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Research Paper

Development of cytoplasmic male sterile lines in chilli (*Capsicum annuum* L.) and their evaluation across multiple environments

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A breeding program was initiated in 2009 to develop temperature stable CMS lines in chilli. 'CCA 4261' was used as a CMS donor. From the 11 testcross progeny screened, maintainer plants were identified from 'SL 461', 'SL 462' and 'SD 463'. After 6 backcrosses to the maintainer plants, 17 CMS lines in diverse genetic backgrounds were established. The CMS lines were evaluated for stability of sterility over four environments during 2014–15 and 2015–16. The environments E_1 and E_3 represented the low temperature regime, and E_2 and E_4 the high temperature regime. The mean square values due to the genotypes and the environments were significant at $p = 0.01$ for pollen sterility (%), pollen release score, fruit setting (%) and number of seed fruit^{-1}. The G × E interaction effects were significant for pollen sterility (%), fruit setting and number of seed fruit^{-1} and non-significant for pollen release score. Ten lines namely 'CMS4611A', 'CMS4614A', 'CMS4622A', 'CMS4624A', 'CMS4626A', 'CMS46213A', 'CMS463D2A', 'CMS463D13A', 'CMS463D14A' and 'CMS463L5A' were completely male sterile across the environments. Under open pollination conditions, the fruit and the seed setting ability of these lines was normal. The CMS transferred into the diverse genetic backgrounds would broaden the CMS germplasm resources in chilli.

Key Words: *Capsicum annuum*, cytoplasmic male sterility, G × E interaction, maternal breeding.

Introduction

Chilli or hot pepper, an important spice and vegetable crop, belongs to the genus *Capsicum*. *Capsicum annuum* is the most widely cultivated throughout the world, both in the number of cultivars grown as well as the area occupied (Bosland 1992, Wang and Bosland 2006). Global production of dry chilli in 2016 reached 3.91 million tons from 1.79 million hectare area. The productivity has increased by about 51.39% from 1.44 ton ha^{-1} in 2000 to 2.18 ton in 2016 (FAO 2016). Cultivation of high yielding hybrid cultivars in place of the traditional open pollinated cultivars is the primary reason for increased productivity over the years (Singh *et al.* 2014).

Commercial hybrid seed in chilli are produced either by hand-emasculation or by exploiting the male sterility (Berke 2000). Both the nuclear or genic male sterility (GMS) and the cytoplasmic male sterility (CMS) have been reported and utilized for hybrid development. Limitation of the GMS is that the progeny segregates into male sterile and male fertile plants, and the 50% male fertile plants have to be identified and removed from the seed production block. This is tedious and time consuming, and the seed is prone to genetic impurities resulting from improper identification and self-pollination (Dhaliwal and Jindal 2014).

The CMS in *Capsicum* was first reported by Peterson (1958) in 'PI 164835', an introduction from India. Various S-type cytoplasms reported in *Capsicum* spp. might be identical (Shifriss and Frankel 1971, Yu 1990). In CMS, the sterility is determined by interaction between the S-cytoplasm and the recessive nuclear *restorer-of-fertility* (*rf*) gene. The dominant (*Rf*) gene restores the fertility by suppressing the CMS-associated genes (Schnable and Wise 1998). The CMS or A-line (S*rfrf*) is maintained by crossing it with the maintainer or B-line (N*rfrf*), and the progeny are 100% male sterile. This reduces hybrid seed cost and ensures purity of the F_1 seed (Yang *et al.* 2008). However, the CMS in chilli could be influenced by the environmental fluctuations and the genetic background. When temperature drops below 25°C/17°C day/night, fertility of the pollen is, albeit in part, restored (Kim *et al.* 2013, Novak *et al.* 1971, Shifriss and Guri 1979). Expression of CMS could also be altered by the presence of some modifier genes (Yu 1985). A more recent

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report suggested a third haplotype, *Rfls*^{7701} that might be related to the instability of the CMS (Min *et al.* 2008). However, the molecular data revealed that unstable CMS was induced by a gene residing at another locus rather than by the *Rfls*^{7701} haplotype-linked allele (Min *et al.* 2009).

