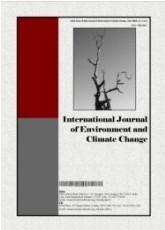

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Genetics Analysis of *Rf* Gene in Chilli Pepper (*Capsicum annuum* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author MKN managed the work, wrote the complete draft of the manuscript, designed the study and performed the statistical analysis. Author VKS supervised the whole work of author MKN to prepare this manuscript. Authors VRS and AKP helped during the experiment. All authors read and approved the final manuscript.

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ABSTRACT

The inability to develop functional pollen is caused by cytoplasmic male sterility (CMS), a maternally inherited trait. Restorer-of-fertility (*Rf*), a nuclear gene, could cause normal pollen production in CMS plants, resulting in fertility in the plant. This paper aimed to study the inheritance of restoration of fertility traits in both Sweet pepper and Hot pepper. The study was conducted in the Indian agricultural research institute, Katrain regional station, India. Genetic analysis of fertility restoration was performed on the progeny of chilli and sweet pepper. KTCA 5 (cytoplasmic-genetic male sterile line-Sweet pepper), KTCA 10 (cytoplasmic-genetic male sterile line-Sweet pepper. F_2 segregation population and back cross BC_1 population obtained from an F_1 hybrid between KTCR 15 (a fertility restorer line). The fertility of the test-crossed lines was assessed under open field conditions using pollen related criteria. The fertility restoration trait segregated in 3:1 and 1:1 F_2 segregation populations and backcrossed BC_1 populations respectively in both Sweet Pepper and Hot pepper backgrounds. This indicates single dominant gene inheritance of the *Rf* gene. There is no effect of CMS background on the restoration trait inheritance.

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1. INTRODUCTION

Chilli (*Capsicum annuum L*.) is a major cash crop as well as an important condiment and spice in India. The chillies are renowned for their pungency (capsaicinoids) [1] and the colour (carotenoids pigments, predominantly capsanthin) [2]. It accounts for about 17% of the global spice trade due to its high cash value and widespread consumption [3]. The primary explanation for improved production over time is the use of high yielding hybrid cultivars instead of conventional open pollinated cultivars [4]. Chilli hybrid seed is generated either through hand-emasculation or through taking advantage of male sterility [5]. GMS (genetic male sterility) and CMS (cytoplasmic male sterility) have also been documented and used for hybrid growth. GMS is tedious, and the seed is vulnerable to genetic impurities due to sib pollination, inaccurate detection and self-pollination [6]. Peterson [4] documented the CMS in Capsicum for the first time in 'PI 164835,' an introduction from India.

Cytoplasmic sterility guarantees 100 percent sterility in the female parent due to maternal inheritance, and it is still the best mechanism for hybrid seed development if sterility is stable and restorer genes are available. CMS systems, on the other hand, are only used in seed crops if a nuclear restorer gene (*Rf*) is used to inhibit male sterility in hybrid plants [7-9]. Martin and Grawford [10] discovered nuclear genetic male-sterility in pepper, and Peterson [11] discovered cytoplasmic male-sterility (CMS). In F_1 hybrid seed production of hot pepper (Capsicum annuum L.), cytoplasmic male sterility (CMS) has been utilized progressively because it can reduce production cost by as much as 47% [12]. But, its use in sweet pepper is circumscribed because of the lack of good CMS-fertility restorer lines to perform as pollen parents.

A single nuclear recessive gene designated as *rf* interacts with the sterile (S) plasmatypic to produce sterility, whereas the restorer dominant allele *Rf1* controls the fertility restoration [13]. Genetic studies on fertility restoration revealed dominant monogenic control of *Rf* allele in hot pepper cultivar 'Pant C-1'. The dominant (*Rf*) gene restores fertility by suppressing the CMS-associated genes [9]. Fertility restoration of the Peterson cytoplasmic male sterility in pepper is more likely a quantitative trait that is controlled by both major and minor genes [14]. The modifier genes in different backgrounds are also affecting the strength of fertility restoration in pepper [15]. The dominant fertility restorer (*Rfs*) alleles are widespread in several hot and small-fruited pepper genotypes, whereas many sweet and large-fruited genotypes possess recessive maintainer alleles [16]. The genetic information, such as the pattern of inheritance is very useful in the selection process so that the selection can be more effective and efficient. In this regard, there is a need to elucidate the inheritance pattern of fertility restoration for chilli. This research was carried out to find out the impact of hot pepper and sweet pepper CMS background on Rf gene segregation, in addition to learning the nature of Rf gene inheritance.

