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Polyembryony

Kundan Kishore

ABSTRACT

Polyembryony is a type of apomixis wherein autonomous development of supernumerary embryos takes place in the seed and consequently genetically similar progenies are developed. Supernumerary embryos are produced in different frequencies singlet, duplet, triplet, quadruplet, quintuplet, sextuplet and so on. Among different types of polyembryony, nucellar embryony ($2n$) is the most common. However formation of multiple embryos from cleavage of proembryos has also been observed in some plant species. The degree of polyembryony is influenced by pollen source and environmental factors; however it is controlled by a dominant gene having heterozygous allele (Pp). Morphologically zygotic embryos are usually larger than the other embryos; however morphological identification of nucellar seedlings is practically difficult due to the availability of very few morphological markers. On the other hand, biochemical and molecular markers are reliable tools to distinguish zygotic and nucellar seedlings. Nucellar and zygotic seedlings of citrus and mango have been identified through RAPD markers. In the recent development of molecular biology, genes like *msg-2* and *SERK* have been linked to somatic embryogenesis in many plant species. Polyembryony has great importance in generation of true-to-type quality plants; however it creates hurdle for hybridization programme as it hampers the production of zygotic seedling.

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Introduction

Generally a seed contains a single embryo which germinates into a seedling, however, in some angiospermic family multiple embryos are developed in an individual seed and consequently multiple seedlings are produced which is known as polyembryony, whereas the term 'monoembryony' has been used to refer to a single seed that contains one embryo to describe strictly sexual seed parents. Thus polyembryony is the occurrence of two or more embryos in a developing ovule and the additional embryos result from the differentiation and development of various maternal and zygotic tissues associated with the ovule of the seed (Tisserat et al. 1979).

Polyembryony was first reported by Leeuwenhoek in citrus as early as 1719 and the different cases of polyembryony were studied by Braun in 1859. In 1878 Strasburger demonstrated the formation of plural embryos in many genera of angiosperms. In 1901, Earnst summarized the works done in polyembryony and classified the various means by which adventitious embryos are derived. Following Ernst's work, it soon became apparent that polyembryony is not an abnormal feature, which it was considered earlier, but rather a desirable character. About 255 genera belonging to 153 families are reported to exhibit polyembryony (Carman 1997).

In normal sexual cycle (amphimixis), diploid sporophytic cell (Megaspore Mother Cell) of ovule transforms to haploid gametophytic cells (embryo sac) through meiosis which contain egg cell that forms embryo after fertilization with male gamete (syngamy). But in some plants meiotic division and syngamy are eliminated and still a viable embryo is formed inside the ovule. The formation of embryo with asexual means is called apomixis (Apo=away from+ mixis=act of mixing) and seed is called apomictic seed. Thus apomixis refers to substitution of the usual sexual reproduction by a form of asexual reproduction which does not involve meiosis and syngamy for embryo formation (Bhojwani and Bhatnagar 1999). Polyembryony is a type of apomixis which initiates autonomous development of embryos through asexual mode and the resulting progenies are the genetic replicas of the mother plant. Polyembryony in plants occurs as facultative apomixis wherein simultaneous growth of multiple embryos of somatic origin co-exist in the same seed containing sexual embryo resulting from self or cross pollination. Polyembryony is exhibited by a number of plants, yet little is known about the origin of this reproductive phenomenon.

To understand the origin and formation of multiple ovules in angiospermic seed, the anatomical structure of ovule is imperative.

OVULE

The female reproductive apparatus in a flower is the gynoecium and its functional unit is carpel. A typical carpel is comprised of swollen ovary, style and stigma. The ovule forms inside an ovary which in turn forms seed. A well developed ovule consists of embryo sac, nucellus enclosed almost completely by one or two integuments leaving a small opening at the apical end and micropyle. The micropyle is the main passage for entry of pollen tubes in embryo sac. The base of the ovule is called funiculus which is attached with ovary wall through placenta. On the basis of the positioning of funiculus and micropyle ovule is divided into five types; anatropous, orthotropous, campylotropous, hemianatropous, amphitropous. Anatropous type of ovule is common in horticultural crops, in which micropyle lies close to funiculus and ovule looks inverted (Fig. 1). The central portion of the ovule is occupied by embryo sac which is surrounded by sporophytic nucellar cells. The nucellar region opposite to the micropyle is called chalaza. In horticultural crops, the embryo sac, nucellus and chalaza are surrounded by two integuments thus make the ovule bitegmic (Frost and Soost 1968; Bhojwani and Bhatnagar 2000).