The commercial hybrid seed production demands that the CMS is highly stable to ensure genetic purity of F_1 seed. Therefore, the available CMS lines should be constantly improved and new CMS lines should be developed to meet the emerging requirements. The stable CMS lines could be developed by selecting the maintainer plants that are tolerant to temperature fluctuations, by eliminating the modifier gene(s) through maintainer plant selection and by screening the progeny under low temperature conditions for maximum expression of the trait (Gniffke *et al.* 2009). This breeding program was initiated to develop temperature stable CMS lines in chilli suitable for hybrid development.

Materials and Methods

Identification of maintainer plants and conversion of maintainer plants to CMS A-lines

The CMS source 'CCA 4261' was introduced from the World Vegetable Center, Taiwan. 'CCA 4261' is sensitive to temperature and the fertility is partially restored when exposed to low temperature (Gniffke *et al.* 2009). To develop temperature stable CMS lines, testcrosses were attempted in 2009 between 'CCA 4261' and 11 elite chilli breeding lines. Salient features of the CMS donor and the recipient parents are listed in **Table 1**. The tester plants from within the genotype were tagged individually and maintained by self-pollination. F_1 seed generated by each tester plant were harvested separately. The single plant hybrid progeny comprising of about 60 plants each were grown in 2010 and screened for male sterility on individual plant basis. The tester plants which produced at least 70% male sterile progeny were considered as possessing the maintainer (N*rfrf*) genotype (**Table 2**). From each male sterile progeny, 20 F_1 plants were backcrossed to their respective maintainer plants. The single plant progeny (designated B-line suffixed with the progeny number) were carried further to establish CMS A-lines. Sixty single plant progeny generated from three crosses constituted the three testcross groups. The identified maintainer plants were maintained by self-pollination to develop homozygous B-lines. The developed CMS B-lines had undergone a minimum of ten cycles of self-pollination (S_{10}) and single plant selection within the progeny rows.

Experimental conditions and layout of the design

The investigations were conducted at the Vegetable Research Farm, Punjab Agricultural University, Ludhiana (India). The farm is located at 30°54′ N, 70°45′ E, and 247 m above sea level. The CMS lines were evaluated over four environments during 2014–15 (E_1 and E_2) and 2015–16 (E_3 and E_4). Environments E_1 and E_3 (sown on 15th September and transplanted on 30th October) represented the low temperature environments and E_2 and E_4 (sown on 1st February and transplanted on 15th March) represented the high temperature environments. The experiment was laid out in a randomized complete block design (RCBD) with seven replications. Each replication accommodated five plants. The plants were protected from insect-mediated cross pollination with 24-mesh net cages. The maximum and the minimum weekly temperature during the period of screening for pollen viability are given in **Fig. 1**.

Evaluation of CMS A-lines for male sterility under caged conditions

CMS A-lines were evaluated for male sterility traits at weekly intervals for 7 weeks, beginning 3rd week of March in E_1 and E_3, and 1st week of June in E_2 and E_4. Observations were recorded on individual plant basis on pollen viability (%), pollen release score (0–2), fruit setting (%) and number of seed fruit^{-1}. For pollen viability, five well-developed unopened flower buds plant^{-1} from different positions were collected in a vial containing 70% ethanol. Anthers crushed on a glass slide and stained with a drop of 2% I_2-KI (prepared by dissolving 2 g of iodine and 4 g of potassium iodide in 100 ml distilled water) were examined under 10× magnification. The round, well-filled and dark stained pollen grains were considered as fertile while the unstained, half stained, shriveled, deformed and empty pollen grains were scored as sterile. Based on the scale of Virmani *et al.* (1997), lines were classified as completely sterile (100% pollen sterility), sterile (90–99% pollen sterility), partially sterile (71–90% pollen sterility), partially fertile (31–70% pollen sterility), fertile (21–30% pollen sterility) and fully fertile (0–20% pollen sterility).

For pollen release score, 10 freshly opened flowers plant^{-1} were observed visually between 9–11 AM at weekly intervals, and scored as per the scale of Liu and Gniffke (2004) where, 0 = no pollen released, 1 = some pollen released but adhering to anthers, and 2 = pollen released freely. For fruit setting (%), 50 well-developed unopened flower buds plant^{-1} were tagged and observed for flower retention. Fruit was considered set if flower bud did not abort 7-days post anthesis. In lines where fruit setting occurred, 20 fruits plant^{-1} were harvested manually, seed extracted, total number of seed counted and number of seed fruit^{-1} worked out.

