigorously. Among animals, polyploidy is often observed in bony fish and amphibians. In general, there is a genetic bias for even ploidy numbers in animals. Polyploidy" is the multiplication of entire sets of chromosomes. In other words, polyploid genotype has more than two homologous sets of chromosomes in its cell. For example, tetraploid plants have four sets of chromosomes in their cells. Polyploidy is common among flowering plants (angiosperms) and is a major force in plant speciation (Grant, 1981). Almost 47%-70% of angiosperms are polyploid (Ramsey & Schemske, 1998 [66].

Traditionally, polyploidy refers to either duplication of a single genome (autopolyploidy) or from the combination of two or more differentiated genomes (allopolyploidy) (Kihara and Ono, 1926; Stebbins, 1947; Stebbins, 1971; Grant, 1981). Wendel and Doyle (2005) noted that polyploids form in many ways, from individual diploids doubling their chromosome complements (strict autopolyploid) to hybridization between individuals from highly divergent species (strict allopolyploid). Thus, there are both taxonomic (the same or different species) and cytogenetic (ability of chromosomes to

pair) dimensions to these terms. Clearly there is broad overlap between the taxonomic and genetic definitions of polyploids, and in actuality these two modes of formation represent endpoints in a taxonomic-genetic continuum. One of the early examples of a natural polyploid was one of De Vries's original mutations of *Oenothera lamarckiana* (mutat. *gigas*) (19). The first example of an artificial polyploid was by Winkler (1916) who in fact introduced the term polyploidy. Winkler was working on vegetative grafts and chimeras of *Solanum nigrum* and found that callus regenerating from cut surfaces of stem explants were teratploid. Digby (1912) had discovered the occurrence of a fertile type *Primula kewensis* from a sterile inter-specific hybrid through chromosome doubling but failed to realize its significance in the context of polyploidy. Though unaware of the 'Primula type" fertile hybrid, Winge (1917), from his studies on the chromosomal counts of *Chenopodium* and *Chrysanthemum* found that chromosome numbers of related species were multiples of some common basic number; he subsequently proposed a hypothesis that chromosome doubling in sterile inter-specific hybrids is a means of converting them into fertile offsprings. This was subsequently verified by various workers in artificial inter-specific hybridizations of *Nicotiana, Raphanobrassica* and *Gaeleopsis*. Finally the colchicine method of chromosome doubling was developed by Blakeslee and Avery (1937) [8] and became an important tool for the experimental study of polyploidy.

Types of Polyploidy

Autopolyploidy

Autopolyploidy are also referred to as autoploids. They contain multiple copies of the basic set (x) of chromosomes of the same genome (Acquaah, 2007; Chen, 2010) [1, 14]. Autoploids occur in nature through union of unreduced gametes and at times can be artificially induced (Chen, 2010) [14].

Natural autoploids include tetraploid crops such as alfafa, peanut, potato and coffee and triploid bananas. They occur spontaneously through the process of chromosome doubling. Chromosome doubling in autoploids has varying effect based on the species. Spontaneous chromosome doubling in ornamentals and forage grasses has led to increased vigour. For instance, ornamentals such as tulip and hyacinth, and forage grasses such as ryegrasses have yielded superior varieties following spontaneous chromosome doubling (Acquaah, 2007) [1]. Due to the observed advantages in nature, breeders have harnessed the process of chromosome doubling *in vitro* through induced polyploidy to produce superior crops. For example, induced autotetraploids in the watermelon crop are used for the production of seedless triploid hybrids fruits (Fig) (Wehner, 2008) [90]. Such polyploids are induced through the treatment of diploids with mitotic inhibitors such as dinitroaniles and colchicine (Compton *et al*., 1996) [20]. To determine the ploidy status of induced polyploids, several approaches may be used. These include, chloroplast count in guard cells, morphological features such as leaf, flower or pollen size (gigas effect) and flow cytometry (Brummer *et al*., 1999; Heping *et al*., 2008) [12, 32].

Allopolyploid

Allopolyploids are also called alloploids. They are a combination of genomes from different species (Acquaah, 2007) [1]. They result from hybridization of two or more genomes followed by chromosome doubling or by the fusion of unreduced gametes between species (Acquaah, 2007; Chen, 2010; Jones *et al*., 2008; Ramsey and Schemske, 1998) [1, 14, 66]. This process is key in the process of speciation for angiosperms and ferns (Chen, 2010) [14] and occurs often in nature. Economically important natural alloploid crops include strawberry, wheat, oat, upland cotton, oilseed rape, blueberry and mustard (Acquaah, 2007; Chen, 2010) [1, 14]. To differentiate between the sources of the genomes in an alloploid, each genome is designated by a different letter. For example, the origin of the cultivated mustards (*Brassica spp*) has been well explained by Nagaharu in the triangle of U with each species represented by a distinct letter (Fig 5.2) (Bellostas *et al*., 2007; Nelson *et al*., 2009).

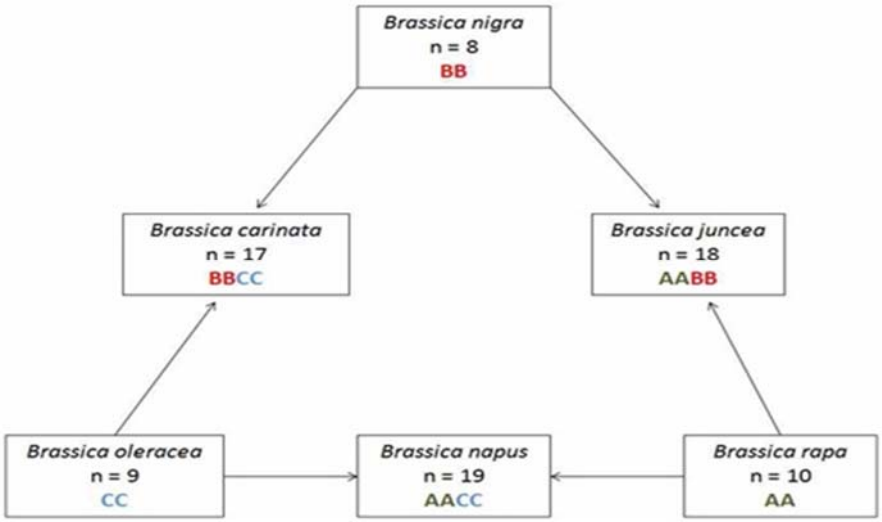

Fig 2: Triangle of U showing the origin of cultivated mustard

The hybridized genomes differ in their degree of homology with some being able to pair during mitosis and/or meiosis while others not. When only segments of the chromosomes of the combining genomes differ, the phenomenon is called segmental all oploidy. These chromosomes are similar but not homologous and are called homeologous chromosomes. Such chromosomes indicate ancestral homology (Acquaah, 2007) [1]. Induced alloploidy is not common. However, it has been used in some Genus such as *Cucumis* to elucidate the molecular mechanisms involved in Diploidization (tendency

of polyploids to act as diploids) (Chen *et al*., 2007) [13]. In this study, an allotetraploid was induced by hybridization between *Cucumis sativus* and *Cucumis hystrix* followed by chromosome doubling. Cytogenetic studies were carried out in the following generations to establish the molecular mechanisms involved stabilization of newly formed allopolyploids which include neo-functionalization and sub-functionalization (Chen *et al*., 2007; Comai, 2005) [13, 19].