2. MATERIALS AND METHODS

A segregating F_2 and a backcross population were developed from a cross of KTCA 5 (Sweet pepper), KTCA 10 (Hot Pepper) and KTCR 15 (Sweet Pepper). The CMS lines KTCA 5 and KTCA 10 have sterile cytoplasm with *rfrf* genotype. The restorer line KTCR 15 has fertile cytoplasm with the *RfRf* genotype. The details of genotypes were given in Table 1. These plants were kindly provided by IARI, Regional Station, Katrain, India. In March 2016, F1 was raised in an insect-proof cage and backcrossed with the KTCA5 sterile parent using F1 plant pollen. From March to October 2017, the F2 and backcross populations were planted in open fields at the IARI Regional Station in Katrain, Himachal Pradesh, India (32.10° N, 77.124° E, 1688 MSL).

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 (Sweet pepper)	IARI, Regional Station, Katrain, India
3.	KTCR 10	R line (Hot pepper)	IARI, Regional Station, Katrain, India

2.1 Fertility Assessment

Visual scoring of the presence of stainable (normal) and unstainable (aborted) pollen was used to assess the male fertility phenotype of all F_2 or BC_1 restorer plants related to testcross progenies. Pollen viability is assessed using three to four well-developed flower buds. Anthers from each flower bud were smeared on a glass slide with a drop of 2% acetocarmine solution. Pollen grains that were round and stained well were considered fertile, while pollen grains that were shriveled, hyaline, or unstained were considered sterile. The germination method is also used to estimate pollen fertility.

Pollen grains were isolated from flowers by forceps and were placed on a glass slide that contains germination media for pollen germination in vitro. The germination media is a liquid medium that consists of 0.01% boric acid + 10% sucrose. The slides with culture media along with pollen grain were kept in a shaker at 10-15 rpm for an hour. The slides were then kept at 25-29°C in an incubator or at room temperature, ideally in the dark. Pollen that has germinated clearly showing the pollen tube is fertile, whereas non germinated pollen is considered sterile. The percentage of pollen stained and germinated was measured in a compound microscope with 10× magnification. The plants were divided into four groups: fully fertile (FF) (61–100% pollen fertility), partially fertile (PF) (31–60% pollen fertility), partially sterile (PS) (11–30% pollen fertility), and fully sterile (FS) (0-10 percent pollen fertility). Male fertility was also examined by visual observation based criteria: male-fertile plants had normal abundant pollen grains adhering to anthers, whereas male-sterile plants had no pollen grains in mature anthers. This is also further confirmed by touching anthers of freshly opened flowers on thumbnails or black paper. The presence of white creamy pollen mass is considered as fertile and the absence of pollen mass is considered as sterile. Pictures of pollen viability and germination were depicted in Fig. 1.

The pollen fertility rate was calculated in accordance with the following formula: (%) = number of germinated/stained pollen grains / total pollen grains x 100.

The agreement of the observed values with the expected phenotyping and genotyping were tested by the chi-square test ($\chi 2$) of the goodness of fit for the understanding of inheritance pattern and confirmation of genetic ratio [17].

3. RESULTS AND DISCUSSION

Understanding the nature of inheritance of restorer gene (*Rf*) trait is essential in the development of the CMS restorer line and its use in hybrid seed production. Pollen fertility analysis is done in 123 segregating plants of the $F_{2\ population}$ of sweet pepper derived from the cross KTCA5 × KTCR15 (Table 2). In the F_2 populations 91 plants showed fertile and 34 plants sterile reaction. The chi-square test indicated that the segregation of the F_2 mapping population does not deviate from the expected mendelian segregation ratio 3:1 at higher stringencies of the P value, which showed a monogenic inheritance pattern of fertility restoration governed by a single dominant gene. The validation was carried out in 66 plants of a backcross population of KTCA5 × (KTCA5 × KTCR15). Out of which, 39 plants were fertile plants and 27 plants were sterile. The chi-square test showed that the segregation of the backcross mapping population does not deviate from the expected ratio of 1:1 (Table 2). These observations validated the monogenic dominant inheritance of the fertility restorer gene trait observed in the F_2 and backcross population. The segregation ratio for fertility and sterility was consistent with the expected ratio of 3:1 and 1:1 in both F2 and backcross populations. Similar observations were seen in the KTCA10 (hot pepper) and KTCR15 (sweet pepper) populations.

A total of 139 F_2 plants of hot pepper cross KTCA10 × KTCR15 along with the parental lines were grown in the Kharif 2016, ICAR-IARI, Regional Station, Katrain. It was observed that out of 139 plants, based on pollen fertility analysis, 107 plants were fertile while 32 plants were sterile (Table 3). When the observed ratio was tested by chi-square test, the x^2 value of 0.29 was less than the tabulated value of 3.84 at a 5 percent level of significance and 1 degree of freedom. The observed c^2 value though quite low and acceptable at higher stringencies of P value 0.59. Thus, the chi-square test showed that segregation of the F_2 mapping population does not deviate from the expected ratio of 3:1. In support of the present results [18, 19, 11, 13, and 20] had also reported single dominant inheritance pattern of fertility restorer gene in pepper.