Evaluation of CMS A-lines for fruit traits under open pollination conditions

The newly developed 17 CMS A-lines were evaluated for their ability to set fruit and seed, and for important fruit traits in open field conditions. Seed were sown in finely prepared nursery beds measuring 7.0 m × 1.0 m × 0.15 m in length, width and height. Sowing and transplanting dates were the same as described above. The experiment was arranged in a RCBD with two replications over four environments during 2014–15 (E_1 and E_2) and 2015–16 (E_3 and E_4). Each CMS line was represented by two rows of five plants each. Row × plant spacing was maintained at 90 cm ×

Table 1. Salient features of the CMS donor 'CCA 4261' and 11 elite chilli pepper breeding lines involved in testcrosses for breeding of new CMS lines

Parental line	Alternate ID	Fertility status	Fruit color	Fruit length	Fruit width	Disease reaction	Species	Source
CC 141	CCA 4261	Cytoplasmic male sterile (S*rfrf*)	Dark green	Long	Medium thick	Susceptible to cucumber mosaic virus, potato virus Y, bacterial wilt and *Phytophthora* blight, resistant to leaf curl virus, moderately resistant to anthracnose (Gniffke *et al*. 2009, Singh 2011)	*Capsicum annuum* L.	WVC[a]
MS 341	MS 12	Genetic male sterile (*ms10ms10*)	Light green	Medium long	Very thick	Resistant to leaf curl virus and anthracnose, moderately susceptible to root-knot nematode, moderately resistant to pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	PAU[b]
SL 461	Sel 11	Male fertile	Light green	Medium long	Thin	Resistant to leaf curl virus, moderately resistant to anthracnose, susceptible to root-knot nematode, and pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	PAU
SL 462	S 217621	Male fertile	Dark green	Long	Thin	Resistant to leaf curl virus, moderately resistant to anthracnose, susceptible to root-knot nematode, moderately susceptible to pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	PAU
DL 161	DCL 524	Male fertile	Light green	Medium long	Thin	Resistant to leaf curl virus, moderately resistant to anthracnose, moderately susceptible to root-knot nematode and pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	IARI[c]
EL 181	ELS 82	Male fertile	Dark green	Long	Thick	Susceptible to leaf curl virus, and resistant to anthracnose (Singh 2011)	*Capsicum annuum* L.	PAU
US 501	US Agri Thick	Male fertile	Light green	Medium long	Very thick	Resistant to leaf curl virus, moderately resistant to anthracnose, susceptible to root-knot nematode, moderately susceptible to pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	Pepsi[d]
PA 401	PAU Selection Long	Male fertile	Green	Medium long	Thin	Moderately susceptible to leaf curl virus, moderately resistant to anthracnose (Singh 2011)	*Capsicum annuum* L.	PAU
SD 463	Selection Dev	Male fertile	Dark green	Very long	Very thick	Resistant to leaf curl virus, susceptible to anthracnose, moderately susceptible to root-knot nematode, and pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	PAU
PP 402	Pepsi 17-2	Male fertile	Dark green	Medium long	Very thick	Resistant to leaf curl virus, susceptible to anthracnose, root-knot nematode, and pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	Pepsi
PS 403	Punjab Surkh	Male fertile	Green	Long	Thick	Resistant to leaf curl virus, moderately resistant to anthracnose, susceptible to root-knot nematode, moderately susceptible to pepper mottle virus (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	PAU
VR 521	VR 16	Male fertile	Dark green	Medium long	Medium thick	Resistant to leaf curl virus and pepper mottle virus, moderately resistant to anthracnose, moderately susceptible to root-knot nematode (Kaur 2015, Singh 2011)	*Capsicum annuum* L.	USDA[e]

[a] WVC = World Vegetable Center, Taiwan; [b] PAU = Punjab Agricultural University, Ludhiana, India; [c] IARI = Indian Agricultural Research Institute, New Delhi, India; [d] Pepsi = Pepsi Foods Pvt. Ltd., USA; [e] USDA = United States Department of Agriculture.

