UTILIZATION OF GENETICALLY DETERMINED SYSTEM AND MANUAL METHOD FOR HETEROSIS BREEDING IN *CAPSICUM ANNUM* L.

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Abstract

The world population is increasing and per capita land resources are decreasing due to the pressure of population growth. Therefore, there is a need to raise the per unit area return. Heterosis breeding is an approach to tackle the challenge of sustaining enhanced yield gains of crops. Hybrid seed production is currently a desired breeding goal, due to its outstanding agronomic performance. Pepper is an important vegetable crop grown and is an excellent source of antioxidant compounds important for human health that aggrandize its economic status. Numerous technologies, ranging from manual emasculation to genetic transformation, are used to produce hybrid seed. In this review, we observe the principles underlying strategies like genetically determined systems (cytoplasmic–genetic male sterility and genetic male sterility) and other methods (manual emasculation) in pepper. Manual method is a labor-intensive task and the production cost of hybrid seed is very high. The characteristics of male sterility is considered as a very important factor for producing hybrid seeds particularly in terms of cost effectiveness.

Key words:

Introduction

Pepper is an important vegetable crop grown for its non-pulpy berry (commonly called fruit or pod), it is an excellent source of antioxidant compounds important for human health that aggrandize its economic status. (Howard *et al*., 2000). It belongs to genus Capsicum from the night shade family. Five species of Capsicum namely *C. annuum L*., *C. frutescens* Mill., *C. baccatum* L., *C. chinense* and *C. pubescens* are domesticated (Csilléry, 2006). *C. annuum* is the most widely cultivated species gaining popularity among farmers throughout the world.

The world population is increasing, and per capita land resources are decreasing due to the pressure of rapidly increasing population. So, there is a need to raise the per unit area return. Good quality seed along with recommended production plan must be assured for maximum production of vegetable crops (Tomar *et al*., 2017). Quality seeds must-have traits like resistance to insects or pathogens, tolerance to abiotic stress and higher nutritional value which ultimately gives greater yield. Vegetable breeding/hybridization is an excellent tool to combine these traits in a single variety (Sweet pepper hybrid 9930417). Hybrids seeds at this moment are comparatively costly, but even then the farmers like and prefer to grow hybrid seeds due to their advantages. Therefore, it seems to be a targeted brand new market for agricultural entrepreneur to produce hybrid seeds at lower costs for interested farmers. Dias (2014) reported that annually 8-10% addendum in the use of hybrid seed in vegetable crops occur worldwide. Hybrid seed production is not simple. There are two main problems that are related to production of hybrid seeds. At first instance it's a labor intensive job, and secondly attentions and skills are required. First problem is resolved in developing countries where labor is very

easily available at lower rates. But second problem is usually need to be addressed with proper training of persons involved.

Heterosis

It is a technique used to produce hybrid seeds. The purpose of production of hybrid seeds is introduction of required variation and qualities in the seeds. Desired qualities include higher yield, rapid growth, resistance to maximum number of pathogens and insects etc. this is also called hybrid vigour (Goulet *et al.*, 2016). Hybrid seed production is facilitated if cross pollination behavior is present in the crops but it is most exploited in those species that has mechanisms like male sterility, self-incompatibility and heterogamy. There are various techniques that are developed and used to make sure the production of hybrids of desired characters. It includes roguing of staminate plants particularly in dioecious lines, hand emasculation, and self-incompatibility, use of predominant female lines, cytoplasmic male sterility, protogyny and genetic male sterility (Janick, 1998). For producing hybrid seeds in capsicum, commonly used techniques are genetic male sterility, manual method (emasculation) and CMS/CGMS (Lee *et al*., 2012).

