

RESEARCH ARTICLE

Mapping and validation of markers linked to *Rf* gene in sweet pepper (*Capsicum annuum* var *grossum*)

Manoj Kumar Nalla^{1*}, Vinod Kumar Sharma², Vinukonda Rakesh Sharma¹

¹Division of Vegetable Science, ICAR-IARI, New Delhi 110012. ²Division of Germplasm Evaluation, ICAR-NBPGR, New Delhi 110012

ABSTRACT

Cytoplasmic male sterility (CMS), is the most valuable system in commercial hybrid seed production in hot pepper. Whereas in sweet pepper (*Capsicum annuum* var *grossum*), the unavailability of a stable restorer's line is one of the major constraints in commercial hybrid breeding. Identifying the markers linked to CMS and nuclear restorer-of-fertility (*Rf*) genes will help in the production of CMS hybrids in sweet pepper. In the present study, we identified seven markers co-segregating with *Rf* genes using bulk segregating analysis (BSA) on the F₂ *Rf*-segregating mapping population. Among these, the CRF-SCAR marker proved to be linked to the fertility restorer gene and it could be used for screening of genotypes to identify restorers and non-restorer lines in future *Capsicum* breeding programs and help in the establishment of CMS system for commercial hybrid seed production.

Keywords: *Capsicum*; CMS; Hybrid production; Restorer-of-fertility (*Rf*) genes; Sweet pepper

INTRODUCTION

Sweet pepper (*Capsicum annuum* var *grossum* L.; *Solanaceae*) is the most economically important vegetable crop which is grown all over the world. It is nutritionally opulent with vitamins, minerals and possess high antioxidant and antimicrobial properties (Alsebaei et al., 2020). Given the economic importance and demand for sweet peppers, improved cultivars of superior quality have to be developed. To feed the world's rising population and to meet global demand, there is a necessity of high yielding hybrid varieties (Luo et al., 2013). Hybrid development through heterosis yields 30-50% high produce than self-pollinated cultivars. The use of male sterility (MS) in pepper can reduce the cost of hybrid seed by up to 50% (Yang et al., 2008). *Capsicum* sps. features a multitude of male sterility systems such as CGMS and GMS which are viable alternatives to hand emasculatation and pollination (Mulyantaro et al., 2014). CMS is more efficient than GMS by providing 100% male sterility of the female parent for F₁ seed production as compared to only 50% for GMS (Dhaliwal et al., 2014).

To expand the genetic basis in the heterosis breeding program, finding a stable fertility restorer and sterility

maintainer for the CMS line is imperious (Sandeep and Sanjay, 2017). The restoration of fertility in chilli pepper is exacerbated by the combination of some major *Rf* genes with sterility-modifying genes and also environmental factors like temperature. Fertility restoration is often linked to genes that encode pentatricopeptide repeat (PPR) proteins, which target mitochondria and suppress CMS gene expression by cleaving or degrading CMS transcripts (Chen and Liu., 2014). Genes that restore fertility are widespread in hot pepper cultivars but scarce in bell pepper cultivars (Kumar et al., 2007).

The inheritance of fertility restoration has been studied by several groups of researchers. Peterson (1958), Yu (1990), Gulyas et al. (2006) and Kumar et al. (2007) had reported that pepper CMS could be restored by one major single dominant nuclear *Rf* gene. Whereas, Lee et al. (2008b) had reported one major dominant nuclear gene (*Rf*), as well as some modifier genes.

Novak et al. (1971) reported that sweet pepper fertility restoration is controlled by two complementary genes. In contrast, Wang et al. (2004) identified one major QTL and four minor QTLs related to restorer-of-fertility in pepper by mapping quantitative trait loci (QTLs). However, the number of *Rf* genes that contribute to restorer-of-fertility

*Corresponding author:

Manoj Kumar Nalla, Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India.
Tel.: +91 70754 79456. E-mail: manojhorti@gmail.com

Received: 07 March 2021; Accepted: 29 May 2021

in pepper is uncertain, implying that restorer-of-fertility inheritance is more intricate. Furthermore, several studies have shown that environmental factors such as low temperatures sometimes causing the breakdown of sterility CMS, indicating the presence of modifying genes (Bueckmann et al., 2014; Lee et al. 2008). There is still no clear understanding of the genetic mechanism of fertility restorer. Understanding the inheritance mechanism for restorer-of-fertility lines will thus be beneficial for breeding fertility restorer lines in pepper.