Natural Mechanism For Polyploidy

Several cytological mechanisms are known to spontaneously induce polyploidy in plants (Ramsey and Schemske, 1998) [66]. One such route involves non-reduction of gametes during meiosis a process called meiotic nuclear restitution. The formed gametes (2n) contain the somatic nuclear condition of cells. Meiotic aberrations related to spindle formation, spindle function and cytokinesis have been implicated in this process (Ramsey and Schemske, 1998) [66]. The subsequent union of reduced and non-reduced gametes leads to the formation of polyploids (Acquaah, 2007; Ramsey and Schemske, 1998) [1, 66]. For example, autotetraploids may be formed in a diploid population through the union of two unreduced 2n gametes as was found in the F1 progenies of open-pollinated diploid apples (Ramsey and Schemske, 1998) [66]. Similarly, spontaneous allotetraploids were formed in 90% of F2 progenies of interspecific crosses between *Digitalis ambigua* and *Digitalis purpurea*, which are common ornamental plants (Ramsey and Schemske, 1998) [66]. Another example is the formation of autohexaploid *Beta vulgaris* (sugar beet) and alfalfa from cultivated autotetraploid varieties apparently from the union of reduced (2x) and unreduced (4x) gametes (Bingham, 1968; Hornsey, 1973) [7].

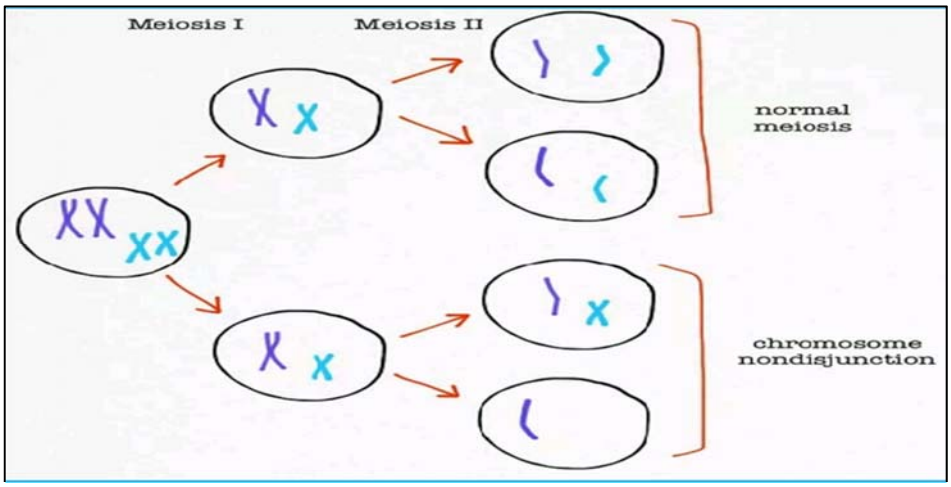

Fig 3: Normal meiosis v/s chromosomal non-disjunction.

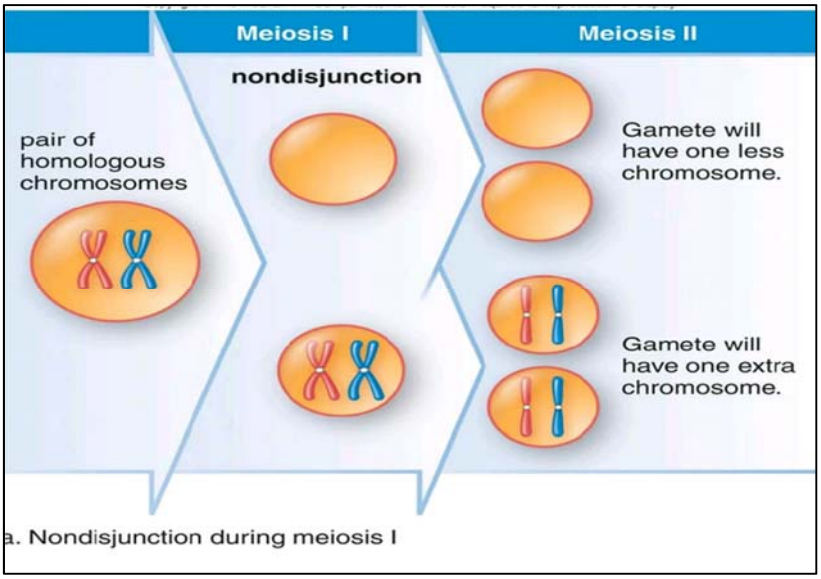

Fig 4: Non- Disjunction during meiosis I and II.

Another major route for polyploid formation is through somatic doubling of chromosomes during mitosis. In nature, the formation of polyploids as a result of mitotic aberrations has been reported in the meristematic tissue of several plant species including tomato and in non-meristematic tissues of plants such as bean (Coleman, 1950; Ramsey and Schemske, 1998) [66]. Artificial inducement of polyploids through the inhibition of mitosis is routine in plant breeding. High temperatures above 40°C have been used to induce tetraploid and octaploid corn seedlings albeit with low success of 1.8% and 0.8% respectively (Randolph, 1932). Currently, chemical mitotic inhibitory agents such as colchicine or dinitroaniles are used to induce polyploidy in crop plants. A typical example is the production of tetraploid watermelon plants for

the production of seedless triploid watermelon (Compton *et al*., 1996) [20].