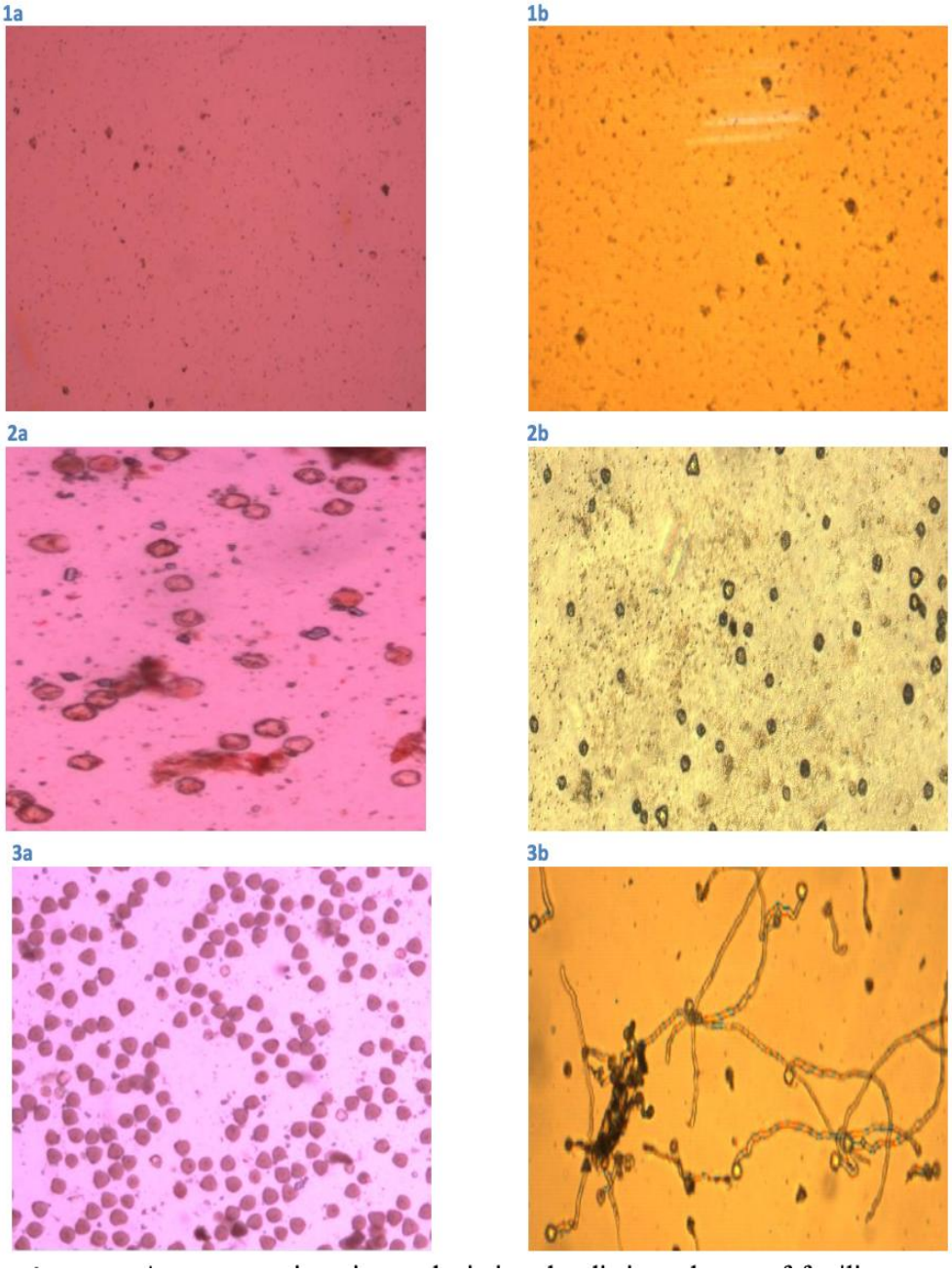

Fig. 1. Picture depicting the distinct classes of fertility observed namely (1a & 1b)-sterile with no pollen, (2a & 2b) - sterile pollen with no stain and germination, (3a & 3b)-fertile pollen with stain and germination in F_2 and backcross populations

Table 2. Segregation ratio for fertility and sterility in F_2 and backcross plants of KTCA5 × KTCR15

Population	Total no. of plants	Plants with pollen fertility		Genetic ratio	c2value	P-value
		Fertile	Sterile			
KTCA5 × KTCR15 F_2	123	90	33	3:1	0.322	0.57
KTCA5×(KTCA5× KTCR15) Backcross	66	39	27	1:1	1.28	0.25

Table 3. Segregation ratio for fertility and sterility in F_2 and backcross plants of KTCA10 × KTCR15