By introgressing the “*Rf*” allele from hot pepper to bell pepper for C line development, the CMS system’s scope can be expanded. As a consequence, the production of successful and stable fertility restorer lines (R-line) is crucial. Furthermore, Marker-assisted selection (MAS) could aid in the identification and development of stable restorer lines. This will assist in the development of CGMS hybrids in sweet pepper.

Molecular markers such as CRF CAPS markers (Min et al., 2009), AFRF8CAPS (Kim et al., 2006), AFRF1CAPS (Zhang et al., 2000), AFRF3CAPS (Min et al., 2009), CRF -SCAR (Gulyas et al., 2006), OPP13CAPS (Kim et al., 2005) and PR-CAPS (Lee et al., 2008), BAC13T7 SCAR marker (Jo et al., 2010), linked to fertility restorer gene were reported in pepper. These will make it easier to screen inbred pepper lines for *Rf* alleles and to pass the *Rf* gene to male sweet pepper parents more easily (restorer breeding). Although several markers such as OPP13 (0.4 Cm) and AFRF4 (0.1 cM) were closely linked to the *Rf* gene in a given population (Min et al. 2009). Most of the markers were unable to correctly detect genotypes for a wider range of pepper lines, inferring that rearrangements have occurred in the *Rf* locus (Jo et al. 2010). The robust *Rf* marker should provide accurate identification of CMS restorer line *Capsicum* sps. The efficacy of these markers, to predict the *Rf* phenotype over a wide range of germplasm is tactlessly, restricted (Jo et al. 2010). The reproducibility of markers is always a major problem as it depends on the mapping population used in the study and also many other factors. So, there is a great need to assess the efficiency of molecular markers, validate them in different mapping populations and report the best markers.

In this regard, the current study is undertaken with the objectives to identify the inheritance pattern of the fertility restoration genes (*Rf*) in sweet pepper and validation of molecular markers that were predicted to be linked with the *Rf* gene in pepper as well as to conduct a linkage analysis for the marker.

MATERIAL AND METHODS

Plant material

The experimental material consists of three generations (F_1 , F_2 and BC_1F_1) of sweet pepper derived from KTCA 5 [Stable CMS line (*rf/rf*)] and KTCR 15 [Restorer line (*R/Rf*)] procured from IARI, Regional Station, Katrain, India (32.10° N, 77.124° E, 1688 MSL) (Table 1). F_1 (KTCA 5 x KTCR 15) and BC_1F_1 (F_1 x KTCA 5) were raised in an insect proof cage. The 125 F_2 and 66 BC_1F_1 populations were planted in the open field of IARI, Regional Station, Katrain from March to October 2017 for phenotyping for pollen fertility.

Evaluation of pollen fertility and germination studies

The male fertility and sterility of genotypes were determined by pollen viability test (Sharma et al., 2019). Male fertility was also examined visually that male-fertile plants that had normal abundant pollen grains, whereas male-sterile plants had no pollen grains in mature anthers (Fig. 1). Pollen grains were germinated invitro in a liquid germination media (0.01% boric acid + 10% sucrose). Petri dishes with medium were kept in a shaker at 10-15 rpm/hour for 30 min. The petri dishes were then covered and incubated at 25-29°C in an incubator for overnight. The pollen grains germination and viability were measured using a compound microscope with a 10× magnification. The pollen fertility rate (%) was calculated with the following formula = (Number of germinated or Stained pollen grains/Total pollen grains) x 100. In this study, the plants were categorized into various pollen fertility classes as per Govindaraj and Vermani (1988).

Genotyping using bulk segregant analysis (BSA) and linkage analysis

BSA was performed with the plants obtained from the F_2 population as described by Michelmore et al. (1991). DNA

Table 1: Details of the parental genotypes used in the present study.