In addition, an uncommon mechanism of polyploid formation involves polyspermy where one egg is fertilized by several male nucleuses as commonly observed in orchids (Ramsey and Schemske, 1998) [66]. The major pathways involved in polyploidy formation are represented in Figure:

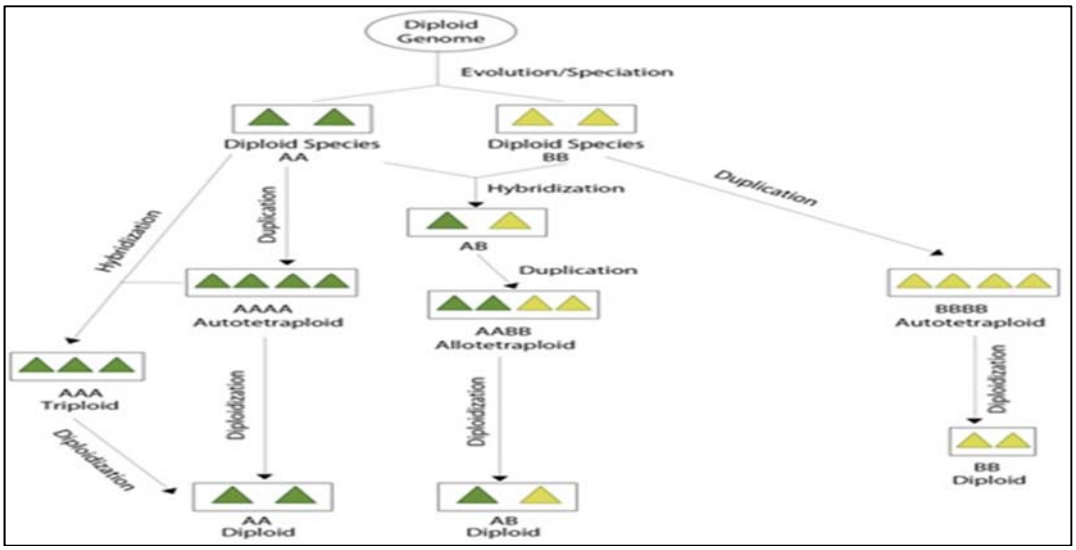

Fig 5: Major pathways in the formation of polyploids

Alterations Associated with Polyploidy

Several changes in the plant accompany spontaneous or induced polyploidy. These may be changes in genetic composition, physiological mechanisms, structural composition and vigor. Some of these changes create the platform for the commercial exploitation of polyploids. Genetic changes following genome duplication involve the rapid loss of chromosomal segments in a process called diploidization. Diploidization describes the process by which a polyploid genome become more 'diploid-like' in character (Clarkson *et al*., 2005; Comai, 2005; Ozkan and Feldman, 2009) [19]. It is necessary to eliminate duplicated genes in a newly formed polyploid to avoid gene silencing as well as to stabilize fertility (Chen *et al*., 2007; Chen, 2010; Clarkson *et al*., 2005; Comai, 2005) [19, 14]. Duplicated genes that are retained often undergo sub functionalization (complementing genes) and neo functionalization (genes with novel functions) (Comai, 2005; Osborn *et al*., 2003) [19]. Diploidization has been described for many genus including *Nicotina* and *Cucumis* (Chen *et al*., 2007; Comai, 2005) [14, 19].

The increase in nuclear ploidy affects the structural and anatomical characteristics of the plant. In general, polyploidy results in increased leaf and flower size, stomatal density, cell size and chloroplast count (Dhawan and Lavania, 1996) [26]. These phenomena are collectively referred to as the Gigas effect (Acquaah, 2007) [1]. It has been mainly applied in forage and ornamental breeding.

Physiological changes are also known to accompany genome duplication. These mainly result from change of metabolism resulting in a general increase in secondary metabolites (Levin, 1983) [43]. This property has found application in the breeding of medicinal herbs in the production of pharmaceuticals. Hybrid vigor resulting from interspecific crosses in allopolyploids is one of the most exploited advantages of polyploid in plant breeding (Ramsey and Schemske, 1998) [66].

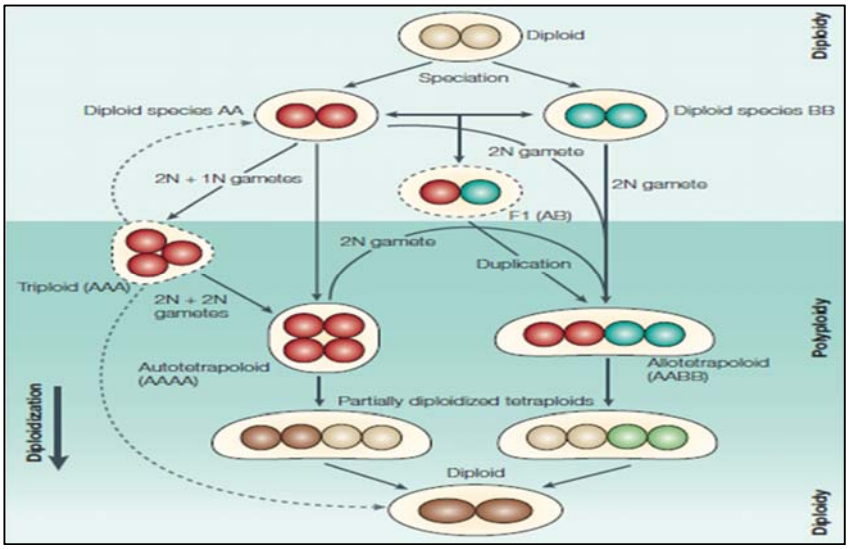

Fig 6: Polyploidy and Diploidization process.

Induced Polyploidy

Herein polyploidy is deliberately induced in the cells to harness it benefits. Induced changes in the chromosome are referred to as polyploid breeding. The method of breeding is entirely dependent on the regulation of chromosome pairing and recombination. The strategies used for such breeding techniques depend on the origin. There are several methods used for induction of polyploidy for commercial applications. Polyploidy is mainly induced by two agents:

2.1 Physical agents

Polyploidy can be induced with sudden variations in the physical and environmental factors such as sudden changes in temperature, dehydration, UV light, X-rays, infections etc., which can cause chromosome doubling.

a. Heat/ cold shock:

Sudden variations in temperature such as heat or cold shock have been found to result in the formation of polyploid cells. Examples include formation of tetraploidy in Datura due to cold shock treatment and tetraploid origins in maize due to heat shocks. Heat treatment has been found effective in generation of polyploid off-springs in wheat and rye also.

b. X-rays

Normal diploid plant cells give rise to polyploid generation on exposure to radioactive substances like radium.

c. Gamma rays

Exposure to damage causing gamma rays has also reported in the formation of polyploid cells.

d. Centrifugation:

Centrifugation of seedlings has been found to produce a higher incidence of polyploid cells in the plant. (http://www.biotecharticles.com/Agriculture-Article/Inducing-Polyploidy-For-Creation-of-Better-Species-924.html)

2.2Chemical agents

Various chemicals have been used to induce polyploidy. Some of them are listed below:

Colchicine

This is the most common chemical used for inducing polyploidy. Its an alkaloid extracted from meadow saffron (*Colchicum autumnale*). This chemical actually suppresses the mitotic spindle fiber production during mitosis as it disrupts the microtubule polymerization. (Blakslee and Avery 1937) [8]. The mitosis that takes place after colchicine treatment is called C- mitosis. In the colchicine treated cells, an S phase of the cell cycle occurs, but neither chromosome separation nor cell division occurs. Interference of the alkaloid colchicine with the "tubulin" molecules arrests the formation of spindle fibers and thus prevents the separation of chromosomes to the poles (Blakeslee and Avery 1937) [8]. As the treated cell enters telophase, a nuclear membrane is formed around the entire doubled set of chromosomes. Thus, treating diploid (2n) cells for one cycle leads to formation of tetraploids (4n) with exactly four copies of each type of chromosome e.g., AaBb =AAaaBBbb.

