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Review Article

The use of genetic, manual and chemical methods to control pollination in vegetable hybrid seed production: a review

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Abstract

Production of hybrid varieties of vegetable crops is currently a desired breeding goal, due to their remarkable agronomic performance and to the possibility of intellectual property protection. However, efficient hybrid production requires a careful pollination control to guarantee the hybrid nature of F_1 seed. Several technologies ranging from manual emasculation to genetic transformation are used to inhibit pollen production in mother plants. In this review, we examine the principles underlying strategies like genetically determined systems (genic male sterility, cytoplasmic–genic male sterility, self-incompatibility) and other methods (manual emasculation, chemical-hybridizing agents) in different species, considering the benefits and drawbacks of their adoption. Finally, we present the current state of the art for vegetable hybrid seed production.

Key words: Hybrid seed production — pollination control — male sterility — vegetables — chemical-hybridizing agents

Hybrid varieties are major components of vegetable production systems due to their vigour, uniformity, horticultural quality, biotic and abiotic stress resistances and high yields. Another important reason is the inherent biological intellectual property protection offered by hybrids. In some cases, hybrid parents are owned by a company. The inbred parents may be trade secrets, and thus, the hybrid has a built in form of protection for seed companies. Worldwide the share of hybrid seed is increasing at a fast pace of 8–10% annually in most of the vegetables crops (da Silva Dias 2014). However, their adoption by vegetable growers is limited by the high cost of hybrid seed. One of the bottlenecks in hybrid seed production is pollination control required to eliminate pollen from the mother line in order to avoid undesirable selfing, thus obtaining true F_1 seed. Different technologies – ranging from manual emasculation to transformation – provide alternative pathways to avoid pollen production in female plants (Fu et al. 2014, Kempe and Gils 2011). The choice among them is made according to the species of interest, the economic value of the final product and the hybrid seed industry characteristics. This review deals with the principles and applications of current available technologies of pollination control for vegetable hybrid seed production. In the first section, different systems are presented and their advantages and restrictions are discussed. The second part describes the current state of the art in the field of hybrid production of vegetable crops.

Types of Pollination Control

Taking into account the mechanism affecting pollen production, pollination control systems can be classified as follows:

Systems determined by genetic control

Male sterility

Male sterility has been defined as the failure of plants to produce functional anthers, pollen or male gametes (Kaul 1988). Anther and pollen development can be considered as a pathway with several stages: anther cell specification – comprising stamen primordial initiation and archaesporial initiation –, mature pollen formation – in which pollen mother cell meiosis and microspore maturation take place – and anther maturation and pollen release, when dehiscence occurs. Many of the key genes involved in anther and pollen formation have been identified (Wilson et al. 2011). In this process, the tapetum plays a central role as supplier of nutrients, proteins, lipids and polysaccharides which are used in microspore release and pollen-wall formation (Parish and Li 2010). Male sterility can be conditioned by nuclear or cytoplasmic factors affecting any of the stages of microsporogenesis or gametogenesis, resulting in genic male sterility (GMS) and cytoplasmic male sterility (CMS), respectively.

Genic male sterility (GMS)

Spontaneous and induced mutants

Nuclear genes affecting normal pollen development have been reported in over 175 species. Most of the male sterile mutants have arisen spontaneously with a high frequency and some have been induced using physical or chemical mutagens, singly or in combination. In all cases, the pattern of inheritance and expression of GMS is Mendelian (Kaul 1988). The majority of the *ms* genes are recessive, in accordance with a loss-of-function mutation. However, some dominant genes have also been reported (Kaul 1988, Fang et al. 1997, Shu et al. 2012). More than 55 recessive *ms* genes have been identified in tomato, over a dozen in pepper and six in broccoli (Kumar 2014). In common bean, faba bean, cauliflower, cabbage and turnip both recessive and dominant *ms* genes have been reported. In other species, like Brussels sprouts, radish, beetroot, Swiss chard, onion and carrot, recessive *ms* genes are available, but they are not used to produce hybrids. Similarly, recessive *ms* genes in spinach, cucumber, pumpkin, zucchini and summer squash have little practical

value due to the use of gynoecious lines or sex regulating chemicals (Kaul 1988, Delourme and Budar 1999, Kumar 2014).

When recessive *ms* genes are used in hybrid seed production, they are maintained by crossing *msms* plants with a heterozygous fertile maintainer line producing 1 : 1 male sterile and male fertile plants. Selection to discard male fertile genotypes before flowering must be carried out, using morphological or molecular markers tightly linked to the *ms* gene (Fig. 1). In tomato, some markers linked to *ms* genes are as follows: absence of anthocyanin in the seedling stem (Gardner 2000), woolly phenotype (Durand 1981), potato leaf shape (Kaul 1988) and enzyme markers (Tanksley et al. 1984). A lack of suitable markers, as well as laborious maintenance processes and poor free outcrossing in certain highly self-pollinated species limit the use of GMS (Dhall 2010).