Emasculation

Whenever a researcher is concerned with production of a good quality hybrid seed, genetic purity of parental lines is a very essential component. Therefore, genetically pure seeds are made by breeders by adopting various techniques. In a bisexual flower androecium is removed manually and this process is called emasculation. Emasculation is widely used in hybrid seed production. But related to this technique, there are problems with capsicum crop including poor fruit set and flower drop. These problems increase the production of seed cost due to intensive labor requirement (Tembhurne and Rao, 2012). It is evident from a study that the pollination time should be selected between 9.00 to 12.00 morning for capsicum. And particularly this selection of time should be made after 24 hours of emasculation. This could cause higher fruit set. It has also been reported that this precaution can cause 85% field emergence rate and germination in capsicum (Kivadasannavar *et al.*, 2009).

Male sterility

Plant failure to develop functional pollen, normal anther or male gametes, this condition is known as male sterility (Kaul, 1988). The success of a hybrid cultivar depends on its production cost. Hand emasculation and pollination is a labor-intensive task and the main reason behind the high production cost of hybrid seed production. Flower drop and poor fruit set after emasculation occur due to the delicate nature of the flower. In 1950's male-sterility was first reported in pepper (*Capsicum annuum* L.) commercially cytoplasmic male sterility and genetic male sterility are types of male sterility. The use of Male sterile plants as a female parent is the best alternative to reduce the production cost (Tembhurne and Rao, 2012). Honey bees are used as natural pollinators. Chilli is one of the self-pollinated crops but natural cross-pollination takes place up to the extent of 7 to 60 % (Aiyadurai, 1996) therefore arrangement of male-fertile and male-sterile lines are planned in such a way that the sufficient movement of bees occurs between them (Abrol, 2011).

Cytoplasmic genic male sterility

Cytoplasmic male sterility also refers to cytoplasmic genetic male sterility (Swamy *et al.*, 2017), which has been used over a wide area for F1 hybrid production of many crops including Peppers (Havey, 2007; Kumar *et al.*, 2009). In sweet pepper the use of stable CMS lines and restorer line would decrease approximately 40% hybrid seed production cost (Lin *et*

al., 2015). Male sterility in Capsicum was first documented by Martin and Grawford in 1951. Later on, Peterson (1958) reported CMS in *Capsicum annuum* L. in USDA accession PI164835. Male sterile flowers are unable to produce functional pollens due to the nutrient shortage to pollens. This is mainly cause of poor pollen development because nutrients don't reach there because abnormal development of the tapetum and callose wall. Innermost layer of cells in anther is known as Tapetum and the cell layer surrounding the tetrads is known as callose wall. Both of these tissues are very important for development of pollen. Developmental defect and abnormal programmed cell death of tapetum cause male sterility (Kim *et al.*, 2013). CMS is a maternally inherited trait, controlled by interaction b/w nuclear genes (*rf1*) and sterile (S-) cytoplasm but the presence of the *Rf1* allele restores fertility. Mendelian inheritance is not followed by them and give hundred percent sterility in the female parent (Shifriss, 1997; Swamy *et al.*, 2017). The *rf1* gene not working alone, there is another nuclear gene *rf2* also found in some genotypes which are needed in addition to inducing sterility in S cytoplasm (Peterson, 1958; Novak *et al.*, 1971). Lee (2001) reported that there are alleles, St^1, St^2 and St^U located in an independent locus from the *Rf* locus and these are responsible for unstable male sterility. Unstable sterile lines could be divided into 2 different types. One whose fertility can be temporarily restored at low temperature and Rf^{TCMS} is the allele that controls thermo-sensitive male sterility, recessive to *Rf* and dominant to *rf* (Kim *et al.*, 2013). The other type produces both normal and aborted pollen grains that remain stuck to anther wall even after dehiscence and this type of partial restoration is genetically controlled by homozygous recessive *pr/pr* alleles (Lee *et al.*, 2008).

Kumar *et al.* (2007) reported that the *Rf* gene is widely distributed in hot pepper lines and 3-line hybridization method involving male sterile line (S-*Rfrf*), maintainer line (N-*Rfrf*) and restorer of fertility line (N/S-*RfRf* or N/S-*Rfrf*) is the best approach to introduce in capsicum for suppression of phenotype of male sterility in their F1 generation plants. Therefore, it is considered very vital to identify co-dominant and reproducible molecular markers that are also linked with mitochondrial (S-cytoplasm) genes and *Rf* genes. This study offers a very rapid and consistent detection of parental lines for the production of F1 hybrid seeds (Swamy *et al.*, 2017).