Other chemicals

Polyploidy can also be induced by the use of other chemicals such as oryzalin, acenapthalene, nitrous oxide, 8-hydroxyquinoline, etc., which have polyploidizing effect. Others which yield similar results include chloral hydrate, veratrine, sulfanilamide, mercury chloride, ethyl hexachlorocyclohexane etc. (www.biotecharticles.com/Agriculture-Article/)

Applications of Polyploidy In Agriculture

Heterosis in allopolyploids

Heterosis or hybrid vigor is the difference between the hybrid and the mean of the two parents and is characterized by increased vigor and superior qualitative or quantitative traits (Chen, 2010; Dhawan and Lavania, 1996; Lamkey and Edwards, 1999) [14, 26, 42]. Over the last several decades, breeders have increased the world food production by utilizing the concept of heterosis in hybrid cultivars (Kempe and Gils, 2011) [40]. For example, following the introduction of hybrid corn (diploid) in the 1920's, there was a six fold increase in corn production between then and 1990 in the U.S (Stuber, 1994). However, unlike diploids which may lose heterosis with each consecutive generation due to segregation, alloploidy and autoploidy imposes pairing of homologous chromosomes, thus preventing intergenic recombination (Comai, 2005) [19]. This concept is called preferential or selective pairing and is the tendency for a doubled set of chromosomes to pair independently of the doubled set of chromosomes of the other species (Acquaah, 2007) [7]. In this way, heterozygosity is maintained throughout generations (Acquaah, 2007; Comai, 2005) [1, 19]. Generally, the parents used in hybrid formation should be within subspecies or between subspecies. An example of a man-made interspecies allopolyploid hybrid is triticale. It is derived from crossing two cereals, hexaploid bread wheat (*T. aestivum*) and rye (*Secale cereale*). Triticale was developed to combine good qualities of wheat including high yield and grain quality with the hardiness (disease and stress tolerance) of rye (Acquaah, 2007; Chen, 2010; Haesaert and De Baets, 1994; Wolski and Pojmaj, 1994) [1, 14].

The process of hybrid formation for polyploids is not without setbacks. Many interspecific hybrids have low fertility and viability due to hybrid incompatibilities (Chen, 2010; Orr, 1996) [14, 55]. Hybrid incompatibility results from genes that are functionally diverged in the respective hybrid forming species. This may lead to silencing of protein encoding genes and has been reported in interspecific hybrids of *Arabidopsis* (Chen, 2010) [14]. To increase the heterosis, fertility and viability of interspecific hybrids, several factors should be considered. The parents used should be of diverse genetic background and preferably heterozygous (Acquaah, 2007; Chen, 2010) [1, 14].

Overcoming Barriers to Hybridization

In some cases, desirable crosses are difficult to obtain due to differences in ploidy levels between prospective parents. Such interploid barriers appear to arise from abnormal endosperm formation. In species where there is an interploid block, seeds will often only develop normally if there is a 2 maternal: 1 paternal ratio in the genomic makeup of the endosperm, which would be the normal case for two diploid parents (Ramsey and Schemske, 1998) [66]. Seeds that don't meet this criterion are often underdeveloped or abort. In some cases this ratio is not exact, but the greater the disparity, the lower the viability of the seeds. In cases where interploid blocks exist, barriers to hybridization may be overcome by manipulating

the ploidy levels to match prior to hybridization (Ramsey and Schemske, 1998) [66].

Aplication of gigas effect in ornamental and forage breeding

One of the immediate and obvious consequences of polyploidy in plants is an increase in cell size which in turn leads to enlarged plant organs, a phenomenon termed gigas effect (Acquaah, 2007; Levin, 1983; Stebbins, 1971) [1, 43]. For example, the volume of tetraploid cells usually is about twice that of their diploid progenitors (Acquaah, 2007; Emsweller and Ruttle, 1941; Levin, 1983; Schepper *et al*., 2001) [29, 1, 43, 69]. The increase in cell volume however is mainly attributed to increased water and not biomass. Therefore, its application is limited for breeding agronomically important crops such as cereals. Although chromosome doubling may result in significantly larger seeds and increased seed-protein content in cereal crops, this advantage is offset by low seed set (Dhawan and Lavania, 1996) [26]. In contrast, the gigas effect has been explored in tree, ornamental, forage crop and fruit breeding (Emsweller and Ruttle, 1941; Schepper *et al*., 2001) []6 [29]. For example, through induced polyploidy, breeders have developed Bouschet tetraploid grapes with more yield and juice content than the diploid progenitor Alicante (Olmo, 1952) [54]. Ornamental crops such as snapdragons and marigolds have been bred through chromosome doubling to improve the quality and size of their blossoms (Emsweller and Ruttle, 1941) [29]. A strong inverse correlation between DNA content and development rates in plants has been reported by several authors (Levin, 1983; Smith and Bennett, 1975) [43]. It has been attributed to lower auxin levels, reduced surface to volume ratio and altered nuclear surface to cell volume ratio (Acquaah, 2007; Levin, 1983) [1, 43]. The slower growth rate of polyploids allows them to flower later and for a longer period of time than their diploid progenitors (Levin, 1983) [43]. This quality may be of interest especially in ornamental breeding.