Some male sterility genes change their expression under different environmental conditions like temperature and photoperiod inducing thermosensitive genic male sterility (TGMS) and photoperiod-sensitive genic male sterility (PGMS), respectively. This means that *msms* plants will be male sterile at a particular temperature or photoperiod at a sensitive stage, whereas they will be male fertile at another condition (Virmani and Ilyas-Ahmed 2001). When grown under restrictive environments, TGMS and PGMS lines serve as the male sterile female parent; the same lines grown in permissive conditions are fertile and allow the propagation of the sterile line. This reversible system eliminates the requirement of crossing to propagate the male sterile line and allows for the efficient development of the 'two-line' hybrids (Chen and Liu 2014). TGMS and PGMS lines have been reported in several horticultural crops (Dhall 2010). Production of hybrids using GMS has been shown in pepper, chilli, cabbage, cauliflower and tomato (Dhall 2010, Radkova et al. 2009).

Genetically engineered male sterility

In the last 25 years, genetic engineering has become a new source of dominant genic male sterility, especially in those vegetable crops where other efficient sources of male sterility are not available. The objective of this technology is to disrupt any step during microsporogenesis or microgametogenesis by inserting cloned gene sequences through genetic transformation. The first report was based on the tapetum specific expression of the toxic enzyme *barnase*, a chimeric ribonuclease gene from *Bacillus amyloliquefaciens*, which leads to the precocious degeneration of the tapetum cells, the arrest of microspore development and male sterility (Mariani et al. 1990). Male fertility restoration is achieved by expressing the *barstar* gene, encoding an intracellular inhibitor of *barnase* (Mariani et al. 1992, Reynaerts et al. 1993), thus allowing hybrid seed production. This strategy has been successfully developed in cauliflower and chicory (Reynaerts et al. 1993), tomato (Zhang et al. 1998, Bai et al. 2002), Chinese cabbage (Yu et al. 2000), cabbage (Shen et al. 2001), flowering Chinese cabbage (Cao et al. 2008) and eggplant (Cao et al. 2010). Male sterility has been successfully engineered in different vegetable crops using different approaches, like down-regulation or over expression of essential genes (Sinha and Rajam 2013, Nandy et al. 2013), transcription factor gene silencing (Toppino et al. 2011), callose degradation (Curtis et al. 1996) and metabolic engineering (Goetz et al. 2001, Cheng et al. 2015). Factors affecting the wide adoption of genetically engineered male sterility are as follows: availability of efficient gene constructs, possible dispersion of transgene to other related species, availability of efficient transformation techniques, very high initial investment and biosafety and regulatory matters (Singh et al. 2012, Ananthi et al. 2013). At present, only the *barnase-barstar* system has been used at commercial level in vegetable crops to produce chicory hybrids (Singh et al. 2012). However, the licence to produce F_1 hybrids using this system is no longer valid, and the marketing of these transgenic plants is not allowed in the European Union (Klocke et al. 2010).

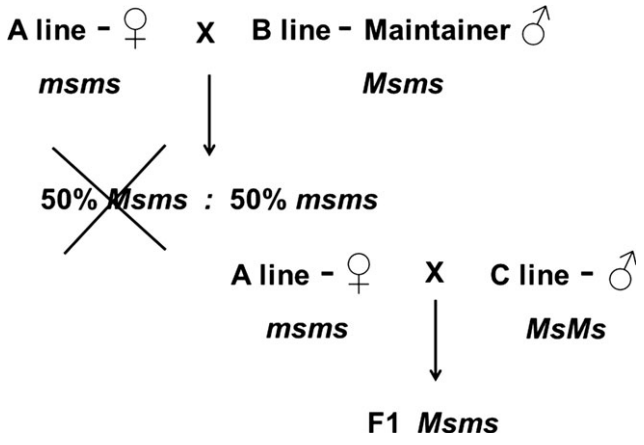

Fig. 1: Hybrid seed production using recessive GMS

Cytoplasmic and cytoplasmic–genic male sterility (CMS-CGMS)

Cytoplasmic male sterility (CMS) is determined by mitochondrial genes resulting from mitochondrial DNA rearrangements which disturb the normal development of pollen. This maternally inherited character has been described in more than 140 species of higher plants. Although the mechanism involved in CMS has not been elucidated, evidence supports roles for energy deficiency, programmed cell death (PCD) and reactive oxygen species (ROS) (Chen and Liu 2014, Horn et al. 2014, Hu et al. 2014, Touzet and Meyer 2014). Cytoplasmic–genic male sterility (CGMS) results from the interaction of mitochondrial genes causing male sterility and nuclear genes, which specifically restore male fertility – *Rf* genes –, thus representing an example of the crosstalk between mitochondrial and nuclear genomes.

Besides spontaneously arising CMS, the character has been obtained through wide crosses as a result of intra/interspecific or intergeneric nuclear/cytoplasmic incompatibilities (Hanson and Bentolila 2004, Kubo et al. 2011, Kaminski et al. 2015). Another source of new CMS lines is protoplast fusion, where recombination between the two parental mitochondrial genomes occurs prior to cytoplasmic segregation in the somatic hybrids (Rambaud et al. 1993, Carlsson et al. 2007). Recently, CMS was achieved by genetic engineering of the chloroplast genome using the b-ketothiolase gene, a component of the polydroxybutyrate pathway (Ruiz and Daniell 2005). Additionally, reproducible transgenic induction of mitochondrial rearrangements leading to CMS was accomplished by disrupting the expression of *Msh1*, a nuclear gene involved in the suppression of mitochondrial DNA rearrangements in tomato and tobacco (Sandhu et al. 2007). So far, no mitochondria transformation technology has been developed in higher plants. Although these biotechnological approaches offer a vast range of possibilities, they have not been adopted in commercial production of hybrids yet.