The embryo sac, the female gametophyte, is the most important structure wherein fusion of male gamete takes place and in turn zygotic embryo and endosperm form. Embryo sac is a 7-celled structure containing two polar nuclei in the centre, egg apparatus at the micropylar region containing one egg cell and two synergids and three antipodal cells at the chalazal end (Fig. 2). Cells of egg apparatus and antipodal cells are uninucleate and haploid whereas the central cell (polar nuclei) is binucleate or diploid. In angiosperm, double fertilization is the rule wherein one male gamete fuses with egg cell and forms embryo ($2n$) whereas other gamete fuses with the polar nuclei and forms endosperm ($3n$). In case of polyembryonate species,

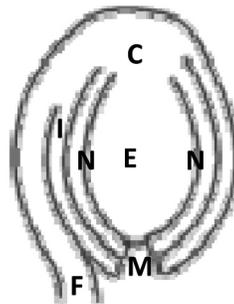


Figure 1. Anatropous ovule of citrus. E—Embryo sac, N—Nucellus, M—Micropyle, I—integument, C—Chalaza, F—Funiculus

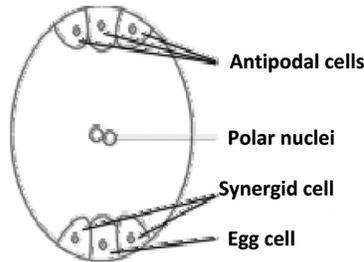


Figure 2. A typical embryo sac.

extra embryos are formed either by nucellar cells (2n), integuments (2n), synergids (n) or antipodal cells (n) (Pullaiah et al. 2001; Bhojwani and Bhatnagar 2000).

Different Ways of Polyembryony

The development of additional embryo may occur by different ways (Fig. 3). In plants polyembryonic cases arise from maternal tissue (adventitious polyembryony) is the most common followed by cleavage of the fertilized embryo (Bhatnagar and Bhojwani 1999; Ganeshiah et al. 1991) whereas formation of additional embryo sac and participation of haploid cells in embryo formation is less common. The different ways of polyembryony are;

- i. Formation of embryo by sporophytic/maternal tissue (2n) of ovule
- ii. Formation of embryos by cells of embryo sac other than egg cell
- iii. Development of more than one embryo sac within the same ovule
- iv. Cleavage of proembryos

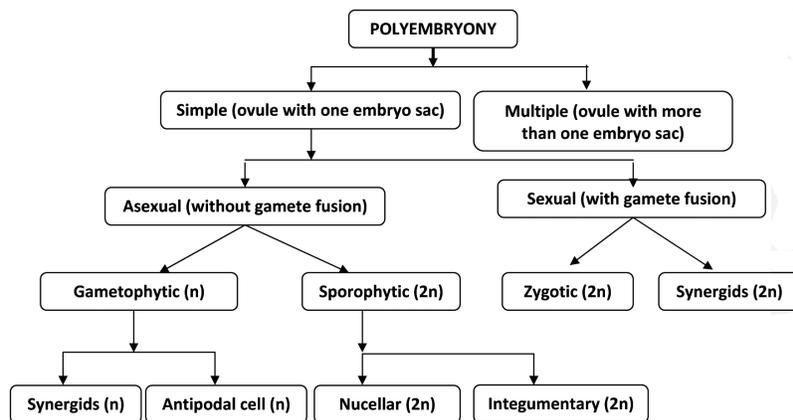


Figure 3. Schematic presentation of different ways of polyembryony.

Activation of Sporophytic Cells of the Ovule

The activation of sporophytic cells (nucellus and integument) of embryo sac is the most common force for polyembryony and the embryos arise from the sporophytic cells are called 'adventive embryos' and the phenomenon is called 'adventive embryony'. The maternal cells which involve in embryo development are primarily nucellus and integument. 'Nucellar embryony' (embryo develops from nucellus) is the most common feature in the families of horticultural importance (Kobayashi et al. 1979).

