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Maintenance and fertility restoration of CMS system in sweet pepper (*Capsicum annuum*)

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ABSTRACT

CMS based breeding is essentially a 3-line hybridization method which requires stable CMS line, maintainer line and restorer of fertility line. To identify maintainer (B) and restorer of fertility (R) lines for five stable CMS lines in capsicum, the present experiment was conducted in line × tester mating design at ICAR-IARI, Regional Station, Katrain (HP), India during summer season of 2015 and 2016. The results revealed that among 125 F_1s, pollen fertility percent, pollen germination percent, self fruit setting rate and seed number per fruit in C1 × P1, C2 × P2, C2 × P12, C3 ×P3, C3 × P12, C4 × P4, C4 × P12 and C5 × P5 were recorded nil. It revealed that testers in these crosses could be maintainer line (N *rfrf*) for maintaining sterility in their maternal parent in the cross, whereas in C1 × P6, P15, P10, P9, P14, P8, P7; C2 × P8, P14, P15, P9, P10, P13, P7; C3 × P7, P9, P10; C4 × P23, P24, P7, P21, P6, P9, P10 and C5 × P21, P9, P10 crosses pollen fertility percent were recorded in range of 80.3 to 91.3 and pollen germination per cent were in range of 80.2 to 93.8 and these results indicated that nucleus of pollen parent in these crosses must have dominant *Rf* allele in homozygous/ heterozygous state and could be exploited as R- line (S/N *RfRf* / *Rfrf*) in specific CMS genotype. Our findings would be useful for developing new CMS and R-lines in sweet pepper (*Capsicum annuum* L. var. *grossum*).

Key words: Capsicum, CMS, Maintainer line, Restorer of fertility line, Pollen germination

Sweet pepper (*Capsicum annuum* L. var. *grossum*) is an important commercial crop cultivated for its non-pungent fruits. It is rich in vitamin C and B-complex, minerals, essential oils, carotenoids and capsaicin. Now a day's, farmers thrust for hybrid seeds in sweet pepper cultivation has magnified manifold owing to their excellence in quality, yield and biotic stress management. It is forecasted hybrid seeds in bell pepper cultivation will cover more than 80% of the total cropped area (Swamy *et al.* 2017). At present, commercial hybrid seed production is carried out by hand emasculation and pollination resulting in increased hybrid seed cost rendering it inaccessible to growers at affordable price level. In view of this constraint, utilization of male sterility (*ms*) trait in hybrid seed production offers immense scope. Cytoplasmic male sterility (CMS) in pepper first was identified by Peterson (1958) in accession USDA P.I. 164835 introduced from India. Shifriss (1997) in his experimental studies identified that expression of cytoplasmic male sterility (CMS) in pepper due to interaction of a single recessive nuclear gene, designated as *rf1*, with sterile (S) cytoplasm; and the restorer dominant allele *Rf1* controls the fertility restoration. CMS is now commercially exploited in pepper hybrid seed development technologies in countries like China, South Korea and India (Kim and Kim 2005).

Exploitation of CMS in pepper hybridization is possible only after identifying suitable B-line (maintainer) and R-line (restorer of fertility) for stable CMS line (A-line). Identified B-line would be used for seed multiplication of A-line and also for conversion into new CMS line after repeated backcrossing and R-line is subsequently used as male parent in hybrid development programme. For producing desired *Capsicum* hybrid breeders need wide range of CMS lines. Therefore, it is necessary to transfer available CMS system into elite breeding lines to develop broad genetic base (Kumar *et al.* 2009). Hence, the present investigation was undertaken to identify maintainer line and restorer of fertility line from 25 diverse pepper genotypes against five CMS lines procured from World Vegetable Centre (WVC), Taiwan.

MATERIALS AND METHODS

Planting material and crossing test: The present

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experimental trial was conducted to identify B-line (N *rfrf*) and R-line (N/S *RfRf / Rfrf*) for five stable CMS lines (S *rfrf*) procured from World Vegetable Centre (WVC), Taiwan at ICAR-IARI, Regional Station, Katrain (HP), India. Twenty five lines (Table 1) were used as tester with five identified CMS lines in Line × Tester mating design to generate 125 F_1 cross combinations during summer (April-September) season 2015. For identification of B and R lines all the generated F_1 hybrids and the respective parents were grown under protected conditions in next cropping season (April-Septmeber 2016). Standard package of practices were followed for cultivation of the crop. The following criteria were adopted for identification of B and R line.