Table 2. Sterile and fertile phenotypes in F_1 generation of 11 testcrosses during 2010

Testcrosses	Number of individuals/plants			
	Total	Sterile	Fertile	% Male sterile plants
CC 141 × MS 341	60	7	53	11.67
CC 141 × SL 461	60	43	17	71.67
CC 141 × SL 462	60	47	13	78.33
CC 141 × DL 161	60	32	28	53.33
CC 141 × EL 181	60	25	35	41.67
CC 141 × US 501	60	0	60	0.00
CC 141 × PA 401	60	0	60	0.00
CC 141 × SD 463	60	44	16	73.33
CC 141 × PP 402	60	0	60	0.00
CC 141 × PS 403	60	0	60	0.00
CC 141 × VR 521	60	4	56	6.67

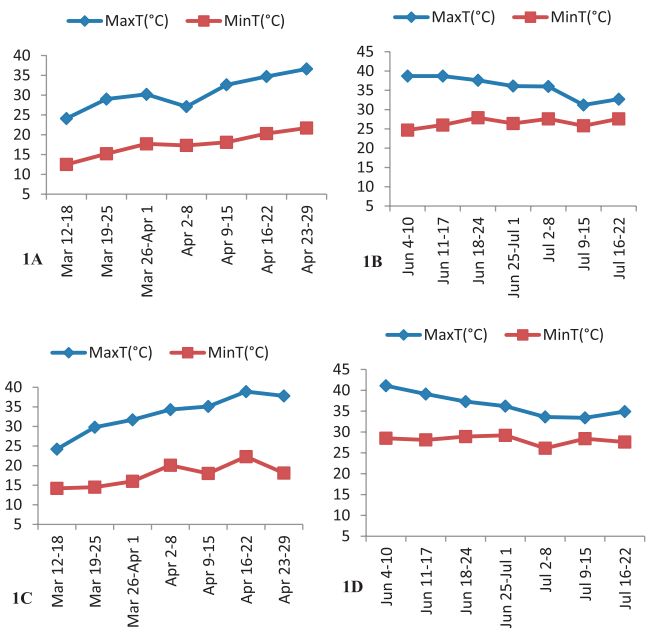

Fig. 1. Maximum and minimum weekly temperature recorded during period of pollen viability study; A. during low temperature of 2014–15 (E_1); B. during high temperature of 2014–15 (E_2); C. during low temperature of 2015–16 (E_3); and D. during high temperature of 2015–16 (E_4).

45 cm. Three rows of a restorer line 'SL 475' was planted as a pollen source on either side of the replication block. Row × plant spacing in 'SL 475' was maintained at 75 cm × 45 cm. Row direction of the CMS lines was from East to West and of the restorer line was from North to South. The scheme of planting the experiment is illustrated in **Supplemental Fig. 1**. The CMS A-lines were allowed to cross pollinate with the restorer line by natural means such as insect pollinators.

Plants of the restorer line 'SL 475' are dark green, compact and 64.5 cm tall. Fruits are 7.38 cm long, 1.37 mm thick, moderately pungent with capsaicin content of 0.72%, dark green when immature and deep red when mature. Average fruit weight is 5.1 g with 58.9 seed fruit^{-1}. The line is resistant to leaf curl virus disease and the total yield of red ripe fruit is 700 g plant^{-1}.

Cultural practices such as plant nutrition, irrigation, weed control, diseases and insect-pest management were adopted as per the University recommendations (Anonymous 2013). Fruit setting and number of seed fruit^{-1} under the open pollination conditions were recorded as described previously. Red ripe fruits were harvested from each line, added over harvests and number of fruit plant^{-1} was worked out. Ten representative fruits from each line were taken from the third harvest to record observations on fruit weight (g), fruit length (cm), fruit width (mm) and pericarp thickness (mm). Fruit weight was recorded on Eagle electronic weighing scale (EG Kantawalla Pvt. Ltd., Pune, Maharashtra, India). Fruit length, fruit width and pericarp thickness were measured with Mitutoyo digital 'vernier caliper' (Mitutoyo, Kawasaki, Kanagawa, Japan). Fruit length was measured from base to tip of the fruit. Fruit width and pericarp thickness were recorded at middle portion of the fruit.