Cleavage of Proembryo

In this case the embryo that forms after normal fertilization divides irregularly and forms mass of cells that proliferate and develop into many embryos. The cleavage polyembryony is quite common in Orchidaceae, Poaceae and gymnosperm (Maheshwari 1950).

Embryos from other than the Egg Cell of Embryosac

In this category the most common source of additional embryo is the synergids (n). If embryo develops from unfertilized synergid the embryo will be haploid whereas fertilized synergids will give diploid embryo. The formation of diploid embryo from synergid is brought about by the entry of more than one pollen tube into the embryo sac or by the presence of additional male gamete in the same pollen tube. Embryos arising from unfertilized synergids are known in French bean (*Phaseolus vulgaris*). On the other hand, the development of embryo from antipodal cells and endosperm cells is rather rare.

Development of Additional Embryo Sac in the Same Ovule

Multiple embryo sac may arise in an ovule either from the same megaspore mother cell or from other megaspore mother cells or from any other sporophytic tissue of ovule.

Classification of Polyembryony

Polyembryony may be classified on the basis of source of origin, frequency of occurrence and ploidy level.

Camaron and Soost (1979) classified the polyembryony on the basis of frequency of polyembryony.

- i. Strictly monoembryonic—Plant species in which the frequency of multiple embryos in less than 6% is described as strictly monoembryonic.
- ii. Nearly monoembryonic—In case of nearly monoembryonic plant species the frequency of polyembryony varies between 6–10%.
- iii. Polyembryonic—If the per cent of multiple embryo formation is more than 10% the condition is called polyembryony and plants are called polyembryonate.

On the other hand, Ernst (1910) divided polyembryony into two categories on the basis of embryogenesis.

- i. True polyembryony—When two or more embryos arise in the same embryo sac from nucellus (citrus, mango, jamun), integument, synergid, etc.
- ii. False polyembryony—In this case more than one embryo sac is formed in an ovule (*Fragaria* sp.) which is followed by the formation of multiple embryos.

Yakovlev (1967) proposed a genetic basis of classification of polyembryony.

- i. Gametophytic—Multiple embryos arise from the gametic cells of the embryo sac (synergid, antipodal) after or without fertilization. In this case haploid/diploid embryos are formed.
- ii. Saprophytic—When multiple embryos arise from sporophytic cells of the ovule (nucellus, integument) without fertilization and the resulting embryos are diploid and akin to parent.

Nucellar Embryogenesis

Nucellar embryony is the most common phenomenon of polyembryony in plants. It is an adventitious form of apomictic reproduction wherein the somatic cells of the nucellus tissue are initialized to enter into an embryonic pathway of development Koltunow et al. (1995). In polyembryonic seed, many nonzygotic nucellar embryos are initiated directly from the maternal cells surrounding the embryo sac containing a developing zygotic embryo. During embryo sac expansion, embryogenic nucellar cells obtain access to endosperm and develop into embryo along side the zygotic embryo that may or may not develop completely. Nucellar embryo gives rise to seedlings that are of the same genotype as the female parent.

The adventive embryogenesis is completed in four steps: (I) formation of adventive Embryo Initial Cells (AEICs), (II) differentiation of AEICs, (III) division of AEICs and (IV) development of adventive embryos (Wakana and Uemoto 1988). AEICs generally appear before anthesis and are characterized

by their homogenous cytoplasm, a large nucleus and irregular plastids. After pollination the cell composition is changed and starts synthesizing more energy. In fertilized seeds the division of the AEICs generally occurs before the division of zygote but after endosperm division (Kobayashi et al. 1979; Koltunow et al. 1995). The recent studies show that the initiation of adventive embryos in Citrus occurs autonomously and not affected by pollination, fertilization or the development of zygotic embryo or endosperm (Wilms et al. 1983). However the development of adventive embryos is greatly influenced by endosperm development. Lack or poor development of endosperm results in poor development of adventive embryos. Therefore, in unfertilized seeds the nucellar embryos fail to develop beyond certain stage and are incapable of normal germination (Wakana and Uemoto 1987). The presence of endosperm promotes the development of adventive embryos at the micropylar end, but suppresses their development towards chalazal end. The degree of suppression is directly related to the distance of embryo from the micropylar end. In normal seeds the AEICs at the chalazal end generally do not develop beyond the initial celled stage.