S. No.	Name of genotype	Material type	Source
1.	KTCA 5	CMS Line (Sweet pepper)	IARI, Regional Station, Katrain, India
2.	KTCR 15	R line (Hot pepper)	IARI, Regional Station, Katrain, India



Fig 1. Fertile and sterile flowers.

was extracted from young leaves using CTAB method (Murray & Thompson, 1980) and the concentration of genotypes was adjusted to 25 ng/μl using Nanodrop (Thermo scientific Inc) for further analysis. A total of seven primer pairs that showed parental polymorphism were used to screen the bulks (Table 2). Among them, six were CAPS and one SCAR marker (Zhang et al. (2000), Kim et al. (2006), Kim et al. (2005), Gulyas *et al.* (2006), Lee et al. (2008) and Min et al. (2009) (Table 2). DNA from the ten individuals each with high fertility and high sterility were pooled in an equimolar concentration to form fertile bulk and sterile bulk respectively. Markers showing polymorphism both in the parents and bulks were then used to genotype all the individuals of the F_2 population. The genotypic data of F_2 individuals is used for the linkage analysis. While analyzing the data, letter 'A' was assigned to plants homozygous male sterile, 'B' for homozygous male fertile, 'H' for heterozygous fertile plants. The MAPMAKER v3.0 (Lander et al., 1987) software was used to generate the linkage map. Map distances in centi-Morgans (cM) were calculated from recombination frequencies using the Kosambi function (Kosambi, 1944).

The agreement of the observed values with the expected phenotyping and genotyping were tested by the chi-square test (χ^2) of goodness of fit for the understanding of inheritance pattern and confirmation of genetic ratio (Panse & Sukhatme, 1985).

RESULTS AND DISCUSSION

Segregation analysis was conducted for fertility restoration traits in KTCA5 × KTCR15 F_2 population. Male-fertile (MF) and male-sterile (MS) phenotypes were highly stable and readily observed during the flowering stage of KTCA5, KTCR15, and their F_2 population, backcross population. Based on pollen fertility analysis in the 125 plants, 91 plants were fertile and 34 plants were sterile (Table 3). Plants that were partially fertile and partially sterile were pooled together to form a single category (Chaudhury et al. 2015; Govinda Raj and Virmani 1988). The ratio of MF to MS plants the F_2 populations conformed to a 3:1 segregation ratio. The observed chi-square value was in fact quite low but acceptable at higher stringencies of $p = 0.57$. Thus, the chi-square test indicated that the segregation of F_2 mapping population does not deviate from the expected ratio of 3:1. In support of the present results, Peterson (1958), Yu (1990), Shifriss (1997), Gulyas et al. (2006) and Lee et al. (2008b) had also reported a single dominant inheritance pattern of fertility restorer gene in pepper. In backcross populations of KTCA5 × (KTCA5 × KTCR15) 66 plants were phenotyped, among which 39 plants were fertile and 27 were sterile. The chi-square test showed that the segregation of fertility does not deviate from the expected ratio of 1:1. The frequency distribution of backcross population with respect to pollen fertility showed a monogenic fashion for fertility restorer gene trait. Similarly, single dominant gene inheritance of fertility restorer genes has also been observed by Yu et al. (1990) and Min et al. (2008) in the backcross population. The phenotype data was strongly

Table 2: Primer sequences used to screen parental polymorphism

Marker	Primer sequence	Reference
AFRF8CAPS		
F	GTTGATGCTCTATGGTTGGAGAAC	Kim et al., 2006
R	CACTATTCTATTGGCTTTCTG	
AFRF1CAPS		
F	CTGCGTACCAATTCATAAAGCACT	Zhang et al., 2000
R	TGAGTCCTGAGTAACCAGGTCAATTG	
AFRF3CAPS		
F	GCAATGGAGAAGGTGAAAGTGATTCTGG	Min et al., 2009
R	TTGGACTTCATGTCCTTTTCAGCTTCC	
CRF CAPS		
F	GATTCATCCTCGACGAAAGAGGA	Min et al., 2009
R	TCACTCAAGGAAATCCCCAAATAAGTGTGAACC	
CRF- SCAR		
F	ATTTTCAGATTGTGGCGACG	Gulyas et al., 2006
R	CGACCATCACGACGAGG	
OPP13CAPS		
F	TACAGCTTCAAAGTAAACACAACC	Kim, 2005
R	ATTCGGGTCCCCAAGAAGGTTCTAT	
PR-CAPS		
F	ATGTCACCCCCACACACTCCTTCACCT	Lee et al., 2008
R	TCCCATCTAGCCTCTGCCTTCTCAAATG	

Table 3: Segregation analysis of F₂ population from the cross KTCA5 × KTCR15.