Statistical analysis

The percent data on pollen viability and fruit setting were subjected to 'arcsin' transformation before statistical analysis. Data on pollen release score were transformed using square-root transformation by adding 0.5 to the score. Analysis of variance (ANOVA) was performed following the generalized linear model procedure of SAS version 9.2 (SAS Inst., Cary, NC, USA). Treatment differences were determined using Fisher's Least significant difference (LSD) test at $p = 0.01$. Differences between pairs of means were tested following Duncan's multiple range test (DMRT).

Results

Development of CMS A-lines

From the 11 testcross hybrid progeny screened, maintainer plants were identified in three lines namely, 'SL 461' designated as 'CMS461B', 'SL 462' designated as 'CMS462B', and 'SD 463' designated as 'CMS463B'. From the 60 single plant backcross progeny generated, 43 progeny (8 in BC_1, 6 in BC_2, and 3 in BC_3 from 'CMS461A' testcross group; 7 in BC_1, 4 in BC_2, and 2 in BC_3 from 'CMS462A' testcross group; and 9 in BC_1, 3 in BC_2, and 1 in BC_3 from 'CMS463A' testcross group) were rejected on the basis of sterility performance. Following six cycles of backcrossing and selection, 17 lines derived from the three testcross groups were established. Male sterile flower of one of the CMS A-line 'CMS463D14A' and male fertile flower of its maintainer 'CMS463D14B' line are shown in **Fig. 2**. List of the established CMS A-lines and their respective maintainer B-lines is given in **Table 3**.

Analysis of variance for pollen sterility and associated traits

The mean square (MS) values due to genotypes, environments and genotype × environment interaction for pollen

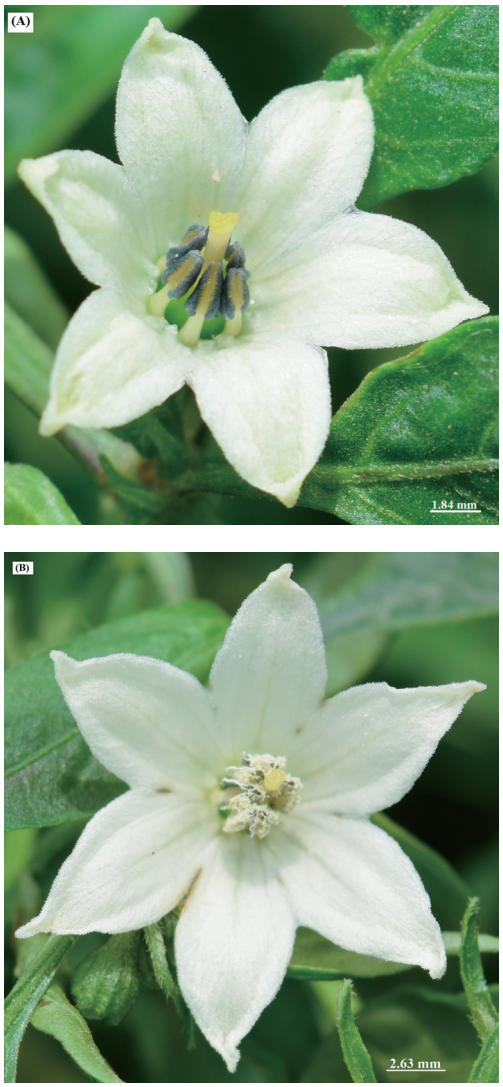

Fig. 2. A. Male sterile flower of CMS A-line 'CMS463D14A' showing purple anthers without pollen grains; and B. male fertile flower of CMS B-line 'CMS463D14B' showing abundant of pollen grains adhering to the green anthers.

sterility and associated traits under the caged conditions are given in **Table 4**. The MS values due to the genotypes and the environments were significant at $p = 0.01$ for pollen sterility, pollen release score, fruit setting and number of seed fruit^{-1}. The G × E interaction effects were significant for pollen sterility, fruit setting and number of seed fruit^{-1} and non-significant for pollen release score. This suggested that there existed significant differences among the CMS lines in their performance for male sterility associated traits. The magnitude of MS values attributed to the genotypes was much higher than the MS values attributed to the environments and to the G × E interaction for all the traits.