Population	Total no. of plants	Plants with pollen fertility		Genetic ratio	c2value	P-value
		Fertile	Sterile			
KTCA10 × KTCR15 (F_2)	139	107	32	3:1	0.290	0.59
KTCA10×(KTCA10× KTCR15) (Backcross)	72	41	31	1:1	1.70	0.19

In the 72 backcross plants 41 plants were fertile and 31 were sterile. The observed chi-square value of 1.70 was less than the tabulated value at a 5 percent level of significance at 1 degree of freedom (Table 3). The chi-square test indicates that the segregation of the backcross population does not deviate from the expected ratio of 1:1. The frequency distribution of the backcross population with respect to pollen fertility showed a monogenic inheritance fashion for fertility restorer gene trait. The single dominant gene inheritance of the fertility restorer gene has also been confirmed in the backcross population has also been reported by [20] and [21]. The above results indicated a monogenic dominant inheritance pattern for the male fertility restoration gene of restorer line (KTCR15) despite the CMS background i.e.; both in sweet pepper (KTCA5) and hot (KTCA10). It was further observed that there was no difference in the genetics of the fertility restoration in sweet pepper (KTCA5) and hot pepper (KTCA10) background; this may be due to the cytoplasm effect that could be Peterson's cytoplasm present in both the plant's background. These two hybrids and their backcrosses were screened in Katrain, Himachal Pradesh, and IARI, New Delhi. Pollen viability, pollen germination, and the number of seeds per fruit were all measured in each environment. We discovered significant variation in pollen fertility locations between both hybrids. We found that hybrid fertility restoration was affected not only by the genetic makeup of the plant, but also by the environmental conditions under which the plants were produced.

According to the data distribution in this cross, the fertility restorer gene trait in the KTCR15 (sweet pepper) line was segregated monogenetically in a KTCA10 (hot pepper) background. The previously observed single dominant gene inheritance pattern is validated again in the KTCA10 KTCR15 F2 population. The marker segregation data of both SSR and SCAR markers, also strongly supported phenotypic results (no data presented). Earlier research on the inheritance of the restoration gene in pepper by [22, 19] using molecular marker segregation endorses the single dominant gene inheritance. In contrast to single dominant gene inheritance, many other studies had reported two complementary genes [23] both major and minor genes [16] and 2 major and 7 minor QTLs [24]. However, previous reports indicated that pepper fertility restoration is likely controlled in a complex manner [11, 13, and 25]. The disparity in segregation ratios observed in different studies could be attributed to the influence of a female parent or, more likely, variable expression of the weaker genes in different genetic backgrounds. Certain modifier genes may also be to blame for the shift in segregation ratio [26, 27]. In most cases, however, the use of different restorer (R) lines with different genetic backgrounds will result in varying fertility restoration ability, and the effect of the environment cannot be ignored.

The results revealed that there is no difference in the inheritance of the fertility restorer gene in sweet pepper (KTCA5) and hot pepper (KTCA10) background. It may be due to the presence of the same Peterson type of cytoplasm in both the plant genetic background as it is the only and most widely utilized cytoplasm source for commercial F_1 hybrid seed production in pepper [22]. Although, their differential expression depends on the genetic background to some extent, but in the present investigation, it was found that both KTCA5 and KTCA10 did not affect restorer gene inheritance. These results are in agreement with the findings of [28], who proclaimed that the same restorer can behave Identifying different CMS sources for a common restorer in the same crop may be useful in practical breeding as the adaptive capacity of the same type of CMS and restorer lines in one crop is limited. Hence, it is necessary to select and breed several types of CMS lines and their related restorers for improving the resistance and stability of yield of F_1 hybrids [29]. Despite all of these contradictory claims, extensive research studies on the inheritance of

the restorer gene from various sources have shown that they all have a common monogenic major determinant of fertility restoration [6] which supports our findings.

4. CONCLUSION

The experiment entitled Genetics analysis of *Rf* gene in chilli peppers (*Capsicum annuum* L.)is conducted to observe the inheritance pattern of fertility restoration gene in sweet pepper and hot pepper cytoplasmic male sterile background. In this study, we observed single dominant gene inheritance of the fertility restoration gene. The fertility restoration trait segregated in 3:1 and 1:1 F_2 segregation populations and backcrossed BC_1 populations respectively in both Sweet Pepper and Hot pepper backgrounds. The results revealed that there is no difference in the inheritance of the fertility restorer gene in sweet pepper (KTCA5) and hot pepper (KTCA10) background. Single gene control of the trait also facilitates its transfer from one genotype to another. In the way forward, gene mapping of the *Rf* gene should be done to identify the markers linked to the fertility restoration trait which will facilitate the maker assisted selection for stable restorer line in pepper hybrid breeding programs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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