In a genotype that produces polyembryonic seeds by nucellar embryony, normal zygotic embryo is also formed and such a genotype can produce different type of seeds (Wakana and Uemoto 1988): i) seeds with one mature zygotic embryo developed by sexual reproduction and in this case seed becomes monoembryonic: ii) seeds with one mature nucellar embryo only and in this case also seed shows monoembryony: iii) seeds with multiple mature nucellar embryos resulting in polyembryonic seed: iv) seeds with one mature zygotic embryo and one or more mature nucellar embryo and in this case seed becomes polyembryonic.

In citrus, nucellar embryony does not prevent normal sexual reproduction and zygotic embryo is also formed (Esan and Soost 1977; Wilms et al. 1983). Thus citrus seed produces both type of seedling having zygotic and nucellar origin. The number of nucellar embryo varies with seed to seed and species to species. So a seed may contain two or more seedlings but not every seed produced by the plant with nucellar embryony has multiple mature embryos.

Regarding the location of embryos in the seed, the positioning of nucellar embryos is not fixed and they may be arranged near micropyle or away from micropyle. The zygotic embryos are generally present at the micropylar end of embryo (Thakur and Bajwa 1971).

Carimi et al. (1998) reported that zygotic embryo developed at faster rate and was at more advanced stage at the micropylar region of the embryo after 105 days of pollination (DAP), while the growth of nucellar embryos was relatively slower and they attained the developmental stage similar to zygotic embryo after 150–200 days of pollination.

Polyembryonate Crops

In fruit tree crops, polyembryony is also common and occurs in many crops; citrus (Frost 1938), mango (Sachar and Chopra 1957), *Syzigium* sp. (Narayanswamy and Roy 1960), kiwi (Crete 1944), almond (Kester and Gordzeil 1996), *Fragaria* sp. (Lebegue 1952) and peach (Toyama 1974). The occurrence of polyembryony depends up on species and varieties, in other words not all species of a genus exhibit polyembryony and not all varieties of a species show polyembryony and the reason could be genetic. Most of the citrus species show polyembryony, while *C. medica* (citron), *C. grandis* (pummel) *C. latifolia* (Tahiti lime) and *C. nobilis* (King mandarin) are monoembryonic. In mango, most of the varieties grown in coastal area show polyembryony. A list of polyembryonic species and varieties are given below.

Crop	Reported by
<i>Citrus</i> sp.	Cameron and Soost 1973; Kultnow et al. 1996
<i>Mangifera</i> sp.	Sabrinbo and Gurgel 1953
Jamun	Van der Pijl (1974)
Kiwi fruit	Crete 1944
<i>Prunus</i> sp.	Toyama 1974
Almond	Kester and Gordzeil 1996
<i>Fragaria</i> sp.	Lebegue 1952
Maize	Erdelska and Vidovencova 1992

Factors Affecting Polyembryony

The number and type of embryo produced may vary from tree to tree and also at different positions on a single tree (Parlevliet and Cameron 1959). The variation has been suggested to be controlled by minor genes, pollen sources and environmental conditions (Khan and Roose 1988). If the zygotic embryo does not survive, the development of nucellar embryo is dependent on the initial development of zygotic embryo or on the process of fertilization of ovule (Tisserat et al. 1979) or on pollination. Thus nucellar embryo apparently may not develop independently of the process of sexual reproduction and in most of the cases it requires the development of zygotic embryo. In citrus, neither the seed setting nor the formation of the nucellar embryos takes place without pollination of flower. The development of nucellar embryos is induced by fertilization (Koltunow 1993). It has been established that pollen plays a definite role in the formation of additional embryos.

Polyembryony is also affected by the type of pollinator (Soares Filho et al. 1995), pollen viability, plant nutrition, temperature, environmental and

soil humidity. The development of endosperm is required for the growth of embryos since endosperm supplies food to developing embryos. The fertilization is prerequisite for the production of matured embryo. The growth of nucellar embryo is arrested at later stage of development if endosperm is not formed (Koltunow et al. 1996). Therefore any factor that affects pollination, fertilization or seed development will also affect the percentage of polyembryony and embryo number per seed.