Estimation of pollen fertility: At the time of anthesis during full bloom stage 5 flowers were randomly selected to evaluate fertility of F_1 hybrid according to the method of Gulyas *et al.* (2006). Anthers were separated from flowers with forceps and placed on glass slide containing 2% potassium iodide (KI) stain. The anthers were gently crushed by using needle to release the pollen grains. By using fluorescent microscope; the round, well stained pollen grains were considered to be fertile, while the shrunken, deformed, amorphous and poorly stained pollen grains considered as sterile. Pollen fertility percentage was estimated using below formula (Prasad *et al.* 2017).

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile pollen grains}}{\text{Total no. of pollen grains}}$$

Virmani *et al.* (1997) classification of pollen parents based on pollen fertility percentage into maintainer (0-1%), partial maintainer (>1-50%), partial restorer (>50-80%) and restorer of fertility (>80%).

Estimation of pollen germination: Pollen germination studies were carried out according to the methodology reported by Ma *et al.* (2013). Five flowers were randomly collected from each F_1 hybrid progeny and anthers were separated from flower and dried under shade. For germination studies pollen grains were isolated with help of forceps and needle and placed on an *in vitro* culture medium (0.01% boric acid + 10% sucrose + 1% agar) at 25°C in the dark under aseptic conditions. The percentage of pollen germination was measured at 24, and 48 h. The pollen germination rate was calculated in accordance with the following formula.

Germination rate (%) = number of germinated pollen grains / total no. of pollen grains × 100

Self fruit setting rate: For assessing self fruit setting rate five flowers were randomly marked on each plant and covered with pollination bag to avoid cross pollination. Mean value is calculated by observing self pollinated fruits in field. Similar method was proposed by Ma *et al.* (2013).

Seed number analysis: Five fruits were randomly selected and their seed numbers per fruit were calculated separately and mean value is used for interpretation of results (Ma *et al.* 2013).

RESULTS AND DISCUSSION

In CMS breeding test cross which resulted into 100% sterile progeny, tester was considered as B (N *rfrf*) line. Whereas, test cross which resulted into more than 80% fertile progeny, tester was considered as R (N/S *RfRf/ Rfrf*) line.

Identification of B-line against established CMS lines: To identify B-line for CMS lines, all 125 F_1s resulting from the crosses were evaluated during April-September 2016. Pollen fertility and pollen germination percentage in each F_1 progeny was studied in laboratory to detect maintainers and partial maintainers. Among 125 F_1s pollen fertility and pollen germination percent in C1 × P1, C2 × P2, C2 × P12, C3 × P3, C3 × P12, C4 × P4, C4 × P12 and C5 × P5 were recorded as nil (Table 2); it reveals that pollen parent in these crosses would be the maintainers (N *rfrf*) for maintaining sterility in their respective female parent used in test cross. Whereas, in F_1 progenies of C1 × P19, C1 × P11, C2 × P11, C2 × P17, C2 × P19, C3 × P19, C4 × P14, C4 × P19 and C4 × P25 crosses pollen fertility was observed less than 1% and pollen germination was practically nil (Table 2); it emphasizes testers in these F_1s are also maintainers (N *rfrf*) though it expressed insignificant pollen fertility (<1%) which may be result of temperature sensitivity of nuclear alleles or mechanical/biological pollen invasion from fertile lines (Dhaliwal and Jindal 2014).

In the remaining test crosses pollen fertility percent recorded between 5.9 to 39.1 and pollen germination was between 6.0 to 47.8 (Table 2) and these results highlighted that pollen parent in the crosses acted as partial maintainers (<50% pollen fertility) and were not appropriate for maintaining sterility trait.

In F_1 progenies where B-line identified through pollen

Table 1 CMS lines (C1–C5) and testers (P1–P25) used in hybridization

Lines	Code	Lines	Code	Lines	Code	Lines	Code
AVPP-9907-S	C1	TC-O6677	P4	PRSM-1	P12	KTC-149	P20
AVPP-9820-S	C2	TC-6308	P5	CW	P13	KTC-152	P21
AVPP-9908-S	C3	AVPP-0504	P6	KTC-131	P14	KTC-157	P22
AVPP-0516-S	C4	AVOO0515	P7	KTP-132	P15	KTC-159	P23
AVPP-1710-S	C5	AVPP-9808	P8	KTC-133	P16	KTC-160	P24
AVPP9907	P1	AVPP-9822	P9	KTP-143	P17	KTC-161	P25
AVPP-9820	P2	AVPP-9904	P10	KTC-145	P18		
AVPP9908	P3	KTPL-19	P11	KTC-148	P19		