_Rf_ gene linked markers

It is reported that Baoxi *et al.* (2000) identified RAPD markers ($OP13_{1400}$ and $OW19_{800}$) by applying bulked segregate analysis (BSA. They found that *Rf* gene was tightly linked with $OP13_{1400}$ and the genetic distance between them was found to be 0.37 cM. whereas they also reported that $OW19_{800}$ on the opposite side of *Rf*, and the genetic distance between these two was found to be 8.12 cM. Further studies indicated that the *Rf* allele is widely distributed in hot pepper lines and Both markers are male-specific and could be useful for hybrid seed purity testing (Kumar *et al.*, 2007).

Kim *et al.* (2005) developed *Rf* linked CAPS marker (AFRF8CAPS, at a genetic distance 1.8 cM from *Rf*) and AFLP markers (AFRF1, AFRF2, AFRF3, AFRF4, AFRF5, AFRF6, AFRF7 and AFRF8 at genetic distance 2.6, 2.6, 4.0, 8.9, 6.3, 29.0, 20.7 and 1.8 respectively) by using AFLP-BSA. In Pepper, restorer lines can be selected easily with the help of these above said markers. Moreover, *Rf* gene can be further coned by map-based cloning by using AFRF8CAPS.

S-cytoplasm linked markers

2 CMS specific SCAR primer pairs were developed (atp6 and coxII) with amplicon size 607 and

708 bp respectively by Kim and Kim (2005). This was built on the variances between the mitochondrial nucleotide sequences at the 3′ region of the N-and S-cytoplasms (Peterson's) by PCR. These sequences are valuable for reliable and quick identification of S-cytoplasmic form at seedling stage for plants/crops.

Limitations in Cytoplasmic Male Sterility

Despite many advantages of cytoplasmic male sterility, sometimes in sweet pepper utilization of technique of male sterility is not much feasible because:

• Sweet pepper or capsicum is usually grown at low temperature as compared to hot pepper. And low temperature is not suitable for onset of male sterility (Kim *et al.*, 2013).

• Stability of expression of male sterility is reduced usually by the presence of modifier genes in capsicum (Lee, 2001).

• Difficulty in locating the restorer of fertility (Rf) line (Kumar *et al.*, 2007).

• Technical complexity involved in seed production and maintenance of parental lines (Swamy *et al*., 2017).

• In the female parent, smaller number of seeds are produced and problem of partial restorers persists (Lee *et al.*, 2008).

Genetic male sterility

The CMS system is limited in the case of *Capsicum annuum* L. due to the high instability of CMS expression at low temperature and the absence of a restorer source in most sweet peppers (Lee *et al.*, 2012; Kim *et al.*, 2013). In CMS system restorer line is required and owing to lack of restorer lines in sweet pepper, GMS system is used. Genetic male sterility is simply inherited and highly stable trait control by *ms* genes. There is a pair of recessive genes, when present in homozygous condition (*ms*/*ms*) determines the male sterility while when their dominant alleles are present in homozygous (*MS/MS*) or heterozygous condition (*MS/ms*) then the plant is totally fertile. Fifty percent of male sterile and fifty percent of male fertile offgeneticc makeup differing only at MS locus as (*Ms*/*ms* and *ms*/*ms*) are crossed. After identification, removal of the male-fertile plants is carried out by manual practice for the purpose of hybrid seed production. Then the desired parental line (*Ms*/*Ms*) is used a s male parent and is crossed with the selected sterile (*ms*/*ms*) plants and hybrid seed are produced. GMS system is less efficient as it is not easy to maintain as compared to CMS system. But it is usually selected due to its advantage of having the ability of being introgressed easily by using the techniques of simple backcrosses. It can give rise to some new elite lines that may have a varied genetic makeup (Lee *et al*., 2010; Ponnam *et al.*, 2018). In general, breeding of new lines or cultivars for a character controlled by a recessive gene is more laborious and time-consuming because within a generation it is very difficult to phenotypically distinguish the genotypes between homozygous dominant plants and heterozygotes. It is very simple to select only heterozygous plants from every generation if a molecular marker linked to the GMS (Lee *et al*., 2010a).