Mutation breeding

High frequencies of chromosome mutations are desirable in modern breeding techniques, such as tilling, as they provide new sources of variation. The multiallelic nature of loci in polyploids has many advantages that are useful in breeding. The masking of deleterious alleles, that may arise from induced mutation, by their dominant forms cushions polyploids from lethal conditions often associated with inbred diploid crops (Gaul, 1958) [30] This concept has been instrumental in the evolution of polyploids during bottlenecks where there is enforced inbreeding (Comai, 2005) [19]. Mutation breeding exploits the concept of gene redundancy and mutation tolerance in polyploid crop improvement in two ways. First, polyploids are able to tolerate deleterious allele modifications post-mutation, and secondly, they have increased mutation frequency because of their large genomes resulting from duplicated condition of their genes (Gaul, 1958) [30]. The high mutation frequencies observed with polyploids may be exploited when trying to induce mutations in diploid cultivars that do not produce enough genetic variation after a mutagenic treatment. This approach has been used in mutation breeding of *Achimenes sp.* (nut orchids) by first forming autotetraploids through colchicine treatment followed by the application of fast neutrons and X-rays. In this study, the autotetraploids were found to have 20-40 times higher mutation frequency than the corresponding diploid cultivar due to the large genome (Broertjes, 1976) [11].

Production of apomictic crops

Apomixis provides another avenue for use of polyploids in breeding. Apomixis provides an avenue for the production of seeds asexually through parthenogenesis. Most apomictic plants are polyploid but most polyploid plants are not apomictic (Otto and Whitton, 2000) [59]. In plants capable of both sexual and asexual reproduction, polyploidy promotes the latter (Dhawan and Lavania, 1996; Levin, 1983) [43, 26]. Obligate apomicts are the most desired of hybrids but little gain has been realized towards their development. However, it has been suggested that obligate apomicts may be induced through development of very high ploidy plants (Levin, 1983) [43]. An example of an obligate apomict achieved at high ploidy level is the octoploid of the grass, *Themeda triandra* (Levin, 1983) [43].

Enhancing Pest Resistance and Stress Tolerance

The influence of polyploidy on adaptability and resistance to biotic and abiotic stresses has been widely studied in crop plants (Levin, 1983) [43]. In some cases polyploids have demonstrated greater resistance to pests and pathogens, greater nutrient uptake efficiency, better drought resistance, and superior cold tolerance. However, polyploidy often results in reduced resistance to these same stresses as well. It should not be assumed that polyploids are necessarily more stress tolerant. There are a number of strategies for inducing polyploids as a means of enhancing adaptability. Increasing the chromosome number and related gene dose can sometimes enhance the expression and concentration of certain secondary metabolites and defense chemicals. However, this is not always the case and little is generally known about the relationship between gene dose, gene silencing, and expression of secondary metabolites. A more promising approach would be to create allopolyploids between plants with diverse endogenous secondary metabolites. A unique and valuable characteristic of allopolyploids is that the secondary metabolites from the parental species are typically additive. That is to say that allopolyploids often produce all the enzymes and metabolites (including defense chemicals) of both parents. This could be particularly effectively for combining the pest resistant characteristics of two species, and potentially contributing to a much broader, more horizontal form of pest resistance. A similar approach may have utility for enhancing tolerance to certain environmental stresses.

Industrial applications of polyploidy

Chromosome doubling is reported to have an apparent effect on many physiological properties of a plant. The most discernable of these has been the increase in secondary as well as primary metabolism (Levin, 1983) [43]. The resulting increase in secondary metabolites, in some cases by 100%, after chromosome doubling has been widely exploited in the breeding of narcotic plants such as *Cannabis*, *Datura* and *Atropa* (De Jesus-Gonzalez and Weathers, 2003; Dhawan and Lavania, 1996; Levin, 1983) [43, 26]. *In vitro* secondary metabolite production systems that exploit polyploidism have also been developed. The production of the antimalarial sesquiterpene artemisinin has been enhanced six fold by inducing tetraploids of the wild diploid *Artemisia annua* L. (clone YUT16) (De Jesus-Gonzalez and Weathers, 2003) [25]. In addition, commercial synthesis of sex hormones and corticosteroids has been improved significantly by artificial induction of tetraploids from diploid *Dioscorea zingiberensis*, native to China (Heping *et al*., 2008) [32]. Attempts have been

made to improve the production of pyrethrin, a botanical insecticide, by chromosome doubling of *Chrysanthemum cinerariifolium* (Liu and Gao, 2007) [45]. Other plants whose production of terpenes has increased following artificial chromosome doubling include *Carum cari, Ocimum kilmandscharicum* and *Mentha arvensis* (Bose and Choudhury, 1962; Levin, 1983) [9, 43]. The enhanced production of secondary metabolites such as alkaloids and terpenes in polyploids may concurrently offer resistance to pests and pathogens. Experiments with diploid *Glycine tabacina*, a forage legume, and its tetraploid forms to measure resistance to leaf rust, *Phakopsora pachyrhizi*, established that 42% of the tetraploid plants were resistant compared to 14% of the diploid plants (Levin, 1983) [43]. Similar results were observed while comparing resistance to insects and the clover eel nematode between *Trifolium pratense* (red clover) tetraploids and diploids (Mehta and Swaminathan, 1957) [50].

Developing Sterile Cultivars

The introduction and movement of invasive species can be a significant threat to certain ecosystems. Development of sterile forms of important nursery crops is an ideal approach for addressing this problem. In doing so, plants can be grown and used for landscaping while minimizing the possibility that these plants could sexually reproduce and become invasive. There are a number of methods available for developing sterile plants. However, one of the most rapid and cost-effective approaches for inducing sterility in a plant is by creating polyploids. In most cases these plants function normally with the exception of reproduction, specifically meiosis. In some cases doubling the chromosomes of an individual plant (autotetrapoid) will result in sterility due to multiple homologous chromosomes and complications during meiosis (as discussed previously). Despite these complications, autotetraploids of some species can produce fertile seeds. In this case, tetraploids can then be hybridized with diploids to create sterile triploids. Triploids have an additional reproductive barrier in that the 3 sets of chromosomes cannot be divided evenly during meiosis yielding unequal segregation of the chromosomes (aneuploids). Even in the unusual case when a triploid plant can produce a seed (apples are an example), it happens infrequently and seedlings are usually abnormal. Development of triploids of some species can be complicated due to the presence of an interploid block that prevents the normal development of triploid embryos. However, embryo culture is an additional technique that can be employed to overcome this problem and produce sterile triploid plants. An alternative approach for creating triploid plants is regeneration of plants from endosperm found in seeds. Although the embryo in most angiosperm seeds is diploid, the adjoining endosperm (nutritive tissue) originates from the fusion of three haploid nuclei (one from the male gametophyte and two from the female) resulting in triploid tissue. This tissue can be excised from developing seeds and cultured in vitro (tissue culture) to eventually give rise to regenerated embryos and plantlets. This approach has been successful for a range of plants including citrus, kiwifruit, loquat, passionflower, acacia, rice, and pawpaw.