KTCA5 × KTCR15	Total Plants Genotyped	Allele			Genetic ratio	c2value
		A	H	B		
F1 (KTCA5 × KTCR15)	3	0	3			
F2	91	34	125	3:1	0.322	0.57
BC1 (KTCA5 × KTCR15)	66	27	39	1:1	1.28	0.25
CRF SCAR	123	33	----	90	3:1	0.21

χ^2 at 0.05 with 1 df=3.84.

supported by molecular data showing 3:1 segregation pattern i.e.; monogenic inheritance of fertility restorer gene. In contrast to single dominant gene inheritance, many other studies had reported two complementary genes (Novak et al., 1971), both major and minor genes (Zhang et al., 2000), one major quantitative trait locus (QTL) and four minor QTLs (Wang et al., 2004) and 2 major and 7 minor QTLs (Wei et al., 2017). These QTL's reports probably correspond to the major restorer gene reported by several authors after phenotypic evaluation (Novak et al., 1971; Yu, 1990). The difference in segregation ratios in different studies could presumably be due to the influence of female parents or probably variable expression of the weaker genes in different genetic backgrounds. Certain modifier genes could also be responsible for changing the segregation ratio (Govinda Raj and Virmani., 1988; Sohu and Phul., 1995). These observations validate the single dominant inheritance pattern of the fertility restorer gene trait.

The selected molecular markers were screened in the parents to observe the polymorphism. Among the markers tested, only Pr-CAPS and CRF-SCAR markers formed polymorphic fragments in both fertile and sterile parents. Other primer pairs linked to the *Rf* gene were found to be devoid of parental polymorphism. Kim (2005); Lee et al. (2008b); Jo et al. (2010) found a low ratio of effective genotyping using these markers, corroborating these findings. Just one of the two primer pairs, CRF SCAR, revealed polymorphism between the F₂ population's parental lines and bulks. Similar results for CRF-SCAR marker were also obtained by Gulyas et al. (2006); Kim et al. (2013); Ma et al. (2013); Lin et al. (2015); Mulyantaro et al. (2014) and Pakozdi et al. (2002).

The two polymorphic markers discovered between KTCA5 and KTCR15 were further screened using bulked segregant analysis (BSA) (Michelmore et al., 1991). BSA approach has been successfully utilized for mapping fertility restorer gene in hot pepper (Chang et al., 2005; Jo et al., 2016; Yanagawa et al., 1996; Pakozdi et al., 2002; Lee et al., 2008a) and in rice (Mishra et al., 2003). BSA's core principle is that if a molecular marker demonstrates

polymorphism between a population's parents and is closely linked to a big QTL/gene regulating a specific trait, it can co-segregate with the QTL (Quarrie et al., 1999). In order to confirm the linkage of the marker to the loci defining the trait, two DNA pools formed from F₂ plants with contrasting traits (high fertile and high sterile) and were evaluated to distinguish polymorphic markers between them. The pictures illustrating the flower morphology, pollen viability and pollen germination of both fertile and sterile bulk individual plants are portrayed in Figs. 2 and 3 respectively. The BSA in our study revealed that, except for the CRF-SCAR marker, none of the reported markers showed linkage with the male fertility gene (*Rf*) in the population. Because the SCAR marker is a dominant marker, the presence of the band represents the fertile bulk, which contains both homozygous and heterozygous fertile plants, and the absence of the band represents the sterile bulk, which is homozygous sterile. The representative gel pictures showing the amplification profile of the parents and the fertile and sterile bulk individual plants with the polymorphic markers CRF-SCAR are presented in Fig. 4. The genotypic data of the F₂ plants were also tested for goodness of fit using χ^2 tests. The observed Chi-square values for CRF-SCAR marker were less than the tabulated value of 3.84 at 5% level of significance and 1 degree of freedom. It is evident from the results obtained that the observed ratio fitted well with the expected ratio 3:1 with respect to the CRF-SCAR marker. The markers which exhibited polymorphism in BSA were taken for analysis of individuals constituting the F₂ and BC₁F₁ populations and their segregation ratios. The amplification profile obtained with the parental polymorphic CRF SCAR markers in the F₂ population is shown in Fig. 5. The genotypic data is in agreement with the phenotypic data which strongly add additional strength and support the monogenic dominant inheritance. However multigene inheritance cannot be ignored. Barchenger et al. (2018) discovered 11 PPR-encoding regions on chromosome 6 that were characterized as candidate *Rf* genes. These findings imply that chili pepper may contain multiple *Rf* genes and that different fertility-restored lines may contain different *Rf* genes for male fertility restoration.