Causes of Polyembryony

Many theories have been proposed to explain the occurrence of polyembryony but most of them are not sufficiently validated. Haberlandt (1921, 1922) proposed the 'necrohormone theory'. The theory advocated that the degenerating cell of the nucellus acts as a source of stimulus for the adjacent cells to divide and form adventives embryo. But the theory could not be validated as adventives embryo could not be induced by damaging the nucellar cells. The monoembryonate conditions in some species of citrus have been ascribed to the synthesis and release of certain volatile and non-volatile embryogenic inhibitors in their ovules which do not occur in the ovules of polyembryonate species. Ethanol and ethylene are important volatile inhibitors produced by the ovule of monoembryonate species of citrus (*Citrus medica*). The ethylene among other substances could be repressing the development of nucellar embryos through its union with an ethylene receptor protein of nucellar cells. The fewer number of adventives embryos in south side of tree than that of north side, might be due to the more synthesis of ethylene, ethanol and abscisic acid due to variation in sunlight and temperature (Garcia et al. 1999). The non-volatile component of the inhibitors was auxin, ABA and GA_3 . But genetic theory is the most accepted theory as the presence of polyembryony is determined by the gene.

Inheritance of Nucellar Embryony

Polyembryony in citrus and mango is generally controlled by a dominant gene having heterozygous allele (Pp) while homozygous recessive gene (pp) is present in monoembryonic citrus species (Parlevliet and Cameron 1959; Aron et al. 1988). In monoembryonic species, these recessive genes may synthesize a potent inhibitor of embryogenesis (Esen and Soost 1977). A variable degree of polyembryony was recorded in polyembryonate offspring obtained by crossing monoembryonic and polyembryonic and between polyembryonic parents that implied the presence of minor genes affecting degree of polyembryony. Moreover, the presence of modifier or duplicate genes should also be considered as in some crosses between monoembryonic

and polyembryonic parents, and between polyembryonic parents, the progeny ratio varied greatly from 1:1 to 3:1 ratios. It may be concluded that mostly polyembryony is controlled by heterozygous dominant gene and the absence of dominant allele leads to monoembryony. But in some of the cases modifier genes and minor genes are also present that tinker with the ratios and degree of polyembryony respectively.

Degree of Polyembryony

The degree of polyembryony is the frequency of occurrence in multiple seedlings in a seed. It varies with species and varieties and the variation also occurs with location, environment and position of fruits on tree. On the basis of degree of polyembryony species/varieties can be divided in to three categories; slightly polyembryonic (polyembryony up to 25%), moderately polyembryonic (polyembryony up to 50%) and highly polyembryonic (polyembryony > 50%). On the basis of number of embryos (1, 2, 3, . . .), embryony may be divided into different morphotypes: singlet (one embryo/seed), duplet (two embryos/seed), triplet (three embryos/seed), quadruplet (four embryos/seed), quintuplet (five embryos/seed), sextuplet (six embryo/seed), heptuplet (seven embryos/seed), octuplets (eight embryos/seed) and so on. In apomictic *Citrus* sp. quadruplet and triplet embryos are more frequent regardless the genotypes (Kishore et al. 2012).



Figure 4

Color image of this figure appears in the color plate section at the end of the book.

Identification of Nucellar Embryo/Seedlings

Morphological identification of nucellar and zygotic seedlings at juvenile stage is practically difficult, moreover it is not an effective method, as the chance of selecting wrong seedling is more. Although, visual recognition is the simplest method and is effective when the male and female parents differ significantly in their growth habit but when the two parents are similar, as is the case with most plant species, separation is more difficult. The difference in zygotic and nucellar seedlings can be made only after fruiting and the plant possessing the characters of mother parent will be nucellar in origin while the plant that shows variation in characters is zygotic in origin. There is considerable variation among zygotic seedlings and visual recognition on the basis of height, leaf size, thorn length, petiole length and stem diameter is especially difficult in non-hybrid cultivars. In citrus the zygotic seedling can be identified morphologically if one variety is crossed with trifoliolate orange (*Poncirus trifoliolate*) as the resultant zygotic seedlings will have trifoliolate leaves as this character is controlled by dominant gene (Khan and Roose 1988).