CMS was first described in onion by Jones and Emsweller (1936), and its use for hybrid seed production was established by Jones and Clarke (1943) who reported that male sterility is originated by the interaction of the male sterile (S)cytoplasm

with the homozygous recessive genotype at the male fertility restoration locus (*Ms*) in the nucleus. The dominant allele *Ms* restores male fertility in plants with S cytoplasm, while plants possessing the normal (N) cytoplasm are male fertile irrespective of the genotype of the restorer gene. For hybrid production, a male sterile inbred line – A line – (S *msms*) and a maintainer line to propagate the A line – B line – (N *msms*) are needed. The A line is pollinated by the pollinator inbred line for hybrid production (Fig. 2). The pollinator line will carry *Ms* alleles if seeds are the product of economic value; on the other hand, nuclear restoration will be not required for vegetable, fruit or forage crops (Havey 2004). Although CGMS is widely applied in hybrid production, it presents some limitations which may restrict its adoption (Dhall 2010).

Some of these constraints are related to adverse effects of the sterile cytoplasm, like (i) pleiotropic undesirable horticultural traits (Pelletier et al. 1983, Kaminski et al. 2012), (ii) poor cross-pollination of male sterile flowers due to changes in flower morphology and chemical composition of nectar (Soto et al. 2013) and (iii) instability of male sterility in different environments, which causes contamination of true hybrid seed with selfed seed (Weider et al. 2009). These topics will be discussed on family basis in the second part of this review. Besides, hybrid seed production using CGMS is technically complex. A, B and pollinator lines must be developed and produced in isolated areas, special distribution of rows in the field is needed, optimal pollination must be ensured, and careful handling of hybrid seed is essential. Finally, male sterile cytoplasms and restorer genes are not always available. Successful application of CGMS to vegetable hybrid production has been achieved in onion, chive, Japanese bunching onion, carrot, pepper, cabbage, cauliflower, broccoli, Swiss chard, beetroot and sweet corn (Dhall 2010, Havey 2004). However, little attention has been paid to diversification of CMS sources. Sustainable breeding should emphasize the search, characterization and introgression of several cytoplasmic types in order to prevent harmful effects associated to uniformity (Kubo et al. 2011, Kumar 2014, Saxena and Hingame 2015). Information about different sources of cytoplasmic male sterility is given on family basis in sections 3.1, 3.2, 3.3, 3.7 and 3.9 of this review.

Self-incompatibility

Self-incompatibility (SI) is a genetically determined prezygotic mate-recognition system preventing self-pollination and is very common in Angiosperms (Kao and McCubbin 1996, Ferrer and Good 2012). SI response is comprised of a self- and non-self-recognition process between pollen and pistil that is followed by selective inhibition of the self-pollen development. In most species, SI is controlled by a single multi-allelic locus, the S-locus, which determines pollen inhibition when the same 'S-allele' specificity is expressed by both pollen and pistil. Current knowledge indicates that S-locus consists of at least two linked genes, each of them coding for the male and female determinants expressed in the pollen grain and pistil, respectively. The variants of the gene complex are called S-haplotypes and the SI response occurs when both determinants are issued by the same S-haplotypes (Takayama and Isogai 2005). SI systems have been classified as gametophytic (GSI) and sporophytic (SSI). In GSI, the most widespread system, the incompatibility type of the pollen is controlled by its own haploid genotype, whereas in SSI, the pollen incompatibility type is controlled by the diploid (sporophyte) genotype of the parental anther in which it was produced (Hiscock and Tabah 2003, Franklin-Tong and Franklin 2003). The identities of female and male determinants have been determined and molecular models for different types of SI have been developed recently (Kaothien-Nakayama et al. 2010, Serrano et al. 2015). A major advantage of using SI for hybrid seed production is that only two self-incompatible lines carrying different S alleles are necessary (Kucera et al. 2006). Besides, in this mechanism, pollen and nectar production are unaltered (Singh et al. 2013). Hybrid seed production requires the maintenance of self-incompatible lines. Several methods have been effectively used to break self-incompatibility like bud pollination (Dixon 2007), manipulation of plant age (Horisaki and Niikura 2008), tissue culture (Razdan 2003), exposure to high temperature (Parkash et al. 2015), carbon dioxide gas treatment (Palloix et al. 1985, Lao et al. 2014) and sodium chloride stigmatic treatment (Monteiro et al. 1988, Kucera et al. 2006). Main difficulties in SI adoption for hybrid seed production are the cost of maintenance of SI lines, depression in SI lines due to continuous inbreeding, effects of environmental factors on the expression of self-incompatibility, pseudo compatibility, lack of synchronization of flowering (Parkash et al. 2015). SI systems have been extensively studied in the *Brassicaceae,* a family carrying SSI, and hybrid seed has been successfully obtained in cabbage, cauliflower and broccoli (Singh 2000, Singh et al. 2013, Parkash et al. 2015). However, self-incompatibility also exhibits some limitations in this family. Some cauliflower genotypes, for instance, present a weak SI at high temperature, resulting into selfing and sibling among the plants of the female parent. This severe limitation can be overcome by the use of parental lines with synchronized flowering and similar morphology or by pollination by stored pollen (Kumar and Singh 2005).