Restoring Fertility in Wide Hybrids

It is not unusual for hybrids between distant taxa (different species or genera) to be sterile. This often occurs due to failure of the chromosomes to pair correctly during meiosis — referred to as chromosomal sterility. By doubling the chromosomes of a wide hybrid, each chromosome has an exact duplicate and chromosomal homology and fertility can be restored. This technique has been used successfully to restore fertility in *Rhododendron* 'Fragrans Affinity' and 5*Chitalpa tashkentensis* (Contreras, 2006; Olsen, 2006). However, in some cases this approach has been unsuccessful in restoring fertility, as was the case with tetraploid hybrids of *Alstroemeria aurea* 5 *A. caryophyllaea* (Lu and Bridgen, 1997).

Gene redundancy

Plants inherit not only the beneficial genes but also potentially harmful ones, from their parents as well- much like genetic disorders in humans. Polyploidy can help mitigate the effects of these conditions, because the organisms inherit multiple copies of each chromosome and hence multiple copies of each gene. If the organism inherited "good" copies of the gene along with the "bad" ones, as the number of copies of that gene increases the effects of bad copy will be masked by others.

Seedless fruits

The seedless trait of triploids has been desirable especially in fruits. Commercial use of triploid fruits can be found in crops such as watermelons and are produced artificially by first developing tetraploids which are then crossed with diploid watermelon. In order to set fruit, the triploid watermelon is crossed with a desirable diploid pollen donor.

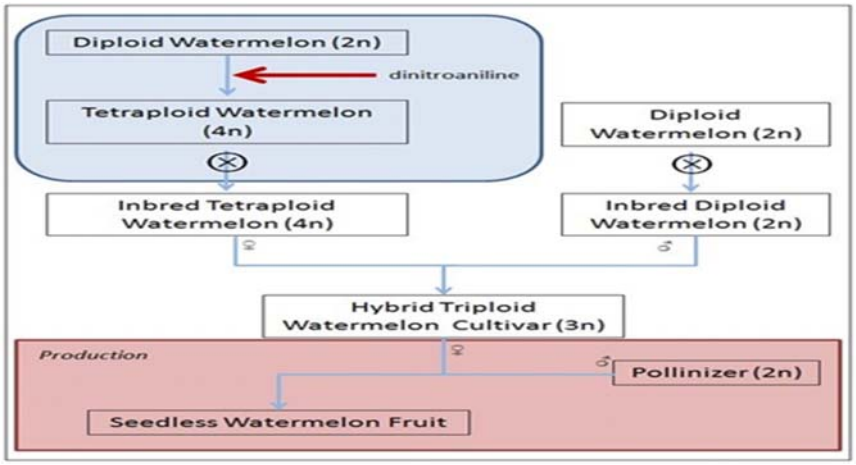

Fig 7: A flow diagram showing the production of seedless triploid watermelon.

Bridge crossing

Another breeding strategy that utilizes the reproductive superiority of polyploids is bridge crossing. When sexual incompatibilities between two species are due to ploidy levels, transitional crosses can be carried out followed by chromosome doubling to produce fertile bridge hybrids. This method has been used to breed for superior tall fescue grass (*F. arundinacea*) from Italian ryegrass (2n=2x=14) and tall fescue (2n=6x=42) by using meadow grass (*Fescue pratensis*) as a bridge species (Fig.5.7) (Acquaah, 2007) [1]. The same principle has been applied in fixing heterozygosity in hybrids by doubling the chromosomes in the superior progeny (Comai, 2005) [19].

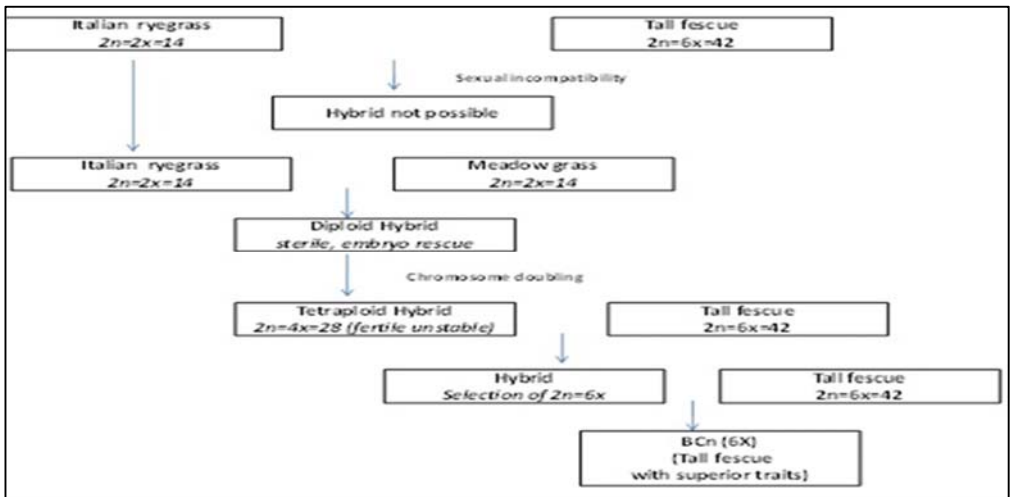

Fig 8: The development of superior tall fescue grass through bridge crossing and induced tetraploidy.

Disadvantages of polyploidy

- Changes in cellular architecture and regulatory implication
- .Difficulties in mitosis.
- Difficulties in meiosis.
- Regulatory changes in gene expression
- Post zygotic incompatibility.

Polyploidy In Mulberry

Sericulture is the science of rearing silkworms for the production of silk fibres. Sericulture is one of the major employment providers in India and several other Asian countries (Vijayan 2010) [79]. Commercially, four major types of silk fibres, namely mulberry silk produced by *Bombyx mori* L., tasar silk by *Anthereae mylitta*, eri silk by *Samia cynthia ricini* and muga silk *Anthereae assamensis*, are used for textile purposes. India has the distinction of harbouring the silkworms of all these four types of silks, though the quantity of the silk produced varies significantly as the mulberry silk occupies a lion share of the total production. It is also interesting to note that *B. mori* can grow well only on mulberry leaves, hence, to enhance sericulture productivity mulberry leaf production has to be increased, which can be made possible by developing new varieties with higher leaf yield and better adaptability. In order to manipulate the genetic constitution of mulberry, it is essential to have adequate information on the genetics and genomics of the plant. In asexually propagated perennial crops, where vegetative organs are of economic use, the polyploidy breeding method has been utilized successfully for their improvement. Being a vegetatively propagated perennial foliage crop with comparatively less number of chromosomes mulberry is suitable for the induction of polyploidy for its improvement. Mulberry (morus spp) is a multipurpose dioecious, heterozygous and out breeding tree. Mulberry is an indispensable crop for the sericulture industry as it is the exclusive sole food source for silkworms. Nearly 70% of the mulberry leaf protein is converted into silk protein through biosynthesis in silkworm. Thus Mulberry leaf protein is the quintessence for synthesis of sericin and fibroin, components of silk protein. Thus, mulberry leaf is the central dogma in sericulture and the increased biomass (leaves) in mulberry variety is the principal determining factor of higher cocoon yield. In India, the cost of mulberry leaf production amounts to nearly 60% of the total expenditure of silkworm cocoon production. Thus there is an immense need for improvement of mulberry varieties in terms of nutritive value and increased biomass (leaves) to ensure profitable production of cocoon.