Cytologically the nucellar cells destined to form adventives embryos can be distinguished from other cells of the nucellus by their dense cytoplasm and starchy contents (Wilms et al. 1983). Zygotic embryo was usually larger than the other embryos and also took the most space in a seed, while, nuclear embryos were tiny, heart shaped and green and were crowded at the micropyle region and most of these could be easily separable (Das et al. 2005). Generally, the most vigorous and first germinating seedling of citrus is considered to be zygotic as it has largest cotyledons and occupies most of the space of seed.

Similarly, in mango, the earlier concept was that the zygotic plantlets are the weakest in polyembryonic mango seed because it probably degenerates due to competition with nucellar plantlets (Sachar and Chopra 1957). The recent findings proved that zygotic plantlets are vigorous and positioned near micropyle and possessed big cotyledons. The vigorosity of zygotic plants can be explained by a heterotic effect of cross between the female parent and unidentified male parent (Maria et al. 2006).

The biochemical techniques are effective and expeditious tools to identify zygotic and nucellar seedlings at an early stage. Among biochemical techniques, isozyme markers are commonly used to distinguish nucellar (true-to-type) and zygotic (off-type) citrus seedlings (Moore and Castle 1988; Anderson et al. 1991). Gill et al. (2002) reported that the nucellar seedlings could be distinguished from the zygotic seedlings on the basis of banding pattern of different isozymes.

Molecular techniques by using molecular markers (RAPD, RFLP, SSR, ISSR, QTL, etc.) are the recent, advanced, effective, expeditious and

reliable tools to study gene expression and to identify zygotic and nucellar seedlings at an early stage as results are not influenced by external factors. Genes show conspicuous difference in expression between polyembryonic (apomictic) and monoembryonic (nonapomictic) genotypes. The *msg-2* gene was highly expressed in the late stage of somatic embryogenesis in monoembryonic cultivars of *Citrus*, whereas, *msg-2* was not expressed in the initiation stage of embryogenesis in polyembryonic cultivar suggesting the suppressing role in initial cell formation of somatic embryos (Nakano 2013). Similarly, genes like *Ig1* in maize (Evans 2007), *OsCem* (Yang and Hwa 2008) and *OsPE* in rice (Puri et al. 2009) associated to polyembryony have been reported. Somatic Embryogenesis Receptor Kinase (SERK) gene, a leucine-rich repeat trans-membrane protein kinase, enhances the ability of the apical meristem to form somatic embryos. SERK genes have been linked to somatic embryogenesis (SE) in a number of species including *Dactylis glomerata* (Somleva et al. 2000), *Arabidopsis thaliana* (Hecht et al. 2001), *Medicago truncatula* (Nolan et al. 2003), *Helianthus annuus* (Thomas et al. 2004), *Ocotea catharinensis* (Santa-Catarina et al. 2004), *Citrus unshiu* (Shimada et al. 2005), and *Theobroma cacao* (de Oliveira Santos et al. 2005). SERK genes have also been described in relation to apomixis in *Hieracium* (Tucker et al. 2003) and *Poa pratensis* (Albertini et al. 2005) as well as zygotic embryogenesis in carrot, *Arabidopsis*, and wheat (Schmidt et al. 1997; Hecht et al. 2001; Singla et al. 2008). The best defined SERK gene in relation to SE is the Arabidopsis SERK1 (AtSERK1) and over expression of this SERK was shown to enhance embryogenic competence in *Arabidopsis* cultures (Hecht et al. 2001). Das et al. (2007) identified the zygotic seedlings through RAPD markers and reported that zygotic seedlings (twin or triplets) usually had one or more extra band than those of the nucellar seedlings they also observed the variability in mandarins of north east by RAPD profiling. Maria et al. (2006) identified the nucellar seedlings of mango through RAPD and also determined the position of zygotic and nucellar embryos in an ovule.

Significance of Polyembryony

Polyembryony or nucellar embryony plays an important role in horticulture, cytogenetics and plant breeding.

- a. Nucellar embryony helps in producing genetically uniform seedlings of the parental type for better clones of scion and rootstock.
- b. Polyembryony helps in the large scale propagation of desired genotype.
- c. The nucellar seedlings show a restoration of the vigour lost after repeat vegetative propagation.

- d. The nucellar embryos are free from diseases as *in vitro* nucellar embryony is the only practical approach to raise virus free clones of polyembryonate citrus varieties in nature.
- e. Haploids can be used for cytogenetic studies.
- f. Homozygous diploids can be raised from haploids by cochicine treatment.

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