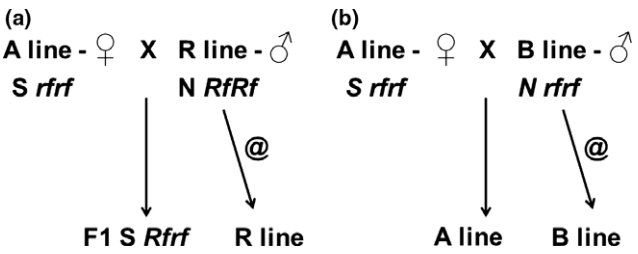

Fig. 2: Hybrid seed production and multiplication of lines using CMS. (a) F_1 seed production and multiplication of R line; (b) multiplication of A and B lines

Systems not determined by genetic control

Manual emasculation

Manual emasculation consists on manual removal of the stamens from hermaphrodite flowers or the elimination of complete male flowers when they are separated from the female ones. This method is labour intensive and requires highly skilful human resources to ensure complete emasculation without affecting female organs (Kumar and Singh 2005, Adhikari 2012, Ozores-Hampton 2014). It is not suitable in species with small hermaphrodite flowers, like carrot and onion, where other methods of pollination control are required. Subsequent pollination after emasculation can be carried out either by hand or by pollinator insects. To be cost-effective, it is appropriate for species that will produce many seeds from each pollination, like solanaceous vegetables and cucurbits as compared to legumes. Nevertheless, availability of a genetic based system of pollination control in tomato or pepper would effectively reduce the cost of hybrid seed production. Hand-pollinated hybrid seed production occurs

mainly in East Asia (China and Taiwan) and South-East Asian highlands (northern Thailand and northern Philippines), India (Karnataka and Andhra Pradesh), Mexico, Chile and, in recent years, Argentina, where local conditions fulfil the requirements of vegetable seed industry (Tay 2006, Gallardo 2012). This system is successfully used in hybrid seed production of tomato, eggplant, pepper, cucurbits and sweet corn (Tay 2006, Kumar and Singh 2005). In Argentina, it is widely used for hybrid squash seed production (Della Gaspera 2013).

Chemical-hybridizing agents (CHAs)

Since the middle of the 20th century, many attempts have been made to find substances effective to selectively disrupt pollen development without affecting the female functionality. These compounds, known as gametocides, pollen suppressors or chemical-hybridizing agents (CHAs) belong to different chemical groups: auxins and auxin inhibitors (NAA, IBA, 2,4-D, TIBA, MH), gibberellins, ethylene (ethephon–ethrel), halogenated aliphatic acids (FW450; Dalapon), arsenicals and brassinosteroids. Not surprisingly, most of the evaluated products are plant growth regulators. Cytokinins, auxin, gibberellins, ethylene, jasmonic acid and brassinosteroids play a role in anther development, and male sterility has been associated with changes in many plant growth regulators, suggesting that normal male development is controlled in concert by multiple hormones (Mc Rae 1985, Huang et al. 2003, Hirano et al. 2008, Ye et al. 2010). The main advantages of this technology are as follows: ease of making and evaluating hybrid combinations, labour efficient seed production and no need for developing male sterile and restorer lines. The major drawbacks are as follows: toxicity effects on the female or F_1 seed, difficulties in field applications due to precise stage of plant development and environmental factors and less effectiveness due to interaction with genotypes and environment (Cross and Ladyman 1991, Adhikari 2012, Fu et al. 2014). In Table 1, products tested in different vegetable crops are presented. Although CHAs efficiency has been verified experimentally, their use at commercial scale in vegetable crops has not been demonstrated (Kumar and Singh 2005). Ethephon is regularly used for squash seed production, mainly for interspecific hybrids between *Cucurbita maxima Duchesne* × *Cucurbita moschata Duchesne*, but the male sterility is never permanent, and several applications of the hormone are required (Della Gaspera 2013).

Table 1: Chemical-hybridizing agents probed for hybrid seed production in vegetable crops

Chemicals	Crops	References
TIBA (Triiodobenzoic Acid)	Tomato	Rehm (1952)
	Squash	Wittwer and Hillyer (1954)
MH (Maleic hydrazide)	Pepper	Chauhan (1980)
	Onion	Chopra et al. (1960)
	Tomato	Rehm (1952)
	Squash	Wittwer and Hillyer (1954)
	Coriander	Kalidasu et al. (2009)
NAA (Naphthalene acetic acid)	Tomato	Mc Rae (1985)
	Squash, cucumber	Wittwer and Hillyer (1954)
Dalapon (Dichloropropionic acid)	Pea, tomato	Brauer (1959)
Dalapon and α-chloropropionate	Pepper	Hirose and Fujime (1973)
FW-450 (sodium 2,3-dichloroisobutyrate)	Tomato	Moore (1959)
Gibberellic acid	Lettuce	Eenick (1977)
	Pepper	Sawhney (1981)
	Tomato	Chandra Sekhar and Sawhney (1990)
	Onion	van der Meer and van Bennekom (1976)
	Brussels sprouts, cabbage, cauliflower and kale	van der Meer and van Dam (1979)
CCC ((2-chloroethyl) trimethylammonium chloride)	Tomato	Rastogi and Swahney (1988)
Etephon–ethrel (2-Chloroethyl phosphonic acid)	Lettuce	Han and Lee (1972)
	Eggplant	Helal and Zaki (1981)
	Squash	Della Gaspera (2013)
ABA (Abscisic acid)	Tomato	Rastogi and Swahney (1988)