Table 2 Identified B and R line through fertility analysis of F1 crosses

Cross combination	Pollen fertility % (mean±SE)	Pollen germination % (mean±SE)	Self fruit setting rate (mean±SE)	Seed number (mean±SE)
C1 × P1	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C1 × P6	81.0±0.23	84.3±0.69	79.1±0.57	110.0±5.70
C1 × P7	89.9±0.34	81.6±0.40	82.8±1.10	121.3±6.11
C1 × P8	87.3±0.17	85.0±1.10	84.6±0.46	112.6±5.77
C1× P9	85.6±0.63	80.2±0.34	81.4±0.86	103.8±0.80
C1 × P10	85.3±0.46	91.1±0.57	78.6±0.34	124.4±5.77
C1 × P11	0.72±0.04	0.0±0.00	1.00±0.11	8.0±0.92
C1 × P14	86.5±0.51	81.4±0.34	78.6±0.28	104.2±2.30
C1 × P15	82.5±0.23	90.4±0.46	87.6±0.34	111.6±5.77
C1 × P19	0.45±0.05	0.00±0.00	0.00± 0.00	0.00±0.00
C2 ×P2	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C2 ×P7	10.6±0.29	90.2±0.17	64.1±0.58	118.2±5.77
C2 ×P8	82.5±1.15	85.0±0.64	71.4±0.64	98.6±2.31
C2 ×P9	84.5±0.64	86.3±1.15	69.8±0.69	106.0±2.89
C2×P10	87.3±0.58	84.1 ±1.15	77.6±0.29	118.0±3.46
C2 × P11	0.26±0.02	0.0±0.00	0.0±0.00	0.0±0.00
C2× P12	0.0±0.00	0.0 ±0.00	0.0±0.00	0.0±0.00
C2 × P13	87.3±1.15	90.8±1.39	81.0±1.15	112.2±5.77
C2 × P14	83.5±0.52	80.2±0.58	82.3±1.15	116.0±2.89
C2 × P15	84.1±0.58	88.0±1.15	84.8±1.15	98.6±1.15
C2 × P17	0.35±0.05	0.00±0.00	0.0±0.00	0.0±0.00
C2 × P19	0.38±0.02	0.00±0.00	0.00±0.00	0.00±0.00
C3 ×P3	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C3 × P7	82.5±2.31	84.6±1.15	81.3±0.58	96.4±1.73
C3 × P9	84.5±2.31	86.2±1.73	75.3±2.31	89.5±0.58
C3× P10	86.5±2.02	90.4±1.15	81.0±2.31	94.6±2.31
C3 × P12	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C3 × P19	0.37±0.03	0.0±0.00	0.0±0.00	0.0±0.00
C4 × P4	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C4× P6	82.5±2.02	84.7±1.73	76.4±0.35	87.4±2.89
C4 × P7	81.5±1.73	87.8±1.73	71.0±2.31	73.1±0.06
C4 × P9	83.5±1.15	86.2±1.73	75.2±0.69	113.5±0.87
C4× P10	85.3±0.06	81.0±2.31	56.4±0.23	91.1±0.64
C4× P12	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
C4× P14	0.45±0.03	0.0±0.00	0.0±0.00	0.0±0.00
C4× P19	0.65±0.01	0.0±0.00	0.0±0.00	0.0±0.00
C4× P21	81.5±1.73	80.6±1.73	70.3±1.73	81.0±0.58
C4× P23	80.3±2.31	88.4±0.58	69.4±0.35	116.2±5.77
C4× P24	81.3±0.58	82.7±1.15	70.2±1.15	121.6±1.15
C4× P25	0.35±0.06	0.0±0.00	0.0±0.00	0.0±0.00
C5 × P5	0.0±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C5 × P9	87.3±3.46	90.4±0.58	76.4±0.23	85.8±0.12
C5× P10	91.3±0.58	93.8±0.46	79.1±0.06	93.2±0.46
C5× P21	86.7±1.73	90.4±4.04	78.8±4.62	102.4±5.77

fertility and pollen germination studies recorded zero self fruit setting rate and seed number per fruit, these results are in correlation with pollen fertility and pollen germination studies. Whereas partial maintainer line has fertile pollen, active pollen germination, presence of self fruit setting and viable seeds renders it unsuitable as maintainers.

Our experiment on identification of B-line for maintaining CMS parent revealed that maintaining ability of B-line varies in different CMS lines containing different sources of cytoplasm, i.e. CMS lines has specific maintainers and same maintainer line reported different maintaining ability among five CMS lines under investigation. For example P1, P2, P3, P4 and P5 are able to maintain sterility loci only in C1, C2, C3, C4 and C5 respectively and in rest of the crosses these testers recorded as partial maintainer. These findings revealed that a CMS line has specific maintainers and same maintainer line has different maintaining ability due to diverse source of cytoplasm and nucleus. These results were similar to the findings reported by Ma *et al.* (2013). Partial maintaining ability recorded by more number of testers in the crosses might be due to genetic factors like heterozygous nature of nuclear allele in testers, modifier genes and/or environmental factors, i.e. CMS in *Capsicum* is more sensitive to low temperature where fertility restoration is reported in sterile parents. Similar results are reported by Kumar *et al.* (2007).