Many mulberry genotypes are available in nature, but all are not utilized for rearing silkworms since they lack in one or the other required beneficial trait. In case of mulberry, triploids are usually preferred over diploids and tertraploids (Venkatesh 2013a) [85] because of the desirable traits like higher foliage yield, better silkworm palatibility, better quality and better adaptability to environmental stimuli and resistance to cold and stress (Das and Prasad,1970; Tojyo, 1985; Yang and Yang, 1989, Venkatesh 2013b) [86]. It has also been found that silkworms fed with the leaves of polyploid mulberry plants resulted in higher egg production, larval weight, good cocoon harvest and longer cocoon fiber length (Seki and Oshikane, 1959) [70]. Triploids are usually produced by crossing diploids and artificially induced tetraploids. The artificially induced tetraploids are also used for silkworm feeding however, they are actually used as a source of breeding material for the production of triploid varieties. Furthermore, the frequency of stomata per unit area is significantly less in triploid and tetraploid compared to diploids. Stomatal frequency is an important parameter in selecting moisture retenting varieties and drought resistant genotype (Hatalli *et al.* 1993; Nautiyal *et al., 1994)* [51]. Most of the species of *Morus* are diploid having 28 chromosomes, but a few polyploid species, namely, *M.tiliaefolia (84), M. cathyana (56, 84), M. nigra (28,* 308), *M. serrata (28, 42, 56, 84), M. laevigata (28,* 42, 56) and even haploid mulberry are available under natural condition (*M. notabilis = 14) (Maode,*

1996, Darlington and Wylie 1937,Dwivedi *et al*., 1986 &Venkatesh; 2007) [87, 28] *M. laevigata Wall.* is a natural tetraploid occurring in the wild and in the cultivated forms in the eastern Himalayas (Datta 1954; Das 1961); its leaves are unsuitable for silkworm feeding. The occurrence of natural tetraploids of *Morus* has not been reported from any other part of the world other than India.

Generally, mulberry is a diploid plant with 28 chromosomes (2n=28). However, it is rich in ploidy and a lot of triploid varieties have been found especially among *Morus bombysis* Koidz. It is said that*M. cathayana* Hemsl. has tetraploid, pentaploid and hexaploid varieties. Both *M. serrata* Roxb, indigenous to India, and *M. tiliaefolia* Makino, originally from Japan and Korea, are known to be hexaploid. *M. boninensis* Koidz. is a tetraploid being endangered due to cross contamination with *M. acidosa* Griff. *M. nigra* L. is dexoploid (2n=308), the largest number of chromosomes among phanerogams.

Methods of inducing polyploidy in mulberry

a. Treatment of seeds and protocorms

Seeds plants are either pre-soaked in aqueous solutions of colchicine and sowed in nutrient agar or sowed without pre-treatment into colchicine-incorporated nutrient agar. Different aqueous colchicine concentrations for pre-treatment can be used especially in the range of 0.05 to 1.0 percent. All seeds should first be sterilized and then used for sowing. In the case of pro to corm treatments, seeds should first be sown and germinated in nutrient agar. When green protocorms are formed, required quantities of sterilized colchicine should be swirled around in the flask each day to wet the protocorms. When seedlings become approximately 1.5 to 2 cm. high with sufficient roots and leaves, they should be removed from the flasks and transplanted into plots containing disinfected peat moss as the planting medium.

b. Treatment of seedlings

Seedlings about 3 to 4 cm tall are soaked in aqueous colchicine of different concentration as per requirement of the experiment. This can be done by two methods. The first method involves the immersion of seedlings for 3 hours in vials containing colchicine solution. The second method, designated as infiltration method after Braak and Zeilinga, consists of immersing the seedlings in vials containing colchicine solution and placing the vials in an exsiccator in which a vacuum is created by means of a water vacuum pump. In 7 minutes the solution began to bubble. The plants are allowed to remain in vacuum for 10 minutes. This procedure is supposed to evacuate the air from the plants and allow the solution to penetrate the tissues more readily than by soaking without the vacuum. At the end of the treatment period for both methods, plants are removed from the vials, washed under tap water, and planted in peat-moss flats for later chromosome counts.

c. Treatment of inflorescence, cuttings, young shoots, and apical meristems of mature plants:

1. Treatment of inflorescence

Immature inflorescence of the plant should be selected and the apical section of the *inflorescence* should be covered with absorbent cotton saturated with aqueous colchicine solution ranging in concentration as per requirement. Polyethylene bags are to be placed over the cotton to prevent drying. Duration of treatments may extend from 8 hours to 5 days. At the end of the treatments, the cotton applicators should be removed and the inflorescence is left to develop.

Treatment of tip

Tip cuttings of the plant should be selected. The cuttings should be approximately 8 to 10 inches long, and the basal ends are then to be immersed in vials containing aqueous solutions of colchicine. Different Concentrations as per requirement can be used and the duration of treatments may range from 1 to 20 days. At the end of the treatment period, cuttings are to be planted in cutting boxes containing wood shavings as medium. The Root tips for chromosome counts can be taken from the roots which will develop on new growth above the original treated apices. In some instances, apical growth after treatment may be arrested for prolonged periods and axillary shoots will emerge from the first or second node below the treated apex. In these cases, root tips can be taken from the axillary growth. In a few cases, chromosome counts can also be made from bud materials.

Treatment of young shoots

Young shoots about 3 to 4 inches long, should either be excised at the base or with part of the parent stem still attached to the base of the young shoots. The base of these shoots or of the mature stems is immersed in colchicine solutions of various concentrations for different durations. At the end of the treatment period, each cutting is to be planted in a 5-inch clay pot for growth and further observations.

Treatment by incision of apical area-

The apical region of the plant should be incised longitudinally to expose the apical meristem, and colchicine-lanolin paste of different concentrations has to be administered into the incision with a toothpick. Controls can be treated with lanolin paste without colchicine. Sometimes, instead of using colchicine -lanolin paste, glycerin -colchicine solution can also be applied into the incisions with a camel-hair brush. To account for the possible effects of time of exposure to the colchicine and the possible drying of the solution, colchicine-glycerine solution has to be applied 2, 4, and 6 times, each application being given at 2-day interval.