Pollination Control Methods in Different Vegetable Crops

Brassicaceae

The main vegetable crops belonging to this family are cabbage, cauliflower and broccoli (*Brassica oleracea* var. *capitata* L., var. *botrytis* L. and var. *italica* Plenck, respectively), turnip (*Brassica rapa* L. *spp rapa*) and radish (*Raphanus sativus* L.). F_1 hybrid production is a goal for breeders due to the high heterosis observed in these species as well as greater uniformity. As mentioned earlier, several pollination control systems are available in this family: GMS, CGMS and SI. Hybrids have historically been produced using sporophytic SI, a natural method lacking adverse side effects. This system, however, has been replaced by an efficient CGMS method due to the occurrence of some self-pollination (Singh et al. 2013, Kucera et al. 2006, Havey 2004). Several male sterile cytoplasms exist in this family, arising from intraspecific variation, interspecific or intergeneric hybridizations and cell fusion (Yamagishi and Bhat 2014). Among them, Ogura cytoplasm has been exhaustively studied and is used worldwide in F_1 breeding of *Brassicaceae*. Discovered by Ogura (1968) in a Japanese radish (*R. sativus* L.), Ogura cytoplasm was introgressed in *B. oleracea* by repeated backcrosses to cabbage and broccoli (Bannerot et al. 1974, Mc Collum 1981) and later from broccoli to cauliflower (Dickson 1975, Hoser-Krauze 1987). However, these first alloplasmic male sterile lines carrying Ogura cytoplasm presented chlorophyll deficiency at low temperatures, underdeveloped nectaries and malformed ovaries and pods which reduced the seed set. These defects were assumed to be caused by negative interactions between the *Brassica* nucleus and the *Raphanus* chloroplasts and were overcome by somatic hybridization. Protoplast fusion between a normal *B. oleracea* line and a CMS (Ogura-radish) *B. oleracea* line allowed the selection of cybrids carrying only *Brassica* chloroplasts that grew normally (Pelletier et al. 1983). These improved CMS lines are known as Ogu-INRA and are widely used to produce hybrids in *Brassicaceae* (Pelletier and Budar 2015, Kaminski et al. 2015). Extensive research on Ogura cytoplasm has revealed the molecular basis of male sterility and fertility restoration (Bonhomme et al. 1992, Tanaka et al. 2012, Brown et al. 2003, Desloire et al. 2003, Koizuka et al. 2003, Uyttewaal et al. 2008). As a result, genomic data are available for development of molecular markers to assist the selection of CMS and restorer lines (Kim et al. 2007, Yu et al. 2016).

In China, GMS-based pollination control is an alternative to CGMS used to produce hybrids in the *Brassica* family (Fang

et al. 1997). Hybrid cabbage seed is produced at commercial scale using a dominant male sterile gene, *Ms-cd1* (Fang et al. 1997), for which SCAR and SSR are available to assist breeding programmes (Zhang et al. 2011).

Apiaceae

Carrot (*Daucus carota* L.): Commercial production of hybrid carrot was feasible only after CGMS systems were available. The main types of CMS are 'brown anther' (Sa), characterized by shrivelled, yellow-to-brown anthers with no pollen (Welch and Grimball 1947) and 'petaloid' (Sp), in which anthers are replaced by a whorl of petals (Thompson 1961, Eisa and Wallace 1969, Peterson and Simon 1986, Morelock et al. 1996). Both systems show instability due to high temperatures, dry conditions, growing time or long-day conditions. As carrot is partially andromonoecious, the development of CMS lines requires stringent selection. Hybrid seed production is largely based on the use of petaloid CMS type because of less frequent reversion to male fertility; however, seed yields on the brown-anther CMS are generally higher (Havey 2004, Dhall 2010). Eleven molecular markers developed by Bach et al. (2002) distinguished all Sp from N cytoplasms and are being used to identify the type of cytoplasm and test seed purity in breeding programmes, to select cybrids after protoplast fusion and to study basic diversity in the genus *Daucus*. Besides these main CMS types, other CMS sources with potential use in hybrid production have been described (Linke et al. 1999, Nothnagel et al. 2000). Regarding fertility restoration, multiple nuclear genes with complex interactions have been involved in different studies (Thompson 1961, Hansche and Gabelman 1963, Börner et al. 1995, Wolyn and Chahal 1998). Recently, Alessandro et al. (2013) found that restoration of petaloid cytoplasmic male sterility was due to a single dominant gene, *Rf1*, and developed a linkage map using molecular markers, some of which can be used to develop PCR-based markers for marker-assisted selection (MAS) in hybrid breeding programmes.

Celery (*Apium graveolens* L.): A recessive single locus involved in genic male sterility was found in an accession from Iran, and efforts to use it for hybrid seed production have been carried out (Quiros et al. 1986, Quiros 1993, Tay 2006). Looking for an alternative system, cytoplasmic male sterility has been experimentally induced by protoplast fusion between celery and CMS carrot (Tan et al. 2009). Recently, the use of CMS to obtain celery hybrids has been patented in China (Zhu et al. 2011, Gao et al. 2015).