Table 3. Pedigree of CMS chilli pepper lines developed in diverse genetic backgrounds

CMS A-Line	Pedigree	B-line	Pedigree
CMS4611A	4261SL461-BC6-P1	CMS4611B	SL461-S10-P1
CMS4614A	4261SL461-BC6-P4	CMS4614B	SL461-S10-P4
CMS46113A	4261SL461-BC6-P13	CMS46113B	SL461-S10-P13
CMS4622A	4261SL462-BC6-P2	CMS4622B	SL462-S10-P2
CMS4623A	4261SL462-BC6-P3	CMS4623B	SL462-S10-P3
CMS4624A	4261SL462-BC6-P4	CMS4624B	SL462-S10-P4
CMS4626A	4261SL462-BC6-P6	CMS4626B	SL462-S10-P6
CMS4627A	4261SL462-BC6-P7	CMS4627B	SL462-S10-P7
CMS46213A	4261SL462-BC6-P13	CMS46213B	SL462-S10-P13
CMS46214A	4261SL462-BC6-P14	CMS46214B	SL462-S10-P14
CMS463D2A	4261SD463DG-BC6-P2	CMS463D2B	SD463DG-S10-P2
CMS463D13A	4261SD463DG-BC6-P13	CMS463D13B	SD463DG-S10-P13
CMS463D14A	4261SD463DG-BC6-P14	CMS463D14B	SD463DG-S10-P14
CMS463L3A	4261SD463LG-BC6-P3	CMS463L3B	SD463LG-S10-P3
CMS463L5A	4261SD463LG-BC6-P5	CMS463L5B	SD463LG-S10-P5
CMS463L9A	4261SD463LG-BC6-P9	CMS463L9B	SD463LG-S10-P9
CMS463L11A	4261SD463LG-BC6-P11	CMS463L11B	SD463LG-S10-P11

Performance of CMS A-lines for pollen sterility and associated traits under caged conditions

The periodical observations at weekly intervals on pollen viability and pollen release score over the four environments are given in **Supplemental Tables 1** and **2**, respectively. Three levels of sterility exhibited by a completely male sterile line (100% pollen sterility), a partially male sterile line (71–90% pollen sterility), and a fully fertile maintainer line (0–20% pollen sterility) is shown in **Supplemental Fig. 2**. Across the environments, 10 lines namely 'CMS4611A', 'CMS4614A', 'CMS4622A', 'CMS4624A', 'CMS4626A', 'CMS46213A', 'CMS463D2A', 'CMS463D13A', 'CMS463D14A', and 'CMS463L5A' expressed 100% pollen sterility. These lines recorded 'zero' pollen release score and did not set any fruit across the environments, and were regarded as completely male sterile. Performance of CMS A-lines for pollen sterility (%) and associated traits under the low and the high temperature environments, and that pooled across the four environments is given in **Table 5**.

Table 4. Analysis of variance for male sterility traits of CMS chilli pepper lines evaluated over environments under caged conditions

Source	Pollen sterility (%)			Pollen release score			Fruit setting (%)			Number of seed fruit^{-1}		
	df	Mean square	F-ratio	df	Mean square	F-ratio	df	Mean square	F-ratio	df	Mean square	F-ratio
Genotypes	16	982.69*	21.93	16	1.463*	15.52	16	59.05*	744.78	16	250.46*	749.1
Environments	3	589.96*	13.17	3	0.487*	5.17	3	11.09*	139.80	3	126.35*	377.91
Genotype × Environment	48	94.31*	2.11	48	0.101^{ns}	1.08	48	3.47*	43.79	48	20.97*	62.72
Error	408	44.81		408	0.094		68	0.079		68	0.334	

* denote significance at $p = 0.01$; ^{ns} non-significant.