The mapping of the fertility restoration gene carried out by using linkage analysis of phenotypic data of pollen fertility of F₂ segregating population and genotypic data generated by CRF-SCAR marker on the F₂ population of the cross KTCA5 × KTCR15 using MAPMAKER ver.3b software. The CRF-SCAR marker mapped at a distance of 7.4 cM using linkage analyses conducted using kosambi mapping function and LOD score 3 (Fig. 6). Jo et al. (2010) and Jo et al. (2016) in their study reported highest genotyping accuracy (70.0–89.1 %) while screening with the CRF-

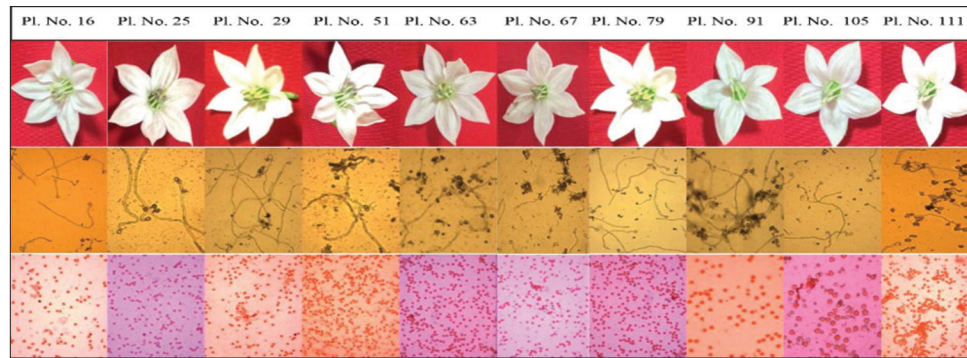


Fig 2. Flower morphology, pollen germination and viability of fertile bulk plants.

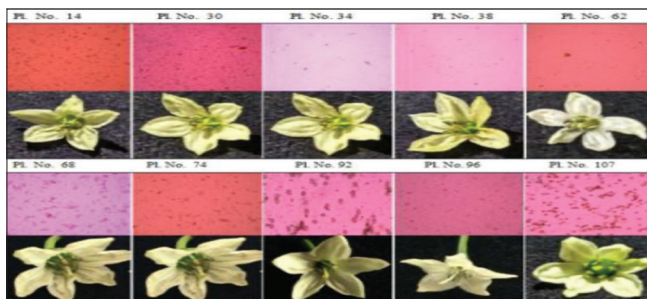


Fig 3. Flower morphology, pollen viability of sterile bulk plants.

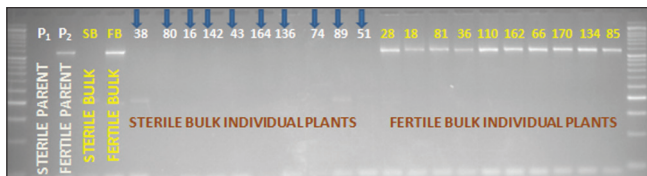


Fig 4. CRF-SCAR marker amplification profile of the parents and the fertile and sterile bulk individual plants with the polymorphic markers.