Amaryllidaceae

Onion (*Allium cepa* L.) hybrid seed has been extensively produced all over the world using CGMS-based systems. There are two main sources of CMS, identified as S and T, which have been genetically characterized. S type (Jones and Emsweller 1936) results from the interaction of a cytoplasmic factor S and a single nuclear restorer gene *Ms* (Jones and Clarke 1943). T type is controlled by the interaction of the cytoplasmic factor T and three independent restorer genes (Berninger 1965, Schweisguth 1973). Both cytoplasm types have been independently isolated from different sources (Havey 2000). S type is the most widely used due to the relatively common occurrence of the recessive allele at *Ms*, the stability of male sterility over environments and no reduction of female fertility. Besides, it was the first source of CMS available in different germplasms (Goldman et al. 2000, Leite et al. 1999). On the other hand, T cytoplasm is commercially used in Europe and Japan (Havey 2000) and is present in Brazilian onion populations (Fernandes Santos et al. 2010). To diversify the male sterile cytoplasms used in hybrid onion production, Havey (1999) introduced the cytoplasm of *Allium galanthum* Kar. et Kir in onion populations. In *galanthum* CMS, complete absence of anthers is observed and nuclear restorer genes appear to be rare or non-existent (Havey 1999).

Determination of cytoplasm types by test crossing demands 4–8 years in onion due to its biennial cycle. Several molecular markers have been developed to identify the different cytoplasm types, thus reducing time and labour (Havey 1995, Sato 1998, Engelke et al. 2003, Kim et al. 2009, Cho et al. 2006, Kohn et al. 2013). Likewise, many attempts have been made to develop molecular markers tightly linked to the *Ms* locus (Gökçe et al. 2002, Bang et al. 2011, Huo et al. 2012, Yang et al. 2013, Havey 2013, Huo et al. 2015, Kim et al. 2015), but their validation is needed before applying them in MAS of maintainer lines (Saini et al. 2015, Khar and Saini 2016).

Hybrid chive (*Allium schoenoprasum* L.) and Japanese bunching onion (*Allium fistulosum* L.) are also produced using CGMS systems (Havey 2004). In leek (*Allium ampeloprasum* L.), hybrids were initially produced using genic male sterility and '*in vitro*' propagation of male sterile lines (Smith and Crowther 1995).

Asteraceae

In chicory (*Cichorium intybus* L.), hybrid seed has been obtained on the basis of a male sterile nuclear mutation known as 'Edith' (Desprez 1993, Desprez et al. 1994, Gonthier et al. 2013). Recently, Barcaccia et al.(2011) and Barcaccia and Tiozzo(2014) reported four new male sterile mutants of red chicory and developed molecular markers to assist hybrid breeding. An attempt to develop an alternative source of male sterility was made by somatic hybridization through protoplast fusion between chicory and the CMS line Pet 1 of sunflower (Rambaud et al. 1993, 1997, Dubreucq et al. 1999, Varotto et al. 2001, Delesalle et al. 2004, Habarugira et al. 2015), but no reliable production of hybrid chicory was achieved. Another approach, which reached commercial application, was based on genetic transformation using the *Barnase* system (Reynaerts et al. 1993, Kempken 2010).

Amaranthaceae subfamily Chenopodioideae

The former *Chenopodiaceae* family includes important vegetable crops like beetroot (*Beta vulgaris varrubra* L.*)*, Swiss chard (*Beta vulgaris var cicla* L.) and spinach (*Spinacia oleracea* L.). In the case of beetroot and Swiss chard, hybrid production relies on CGMS systems. The most commonly used cytoplasm is Owen type originally described in sugar beet as the result of the interaction between a sterilizing cytoplasm and at least two nuclear restorer genes and environmental factors (Owen 1942, 1945). Restorer gene *Rf1* has been cloned and sequenced (Hagihara et al. 2005, Matsuhira et al. 2012), and molecular markers are available for MAS (Moritani et al. 2013). Recently, Honma et al. (2014) have reported the molecular mapping of *Rf2*. Owen cytoplasm was introduced in beetroot between the 1950s and the 1960s, and hybrids have been produced since then. The associated use of the annual gene B (conditioning annual flowering habit) has helped to efficiently develop sterile inbred lines and the introduction of the S^F allele permitted self-pollination thus

allowing the development of inbred maintainer lines (Bliss and Gabelman 1965, Goldman and Navazio 2008). Three other CMS types – E, G and H – have been characterized in *Beta vulgaris* (Cuguen et al. 1994, Satoh et al. 2004, Darracq et al. 2011), but there is no information about their use in hybrid production.

Spinach (*S. oleracea* L.) is a dioecious species with an even ratio of female-to-male individuals. However, occasional monoecious plants are observed in some populations, among which the proportion of female-to-male (or hermaphrodite) flowers per plant varies widely (Janick and Stevenson 1955, Onodera et al. 2008, 2011, Yamamoto et al. 2014). Hybrids were initially produced alternating female and male rows of two promising dioecious lines and removing the staminate flowers from the female rows as soon as differentiation was possible (Webb and Thomas 1976). At present, it is preferred to use a highly female monoecious inbred as female parent and a highly male monoecious inbred as male parent (Thompson 1955, Janick 1998, van der Vossen 2004) .