Table 5. Performance of CMS chilli pepper lines for pollen sterility, pollen release score, fruit setting and number of seed fruit^{-1} under caged conditions

CMS lines^{+}	E_1	E_2	E_3	E_4	Pooled mean
	a. Pollen sterility (%)†				
CMS46113A	81.83 B^{a}	99.17 A	96.59 A	91.37 AB	92.24 b^{b}
CMS4623A	79.68 B	93.73 A	90.14 AB	87.00 AB	87.64 c
CMS4627A	76.44 B	94.99 A	83.15 B	92.86 A	86.86 c
CMS46214A	92.30 A	98.43 A	100.00 A	100.00 A	97.68 a
CMS463L3A	96.89 A	100.00 A	100.00 A	100.00 A	99.22 a
CMS463L9A	74.37 B	93.05 A	78.48 B	91.48 A	84.35 c
CMS463L11A	82.34 A	92.10 A	82.10 A	90.50 A	86.76 c
	b. Pollen release score				
CMS46113A	0.69 A^{a}	0.00 B	0.37 AB	0.43 AB	0.37 b^{b}
CMS4623A	0.74 A	0.37 A	0.43 A	0.51 A	0.51 ab
CMS4627A	0.71 A	0.14 B	0.74 A	0.34 AB	0.48 ab
CMS46214A	0.29 A	0.11 A	0.00 A	0.00 A	0.10 c
CMS463L3A	0.17 A	0.00 A	0.00 A	0.00 A	0.043 c
CMS463L9A	0.69 AB	0.34 B	0.89 A	0.43 B	0.59 a
CMS463L11A	0.51 AB	0.37 B	0.69 A	0.37 B	0.49 ab
	c. Fruit setting (%)				
CMS46113A	4.66 A^{a}	0.00 C	2.66 B	3.33 AB	2.66 e^{b}
CMS4623A	4.66 B	3.85 B	3.33 B	8.66 A	5.12 d
CMS4627A	7.66 A	1.33 B	8.66 A	5.33 A	5.74 c
CMS46214A	5.33 A	0.00 B	0.00 B	0.00 B	1.33 f
CMS463L3A	2.00 A	0.00 B	0.00 B	0.00 B	0.50 g
CMS463L9A	8.33 A	5.83 A	8.66 A	6.66 A	7.37 a
CMS463L11A	6.33 AB	5.12 B	6.66 AB	9.31 A	6.85 b
	d. Number of seed fruit^{-1}				
CMS46113A	15.66 A^{a}	0.00 C	11.51 B	17.40 A	11.14 c^{b}
CMS4623A	14.38 A	6.06 B	11.60 AB	17.07 A	12.27 ab
CMS4627A	16.87 A	1.15 B	14.84 A	14.25 A	11.78 bc
CMS46214A	14.14 A	0.00 B	0.00 B	0.00 B	3.53 d
CMS463L3A	10.22 A	0.00 B	0.00 B	0.00 B	2.55 e
CMS463L9A	15.72 A	7.95 B	13.70 A	14.30 A	12.92 a
CMS463L11A	15.75 A	8.96 A	12.85 A	14.06 A	12.90 a

† Values are presented as mean.

$^{+}$ Data representing completely male sterile lines not included.

^{a} Between environments means of a genotype with same upper case letter are not significantly different at $p < 0.01$ according to the Duncan's multiple range test.

^{b} Across environments pooled means with same lower case letter are not significantly different at $p < 0.01$ according to the Duncan's multiple range test.

The data pertaining to the completely male sterile lines being static are not included in **Table 5**.

Based on the pollen viability score, three lines namely 'CMS46113A', 'CMS46214A', and 'CMS463L3A' were regarded as male sterile. The overall performance of 'CMS46214A', and 'CMS463L3A' for pollen viability and pollen release score was statistically at par with the completely male sterile lines. The two lines recorded erratic fruit setting only under E_1. The remaining four lines namely 'CMS4623A', 'CMS4627A', 'CMS463L9A', and 'CMS463L11A' recorded fruit setting under all the four environments and were regarded as partially male sterile. In these lines, a small amount of pollen tightly adhering to the anthers was seen during the initial two weeks of screening (**Supplemental Table 2**). Presence of seed in fruits especially in 'CMS46113A' (male sterile), and 'CMS4623A', 'CMS4627A', 'CMS463L9A' and 'CMS463L11A' (partially male sterile) also indicated production of some amount of viable pollen in these lines.