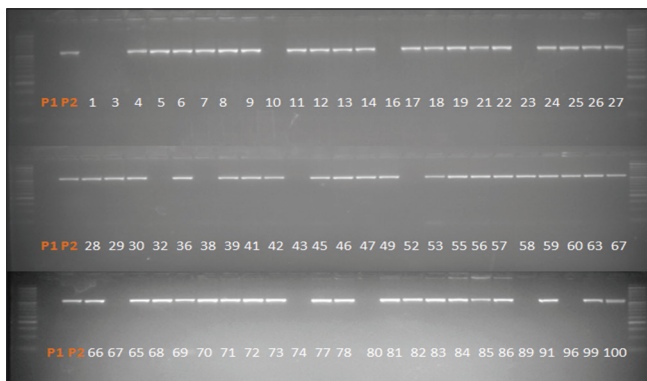


Fig 5. CRF-SCAR marker Amplification profile in the F2 population (KTCA5 x KTCR15).

SCAR marker. Linkage map showing the linkage of *Rf* gene governing male fertility restoration gene with CRF-SCAR marker is depicted in the plate 4.10. The CRF SCAR marker is located on chromosome 6 of pepper. Supporting our statement Wang et al. (2004); Jo et al. (2010); (2016) in their studies have reported *Rf* loci to chromosome 6 on the basis of a genetic linkage map.



Fig 6. Linkage map of the *Rf* gene and CRF- SCAR marker using MAP maker 3.0 software. The cM is for genetic distance based on Kosambi mapping function.

The SCAR marker is found to be placed at a bit more distance 7.4 cM as compared to the reported distance of 4.8 cM by Gulyas (2006). It can be supported by the findings of Jo et al. (2010), who explained that although several markers are closely linked to the *Rf* gene in a given population, most of the markers were unable to correctly detect genotypes for a wider range of pepper lines implying that rearrangements may have occurred in the *Rf* locus. Similarly, several DNA markers distinguishing *Rf* and *rf* alleles have been reported in chili peppers (Pakozdi et al., 2002; Kumar et al., 2007; Lee et al., 2008a; Min et al., 2009; Jo et al., 2010; Lee et al., 2012 and Mol et al., 2020). But these markers closely linked to the *Rf* locus were often inconsistent with the phenotypes in inbred lines (Zhang et al., 2000; Kumar et al., 2007). The disparity in results between *Rf* phenotype and marker could be explained by the fact that they may have used different mapping populations in previous studies or due to the plant genome's frequent recombination frequency. (Lee et al., 2008a) suggested that some insertions may have occurred in the genome of the plant material. In some cases, the effect of modifiers and environment may also be involved (Lee et al., 2008b) or it may be due to nature of

the *Rf* gene (Jo et al., 2010). Moreover phenotyping errors which cannot be overruled. In recent years, the complete pepper genome sequence has been deciphered which may not only assisted in developing STS markers with a very tight linkage to the *Rf* gene but also in map based cloning of the *Rf* gene in pepper (Kim et al., 2007; Jo et al., 2016).

CONCLUSION AND FUTURE OUTLOOK

In this investigation, it was concluded that the fertility restoring trait was controlled by a single dominant gene. We also validated a molecular marker namely CRF-SCAR that has been previously reported to be associated with fertility restoration traits in the F_2 mapping population derived from the cross KTCA5 x KTCR15 through bulk segregant analysis. The linkage analysis shows that the genetic distance of the CRF-SCAR marker with the *Rf* gene is 7.4cM. The current findings may pave the way for more reliable and efficient marker-assisted selection, for the restorer line development in sweet pepper. In the future, incessant efforts are needed to clone and characterize *Rf* gene, and develop functional marker for stable restorer line selection.

ACKNOWLEDGMENT

We are thankful to the Department of science and technology (DST), for providing the Inspire fellowship.

Author's contribution

Conceptualization of research (VK, MK); Designing of the experiments (VK, MK); Contribution of experimental materials (VK); Execution of field/lab experiments and data collection (MK); Analysis of data and interpretation (MK, VR); Preparation of the manuscript (MK, VR).