Through our experimental results we inferred that due to CMS specificity to maintainers, breeders first find the suitable B-lines for maintaining sterility or seed multiplication in CMS lines prior to its exploitation in hybrid seed production. Our findings provide foundation for further exploitation of CMS in breeding programme.

Detection of R-lines for identified CMS line: Based on the result of fertility restoration in 125 CMS based F_1s, pollen parent were identified for their restoration of fertility (>80% pollen fertility) (R-line) and for partial restoration (51-80% pollen fertility). In crosses of C1 × P6, P15, P10, P9,P14, P8, P7; C2 × P8, P14, P15, P9, P10, P13, P7; C3 × P7, P9, P10; C4 × P23, P24, P7, P21, P6, P9, P10 and C5 × P21, P9, P10 testers were confirmed as restorer of fertility line (>80% pollen fertility) based on pollen fertility and pollen germination studies. In these crosses pollen fertility percent were recorded in range of 80.3 (C4 × P23) to 91.3 (C5 × P10) and pollen germination percent were observed in range of 80.2 (C1 × P9) to 93.8 (C5 × P10) (Table 2). Field observation of these crosses had shown >50% self fruit setting and more than 70 seed numbers per fruit which was in accordance with laboratory observations of pollen fertility and pollen germination. These findings revealed the potential restoration of fertility capability of testers in different CMS lines. In other test crosses pollen fertility was recorded in range of 56.8 % to 76.2%, whereas pollen germination was observed in range of 51.3% to 71.4% and self fruit setting rate was recorded between 21 to 50 % and also the seed number per fruit was in accordance with pollen fertility and pollen germination. It was confirmed that testers in these crosses acted as partial restorers and not useful in CMS hybrid breeding as R-line.

Our results indicated that pollen parents in C1 × P6, P15, P10, P9,P14, P8, P7; C2 × P8, P14, P15, P9, P10, P13, P7; C3 × P7, P9, P10; C4 × P23, P24, P7, P21, P6, P9, P10 and C5 × P21, P9, P10 test crosses must have *Rf* gene (nucleolus) in homozygous/heterozygous state for restoring fertility in F_1 progenies and can be exploited as an effective R- line (S/N *RfRf* / *Rfrf*) in their specific CMS parents after testing their combining ability. These results are in correlation with findings of Kumar *et al.* (2007). It was also observed that same tester performing as restorer of fertility line (R-line) and as partial restorer in different CMS background, i.e. different restorers had different restoring ability to the same CMS line. For instance P15 were recorded as R-line for C1 and C2 and in test crosses with C3, C4 and C5 it recorded as partial restorer. P10 pollen parent observed as restorer of fertility (R-line) for all five CMS lines under investigation. It was inferred that variations in *Rf* gene led to these differences. Similarly, Prasad *et al.* (2017) in rice reported that different cytoplasm leading to the differences of restoring ability among the CMS lines with the same nucleolus. Ma *et al.* (2013) in pepper reported the difference in restoring ability of testers due to diversity in nucleolus (*Rf* gene) and cytoplasm sources might lead to different restoring abilities.

In the experiment of searching maintainers and restorers for identified CMS lines we found that CMS lines under study had specific maintainers and restorers, moreover same maintainer and restorer line given different maintaining and fertility restoring abilities in five CMS lines under study. Breeders must select both maintainer and restorer lines for extreme phenotype to guarantee a completely sterile female parent, but a fully fertile hybrid (Wang *et al.* 2004).

Sweet pepper hybrid seeds production using CMS could lower cost by reducing time and labour, and increase the genetic purity of the F_1 seeds. Stable CMS (A-line), maintainer (B-line) and restorer of fertility (R-line) lines are essential for exploitation of the CMS system in hybrid seed production. In the process of searching for suitable B-line and R-line we found that it is difficult to get testers which performed as maintainer for all the five CMS lines under study. Though we got P10 tester as R-line for all the five CMS lines under investigation its exploitation in CMS based breeding depends on combining ability and its stability to different temperature conditions during growth period. Our findings would be useful for developing new CMS lines from identified maintainers through repeated backcross breeding. whereas, testers which showed high fertility (R-line) with CMS lines could be utilized in the heterosis breeding after testing their combining ability and heterosis with different CMS lines.

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