Solanaceae

Pepper (*Capsicum annuum* L.) hybrid cultivars are commercially obtained using manual emasculation with hand pollination or methods based on genetic control of pollen, like GMS and CGMS, with hand pollination or natural pollination (Kumar and Singh 2005, Tay 2006).

Nearly 20 genes for GMS have been found or induced. All of them are highly stable, and a few of them are linked with markers which allow early identification of the sterile plants (Shifriss 1997, Wang and Bosland 2006, Dhaliwal and Jindal 2014). Commercial production of hybrids in India and Hungary are based on the use of the line 'MS-12' (*ms10*/*ms10*) and *ms3* gene, respectively (Kumar 2014). Molecular markers have been developed and will help the introgression of *ms* genes in different backgrounds and selection of sterile plants in hybrid seed production (Bartoszewski et al. 2012, Aulakh et al. 2016).

The only source of CMS in this species is S cytoplasm from the 'PI 164835' line introduced from India (Peterson 1958, Liu et al. 2013). Kim and Kim (2005) developed two CMS-specific SCAR markers to distinguish N cytoplasm from S cytoplasm by PCR. Restoration of fertility for S cytoplasm has been attributed to a single dominant nuclear gene (Peterson 1958, Gulyas et al. (2006) or to one major and four minor quantitative trait loci which were mapped in the pepper genome by Wang et al. (2004). Molecular markers tightly linked to the major restorer gene *Rf* were obtained (Zhang et al. 2000, Kim et al. 2006, Min et al. 2009). CGMS is commercially used to produce hot (chilli) pepper hybrid seed, but it has not been successful for sweet pepper due to the lack of restorer genes or instability of restorer lines in most of sweet pepper genotypes. Recently, Lin et al. (2015) introgressed the *Rf* allele from hot pepper into several sweet pepper lines opening the way to an efficient CMS application for sweet pepper hybrid seed production.

In tomato (*Solanum lycopersicum* L.), commercial hybrid seed production using manual emasculation and hand pollination is economically viable and predominates in the seed industry at present. However, the availability of alternative suitable methods to avoid selfing and optimize crossing will considerably reduce the cost of F_1 seed (Kumar and Singh 2005, Sharma et al. 2015). More than 55 male sterile genes are known, and sterility they confer can be grouped in four groups: pollen sterile (pollen abortive), stamenless (stamens absent), positional sterility (stigma exerted) and functional sterility (anthers do not dehisce). Both pollen abortive type (conferred by the *ms*-series) and functional sterility (conditioned by *ps-2* gene) are often used in hybrid production, generating reductions in the costs of hybrid seed production as compared to manual procedures (Yordanov 1983, Georgiev 1991, Dhall 2010, Atanassova and Georgiev 2002). In particular, the $ms10^{35}$ gene (Rick 1948, Jeong et al. 2014) linked to the marker gene anthocyanin-less *aa* has been widely used due to its stability and lack of growth defects.

Attempts to establish a system based on CMS in tomato have been made by exploring interspecific crosses (Andersen 1963, 1964, Valkova-Achkova 1980) and protoplast fusion (Melchers et al. 1992, Petrova et al. 1999); however, no CMS system is currently available for this crop (Stoeva-Popova et al. 2007).

Eggplant (*Solanum melongena* L.) is another example of the use of manual emasculation and hand pollination to commercially produce hybrid seed (Kumar and Singh 2005). A recessive gene conferring functional male sterility – *fms* – (Phatak et al. 1991) was tested, but the occasional presence of pollen in the indehiscent anthers due to environmental factors inhibited its application to hybrid seed production (Daunay 2008). Well-characterized genetic resources related to CMS systems and molecular tools designed to assist selection will provide new possibilities for eggplant hybrid breeding in the future (Mizanur et al. 2016, Krommydas et al. 2016).

In potato (*Solanum tuberosum* L.), self-incompatibility and genic and cytoplasmic male sterility have been described (Jansky 2009). However, hybrid cultivars have received a strong boost with the recent adoption of *Sli* gene originating from *Solanum chacoense* Bitter which makes diploid potato self-compatible (Hosaka and Hanneman 1998a,b, Phumichai et al. 2005). Using this strategy, Lindhout et al. (2011) obtained inbred lines that combined self-compatibility with good agronomic performance as well as hybrids which were uniform and showed good tuber quality; nevertheless, agamic propagation is mainly used worldwide.

Fabaceae

In common bean (*Phaseolus vulgaris* L.), hand emasculation and pollination have been experimentally used. However, the low outcrossing rate in field conditions yields insufficient number of seeds for large agronomic evaluations, thus limiting the advance of hybrid breeding (Palmer et al. 2011). A male sterile cytoplasm found in several accessions has been characterized at the molecular level (Bannerot 1989, Mackenzie 1991), and its single dominant restorer gene has been identified (Mackenzie and Basset 1987).

Faba bean (*Vicia faba* L.*)* presents considerable levels of allogamy and high heterosis for productive traits (Le Guen et al. 1991). Genetic resources related to male sterility include recessive and dominant male sterility genes and several sterile cytoplasms (CMS 447, CMS 350, CMS 199 and CMS 297), together with their restorer of fertility genes (reviewed in Palmer et al. 2011). However, spontaneous reversion to fertility observed in these male sterile cytoplasms has precluded their use in commercial hybrid seed production.