REFERENCES

- Alsebaei, M., A. K. Chauhan, Arvind and P. Yadav. 2020. Consumption of green chilli and its nutritious effect on human health. In: P. Mishra, R. R. Mishra, Adetunji (Eds.), Innovations in Food Technology, Springer, Singapore, pp. 373-384.
- Bueckmann, H., K. Thiele, A. Huesken and J. Schiemann. 2014. Influence of air temperature on the stability of cytoplasmic male sterility (CMS) in maize (*Zea mays* L.). *Ag. Bio. Forum.* 17: 205-212.
- Chang, C. T., C. G. Wang, C. B. Chen, F. Wu and D. L. Sun. 2005. Identification of molecular markers linked to the fertility restoring gene for the CMS (*Capsicum annuum* L.). *Shi Yan Sheng Wu Xue Bao.* 38(3): 227-232.
- Chaudhari, S., A. N. Tickle, Uttamchand, K. B. Saxena and A. Rathore. 2015. Stability of cytoplasmic genetic male sterility and fertility restoration in Pigeonpea. *J. Crop. Improv.* 29(3): 269-280.
- Chen, L. and Y. G. Liu. 2014. Male sterility and fertility restoration in crops. *Annu. Rev. Plant. Biol.* 65: 579-606.
- Dhaliwal, M. S. and S. K. Jindal. 2014. Induction and exploitation of nuclear and cytoplasmic male sterility in pepper (*Capsicum* spp.): A review. *J. Hort. Sci. Biotechnol.* 89(5): 471-479.
- Govindaraj, K. and S. S. Virmani. 1988. Genetics of fertility restoration of "WA" type cytoplasmic genic male sterility in rice. *Crop Sci.* 28: 787-792.
- Gulyas, G., K. Pakodzi, J. S. Lee and Y. Hirata. 2006. Analysis of fertility restoration by using cytoplasmic red pepper (*Capsicum annuum* L.) lines. *Breeding Sci.* 56: 331-334.
- Jo, Y. D., Y. Ha, J. H. Lee, M. Park, A. C. Bergsma, S. Goritschnig, B. Kloosterman, V. P. J. Dijk, D. Choi and B. C. Kang. 2016. Fine mapping of restorer-of-fertility in pepper (*Capsicum annuum* L.) identified a candidate gene encoding a pentatricopeptide repeat (PPR) containing protein. *Theo. Appl. Genet.* 129(10): 2003-2017.
- Jo, Y. D., Y. M. Kim, M. N. Park, J. H. Yoo, M. Park, B.D. Kim and B.C. Kang. 2010. Development and evaluation of broadly applicable markers for restorer-of-fertility in pepper. *Mol. Breed.* 25: 187-201.
- Kim, D. H. and B. D. Kim. 2006. The organization of mitochondrial atp6 gene region in male fertile and CMS lines of pepper (*Capsicum annuum* L.). *Curr. Genet.* 49: 59-67.
- Kim, D. S. 2005. Development of RAPD and AFLP Markers Linked to Fertility Restorer (Rf) Gene in Chili Pepper (*Capsicum annuum* L.). (Doctoral Dissertation), Seoul National University, South Korea.
- Kim, Y. M., Y. D. Jo, J. K. Kwon, I. T. Kim and B. C. Kang. 2013. Characterization and inheritance of a novel thermo-sensitive restoration of cytoplasmic male sterility in *Capsicum annuum*. *Sci. Hort.* 164: 512-520.
- Kosambi, D. D. 1944. The estimation of map distance. *Ann. Eugenics.* 12: 505-525.
- Kumar, S., V. Singh, M. Singh, S. Rai, S. Kumar, S. K. Rai and M. Rai. 2007. Genetics and distribution of fertility restoration associated RAPD markers in inbreds of pepper (*Capsicum annuum* L.). *Sci. Hort.* 111: 197-202.
- Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, S. E. Lincoln and L. Newburg. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics.* 1(2): 174-181.
- Lee, H. R., H. J. An, D. C. Yang, S. H. Choi, H. J. Kim, H. G. Rhee and Harn, C. H. 2012. Development of a high resolution melting (HRM) marker linked to genic male sterility in *Capsicum annuum* L. *Plant. Breed.* 131: 444-448.
- Lee, J., J. B. Yoon, and H. G. Park. 2008a. A CAPS marker associated with the partial restoration of cytoplasmic male sterility in chili pepper (*Capsicum annuum* L.). *Mol. Breed.* 21: 95-104.
- Lee, J., J. B. Yoon, and H. G. Park. 2008b. Linkage analysis between the partial restoration (pr) and the restorer-of-fertility (Rf) loci in pepper cytoplasmic male sterility. *Theo. Appl. Genet.* 117: 383-389.
- Luo, D., H. Xu, Z. Liu, J. Guo, H. Li and L. Chen, C. Fang, Q. Zhang, M. Bai, N. Yao, H. Wu, H. Wu, C. Ji, H. Zheng, Y. Chen, S. Ye, X. Li, X. Zhao, R. Li and Y. G. Liu. 2013. A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. *Nat. Genet.* 45: 573-577.
- Ma, Y., W. Huang, J. J. Ji, Z. H. Gong, C. C. Yin, S. S. Ahmed and Z. L. Zhao. 2013. Maintaining and restoration cytoplasmic male sterility systems in pepper (*Capsicum annuum* L.). *Genet. Mol. Res.* 12(3): 2320-2331.
- Min, W. K., S. Kim, S. K. Sung, B. D. Kim and S. Lee. 2009. Allelic discrimination of the restorer-of-fertility gene and its inheritance in peppers (*Capsicum annuum* L.). *Theor. Appl. Genet.* 119: 1289-1299.
- Mishra, G. P., R. K. Singh, T. Mohapatra, A. K. Singh, K. V. Prabhu, F. U. Zaman and R. K. Sharma. 2003. Molecular mapping of gene for fertility restoration of wild abortive (WA) cytoplasmic male sterility using a basmati rice restorer line. *J. Plant Biochem. Biotech.* 12: 37-42.