In pepper up to 20 nuclear genes responsible for male sterility have been reported by mutagenesis using X rays or gamma rays and EMS (Wang and Bosland 2006; Dhaliwal and Jindal, 2014). Natural *msk* and *ms2* alleles are reported to be allelic with EMS induced *ms10* allele (Daskalov and Poulos, 1994). But EMS induced *ms10* allele was non-allelic to the *ms1* allele (Shifriss, 1997). There are also molecular markers reported that are associated to *ms10* (Aulakh *et al.*, 2016), *ms8* (Bartoszewski *et al.*, 2012), *ms3* (Lee *et al.*, 2010b), *ms1* (Lee *et al*., 2010a). There is also a reported 1 *ms* that has unknown origin also in associated form (Lee *et al.*, 2012). The ms8 gene mapped to chromosome

4 and ms10 gene mapped to chromosome 1. The ms3 linked with amplified fragment length polymorphism converted CAPS marker was described. But the shortcoming associated with this research were not providing the location on chromosome and basic primer sequence (Lee *et al*., 2010b).

SNPs discovery and genotyping is also associated positively and efficiently with Genotyping by sequencing (GBS) (Elshire *et al.*, 2011; Taranto *et al.*, 2016). GBS has been broadly implemented for high density genome-wide association studies, diversity studies and genotyping for linkage analyses depending upon its cost-effectiveness (He *et al.*, 2014). Due to its co-dominant and bi-allelic nature and the relative abundance in the most genomes, the SNP markers also considered as widely implemented marker system (Mammadov *et al*., 2012). Efficiency of SNP markers depend upon the identification SNPs that are associated with some particular trait. Once SNP they are recognized researchers can develop proper SNPs assays for genotyping of large numbers of individuals (Semagn *et al.*, 2014). It also means that SNPs can act as PCR based dCAPs or CAPs markers (Lee et al., 2010; Schafleitner *et al.*, 2016).

Test of Genetic purity

It is very essential to test the genetic purity of seed production and there is no commercial use of them without determining their commercial purity. Conventional type of genetic purity tests are also adding to cost as they are time-consuming. They are also depending on observing just phenotypic characteristics. It may add to confusion as phenotypic characteristics of hybrid seed may be very much similar to 1 of the parents. It is not possible to determine or estimate the genetic purity of the hybrid seeds on basis of visual analysis only.

Molecular markers gain their importance here, where they can be used with confidence to assess the genetic purity. A few of the molecular techniques have established to be exceptional tools for genotype testing. As compared with hybridization-based markers such as restriction fragment length polymorphisms (RFLP) the PCR-based markers such as RAPD (Williams *et al*.,1990) and ISSR (Gupta *et al*.,1994; Zietkiewicz *et al.*,1994) are comparatively simple and cheap. Randomly amplified polymorphic DNA analysis are effective in varietal identification of several crops including potato (Ford and Taylor, 1997) and Capsicum (Ilbi, 2003).

In crop plants. Inter simple sequence repeats (ISSR) markers have been resourcefully utilizeded for identification of cultivars in crop plants (Ge *et al*., 2003). The role of ISSR in recognition of potato and citrus hybrids has already been reported successfully by Matthews *et al*. (1999) and Scarano *et al*. (2002) respectively.

It has also been reported that RAPD markers are more consistent. It is due to the fact that the RAPD marker system can produce a greater number of markers as compared to the ISSR marker system. Mongkolporn *et al.* (2004) used RAPD marker analysis for three F1 chilli hybrids for determining their genetic purity. They were successful for finding all three F1 hybridity where as ISSR marker system could detect hybridity in only two lines.

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