Cucurbitaceae

Most of the species in this family are monoecious, with big flowers which allow commercial hybrid production by pinching staminate flowers followed by hand or natural pollination usually helped by pollinators like honeybees and bumblebees (Kumar

Table 2: Available (●) and commercially applied (✖) systems for pollination controlling F_1 hybrid seed production of vegetable crops

Pollination control	Systems determined by genetic control				Systems not determined by genetic control	
	Genic male sterility		Cytoplasmic Male Sterility	Self-incompatibility		
Species	S/I	GE			Manual	Chemical
Cabbage	✖	●	✖	✖		●
Cauliflower	●	●	✖	✖		●
Broccoli	●		✖	✖		
Turnip	●		✖	✖		
Radish	●		✖	✖		
Carrot	●		✖			
Celery	●		✖			
Onion	●		✖			●
Chive			✖			
Bunching onion			✖			
Leek	✖		●			
Chicory	✖	✖	●			
Beetroot	●		✖			
Swiss chard	●		✖			
Spinach	●				✖	
Pepper	✖		✖		✖	●
Tomato	●	●			✖	●
Eggplant	●	●	●		✖	●
Common bean	●		●		●	
Faba bean	●		●			
Potato	●		●	●	●	
Cucumber	●	●			✖	●
Squash	●				✖	✖
Pumpkin	●				✖	●
Zucchini	●				✖	●
Sweet corn	●		✖		✖	

and Singh 2005, Gajc-Wolska et al. 2011, Petersen et al. 2013). In cucumber (*Cucumis sativus* L.), gynoecious lines are available and their widespread use has hampered the applicability of GMS based on recessive *ms* genes (Kumar 2014, Call and Wehner 2010). Plant growth regulators are frequently employed in species of this family to increase the number of female flowers. Application of ethylene or ethylene-releasing compounds increases female flower production in zucchini (*Cucurbita pepo* L.), pumpkin (*Cucurbita maxima Duchesne*) and monoecious cucumber (Robinson et al. 1969, Rudich et al. 1969, Hume and Lovell 1981, Manzano et al. 2011). Similarly, auxins and brassinosteroids promote femaleness in cucumber probably stimulating ethylene production (Shannon and De La Guardia 1969, Papadopoulou and Grumet 2005). Hand pollination and applications of ethephon are regularly used for squash seed production, mainly for interspecific hybrids between *Cucurbita maxima Duchesne* × *Cucurbita moschata Duchesne* (Della Gaspera 2013).

Poaceae

Sweet corn (*Zea mays* L.) hybrid breeding has assimilated methods and genetic resources developed in corn hybrid seed production. Hybrid seed is produced by hand emasculation (detasseling) and wind pollination or using CGMS systems. Three male sterile cytoplasms are available in maize, T, C and S, each of them being restored by specific *Rf* genes (Chen and Liu 2014). Male sterile and normal cytoplasms can be easily discriminated by multiplex PCR (Liu et al. 2002). T cytoplasm has been banned from hybrid seed production because of its susceptibility to *Bipolaris maydis (*Nisikado and Miyake) Shoemaker; race T; C and S cytoplasms need to be checked for their stability over genotypes and environments (Weider et al. 2009). However, the relatively short life of sweet corn hybrids makes it very difficult to develop new inbred CMS lines soon enough, thus favouring hand emasculation (Havey 2004). Recent incorporation of transgenic traits like herbicide resistance and insect control in commercial sweet corn cultivars has reduced the use of CMS because of the time necessary to transfer CMS into transgenic lines (Havey 2004, Williams et al. 2015). Nevertheless, CMS has recently been proposed as a tool to prevent transgene dispersal through pollen, thus enabling the coexistence of GM and non-GM crops (Bückmann et al. 2013).

Table 2 presents a summary of the methods for pollination control cited in this review.

Perspectives

The global market of vegetable seeds is expected to expand in future years, due to the increase in world population and consumption. There is a clear association between human health and vegetable consumption that is increasing vegetable demand worldwide. Moreover, markets are getting more refined in terms of quality and yield and there is a clear demand for excellent hybrid vegetable cultivars (da Silva Dias 2014). The exploitation of heterosis is one of the leading causes for that trend, but it is even more important the protection of breeder rights. Although OP cultivars can perform in many cases as well as F_1 hybrids, the latter are preferred by the industry. Any contribution to increase the efficiency of hybrid seed industry will help to reduce seed price and alleviate grower's costs. In this context, the availability of a safe and cost-effective method to control pollination is of major relevance during hybrid seed production.

In the last decades, a trend favouring the incorporation of genetic control of male fertility can be observed. Advanced knowledge in genetics and genomics, microsporogenesis and gametogenesis, plant cell biology and plant biotechnology have allowed the characterization of genetic resources and the development of new technologies for different vegetable crops thus broadening the range of choices. Molecular breeding has also facilitated the introgression of desired characters by the use of MAS and MABC (marker-assisted backcross). On these bases, it is reasonable to predict continuous progress in the adoption of these technologies by breeders and seed producers in the future.

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