- Mulyantoro, S. Y., A. Chen, Wahyono and H. M. Ku. 2009. Modified complementation test of male sterility mutants in pepper (*Capsicum annuum* L.): Preliminary study to convert male sterility system from GMS to CMS. *Euphytica*. 169: 353-361.
- Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8(19): 4321-4326.
- Novak, F., J. Betlach and J. Dubovsky. 1971. Cytoplasmic male sterility in sweet pepper (*Capsicum annuum* L.). Phenotype and inheritance of male sterile character *Z. Pflanzenzücht.* 65: 129-140.
- Pakozdi, K., J. Taller, Z. Alföldi and Y. Hirata. 2002. Pepper (*Capsicum annuum* L.) cytoplasmic male sterility. *J. Central. Euro. Agri.* 3: 149-158.
- Panse, V. G. and P. V. Sukhatme. 1985. *Statistical Methods for Agricultural Workers*, ICAR, New Delhi, pp. 70-72.
- Peterson, P. A. 1958. Cytoplasmic inheritance of male sterility in *Capsicum annuum*. *Am. Nat.*, 92: 111-119.
- Quarrie, S., V. Lazi-Jani, D. Kovaevi, A. Steed and S. Peki. 1999. Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. *J. Exp. Bot.* 50(337): 1299-1306.
- Sandeep, A. M. and L. K. Sanjay. 2017. Inheritance of restorer gene in chilli (*Capsicum annuum* L.) *J. Glob. Biosci.* 6(8): 5201-5204.
- Wang, L. H., B. X. Zhang, V. Lefebvre, S. W. Huang, A. M. Daubèze and A. Palloix. 2004. QTL analysis of fertility restoration in cytoplasmic male sterile pepper. *Theor. Appl. Genet.* 109: 1058-1063.
- Wei., B. Q., L. L. Wang, R. Zhang and J. N. Zhang. 2017. Identification of two major quantitative trait loci restoring the fertility of cytoplasmic male sterility in *Capsicum annuum*. *J. Agri. Biotechnol.* 25(1): 43-49.
- Yang, C. J., B. J. Chen, J. G. Liu and S. Li. 2008. Study on the superiority of hybrid breeding by male sterility in hot pepper. *J. China Capsicum.* 1: 36-38.
- Yu, I. W. 1990. *The Inheritance of Male Sterility and its Utilization for Breeding in Pepper (Capsicum spp.)*. (Doctoral Dissertation), Kyung Hee University, Seoul.
- Zhang, B. X., S. W. Huang, G. M. Yang and J. Z. Guo. 2000. Two RAPD markers linked to a major fertility restorer gene in pepper. *Euphytica.* 113: 155-161.