Treatment by injection of colchicine-

The apical regions young shoots (3 to 15 inches long) are treated by injecting aqueous-colchicine or glycerin-colchicine solution with capillary pipettes. These pipettes are made by stretching glass tubing to a thin point. the excess terminal leaves have to be cut off to facilitate the penetration of the pipette into the meristematic region between the folds of the leaf sheaths. the capillary pipettes can either be inserted and left in the plant for gradual release of the solution or 3 - 6 drops of colchicine solution should be injected and the pipette has to be removed immediately. Injections have to be given 1, 3, and 6 times at 3-day intervals (H. Y. Nakasone and H. Kamemoto, 1961**) [31].**

Case study: Colchicine induced morphological variation in mulberry variety "Kajali

The study was conducted by H. L. Ramesh, and V. N. Yogananda Murthy of V.V. Pura College of Science, Bangalore in 2014.Here a Mulberry variety Kajali was used for the induction of polyploidy through colchicine treatment. Five different concentrations of aqueous colchicine viz., 0.1% - 0.5% were used to treat the vegetative buds. Five buds in each plant were earmarked by tagging for the treatment. The

selected buds were thoroughly washed in distilled water before the application of colchicine. The buds were covered with cotton swabs and colchicine solution was applied from 8am – 5pm for three consecutive days at an interval of one hour (Dwivedi *et al*., 1986) [28]. After the complete treatment, the cotton swabs were removed and the buds were thoroughly washed in distilled water. The buds were allowed to grow in the treated plants by providing needed agricultural inputs.

Results

- Colchicine treated mulberry variety Kajali showed decreased survival percentage, poor rooting, delayed sprouting and exhibited slow growth.
- It was observed that, higher concentrations of colchicine (0.4%-0.5%) not only delayed the emergence of buds but also severely affect survivability.
- It may be due to physico-chemical disturbances of cells and reduced rate of cell division.
- With the increase in colchicine concentration there was a corresponding decrease in rooting.
- Poor rooting was also observed in colchicine induced auto-tetraploids of S_{30} and S_{36} mulberry varieties compared to their diploid varieties (Dwivedi *et al., 1989a).* Similar results were also noticed in mulberry varieties M5 and S54 by Ramesh *et al., (2011)* [64]. 0.3% and 0.4% of colchicine applied for 6hrs and 8hrs for three consecutive days were more effective in the induction of tetraploids in mulberry.
- Tetraploid plants' leaves were thick, large and dark green than diploid plants. Increase in leaf thickness is due to increased palisade, spongy tissues and cell size of the polyploid. The stunted growth with deformities following colchicine treatment was probably due to serious hormonal imbalances resulting in physiological disorder (Behera and Patnaik, 1975) [5]. It was also found to be due to reduced rate of cell division (Swanson, 1965) [77].
- Considerable reduction in stomatal frequency and increase in chloroplast was observed. This was in accordance with Beck *et al.,* (2003) [6] and Cohen and Yao (1996) [17] who observed and reported that, size and number of stomata may change significantly due to chromosome doubling compared to the diploids.
- The lesser number of stomata is a good trait for sericulture as it helps to reduce the transpirational loses and helps to retain moisture for a longer time. It also corelates with drought and disease resistance.
- Occurrence of dark green colored leaves has been attributed to increase in chloroplasts number in tetraploids of Coriander and Foeniculum (Singh *et al*., 1987) [71].
- Increase in the concentration of colchicine concurrently reduces the plant height and lowest concentration of the chemical has minimum effect on the plant height. The reduction in height of colchicine treated plants was found to be due to shorter internodes.
- There was size variation in pollen grains of tetraploids; some were bigger while others were almost of the same size as in the diploids.
- Pollen fertility was much reduced in tetraploids as compared to diploids.
- The researchers opined that this is due to the meiotic abnormalities such as irregular separation and unequal distribution of chromosomes to different poles which resulted in the size variation of pollen and their low fertility which was reduced to half in the induced tetraploids as compared to diploids.

Table 1: Effect of colchicine on propagation and growth parameters of Kajali mulberry variety at C generation.

Treatment	Survivability (%)	Sprouting (%)	Rooting (%)	Plant height(cm)	No. of branches (No.)	Internodal distance (cm)	Leaf area (cm^2)
Control	100.00	92.10	89.00	152.43	5.51	4.04	178.37
0.1	78.00	82.70	81.37	144.29	4.29	3.80	165.27
0.2	69.00	84.19	76.29	129.14	4.64	3.64	167.31
0.3	58.00	69.21	78.40	131.88	4.87	3.75	158.43
0.4	36.00	73.88	67.10	119.10	5.72	3.82	196.11
0.5	27.00	62.42	56.21	106.76	5.28	3.72	174.29
SEM	0.19	0.32	0.21	0.27	0.55	0.09	1.31
CD@5%	NS	NS	NS	NS	1.69	1.92	2.04

Production of Triploids

- The induced tetraploid varieties are then to be crossed with the diploid varieties.
- The progeny however may consist of tetraploids, triploids and mixoploids.
- The best triploids are then identified from studies of leaf size, weight and other desirable qualities and are then used as maternal/Paternal or vegetative propagule.

Production of triploid mulberry plants through Endosperm culture:

The conventional method of triploid production by crossing diploids and tetraploids is lengthy and tedious. The regeneration of triploid plants from endosperm culture provides an easy and direct approach to triploid production (Bhojwani and Razdaan 1996; Thomas *et al*., 2000) [43, 78]. Mature Seeds of *M. alba* are non- endospermic but the Seeds younger than 17 DAP contain jelly like endosperm, during this period the seed coat is also very soft and easy to remove. So seeds should be used during this stage for endosperm culture.

Procedure

- Young fruits are washed with water and surface sterilized with 0. 1% mercuric chloride.
- After drying it, the seeds are separated from it, from which endosperms are extracted.
- These are then cultured on a suitable nutrient medium e.g. MS media.

Conclusion

Although there are good number of breeding techniques available in various agricultural crops; including mulberry, yet each of these methods suffers from one drawback or the other. Polyploidy has been intensively used in improving the mulberry crop, and there are good numbers of reports available which have indicated improvement in various commercial characters of the plant. However the technique has not been popularized in mulberry due to many reasons. The suitability of breeding method usually depends upon the nature of crop. So, I'm of the opinion that selection of the breeding method depends upon the kind and nature of crop which is under study.

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