Evaluation of CMS A-lines for fruit and seed setting under the open pollination conditions

A pre-requisite for a female parent to be used in hybrid seed production is its ability to have good fruit setting with normal seed set under the open pollination conditions. Fruit setting (%), number of fruit plant^{-1} and number of seed fruit^{-1} in CMS A-lines under the open pollination conditions is given in **Table 6**. In general fruit setting, number of fruit plant^{-1} and number of seed fruit^{-1} were not affected by the sterility level of the CMS lines. Comparatively, the genotypic mean values were higher under the low temperature environments than under the high temperature environments. Pooled across the environments, a wide range of genotypic variation was observed for all the traits. The maximum fruit setting, number of fruit plant^{-1} and number of seed fruit^{-1} were recorded in 'CMS463D13A'. The performance of 'CMS46213A', 'CMS463D2A', and 'CMS463D14A' for fruit setting and number of seed fruit^{-1} was significantly better than rest of the lines.

Evaluation of CMS A-lines for important fruit traits

Fruit weight, fruit length, fruit width and pericarp thickness of CMS A-lines under the open pollination conditions is given in **Table 7**. A wide range of variation was observed for all the fruit traits evaluated. The maximum fruit weight was recorded in 'CMS463D14A' and the minimum in 'CMS46113A'. Fruit weight of 'CMS463D2A', 'CMS463D13A', and 'CMS463L5A' was statistically at par with 'CMS463D14A'. Except 'CMS463L5A', the lines which recorded the maximum fruit weight also recorded significantly the longer, wider and thicker fruits compared to the other lines. The maximum pericarp thickness was recorded in 'CMS463D13A' and the minimum in 'CMS4623A'.

Discussion

In chilli, CMS is the most effective method of hybrid seed production (Dhaliwal and Jindal 2014, Ma *et al.* 2013). The system could be unstable due to the environmental fluctuations and the genetic background. Instability of the CMS was attributed to interaction between the temperature and the sterility modifier genes (Kim *et al.* 2013, Min *et al.* 2009, Novak *et al.* 1971, Shifriss and Guri 1979). Gniffke *et al.* (2009), Gong *et al.* (2008) and Shifriss and Guri (1979) developed several CMS lines in chilli with varying levels of male sterility among and within the lines. The commercial use of CMS demands stability of sterility to ensure genetic purity of hybrid seed. Here we report the development of temperature stable CMS lines in chilli suitable for hybrid development.

Table 6. Performance of CMS chilli pepper lines for fruit setting, number of fruit plant^{-1} and number of seed fruit^{-1} in open pollination conditions

CMS lines	a. Fruit setting (%)†				
	E_1	E_2	E_3	E_4	Pooled mean
CMS4611A	29.33 A[a]	12.66 B	36.66 A	11.33 B	22.49 f[b]
CMS4614A	19.33 AB	9.33 C	27.33 A	16.37 BC	18.09 h
CMS46113A	37.33 A	12.66 B	42.66 A	18.66 B	27.82 cd
CMS4622A	24.00 A	22.66 A	21.33 A	24.00 A	22.99 ef
CMS4623A	24.66 A	19.75 A	20.66 A	22.66 A	21.93 fg
CMS4624A	31.00 A	18.66 B	34.66 A	21.33 B	26.41 cde
CMS4626A	22.00 AB	16.66 AB	22.66 A	14.66 B	18.99 gh
CMS4627A	26.66 A	17.33 A	29.33 A	24.66 A	24.49 def
CMS46213A	45.33 A	24.66 B	46.66 A	27.33 AB	35.99 b
CMS46214A	32.66 AB	21.33 BC	35.33 A	19.33 C	27.16 cd
CMS463D2A	43.33 A	27.40 A	37.33 A	28.66 A	34.18 b
CMS463D13A	50.66 A	31.33 A	53.33 A	33.56 A	42.22 a
CMS463D14A	47.33 A	28.66 A	46.00 A	26.66 A	37.16 b
CMS463L3A	22.00 A	26.66 A	31.33 A	27.33 A	26.83 cd
CMS463L5A	27.33 A	23.05 A	24.66 A	22.66 A	24.42 def
CMS463L9A	29.33 A	27.96 A	27.33 A	29.33 A	28.49 c
CMS463L11A	34.00 A	29.07 A	30.66 A	23.23 A	29.24 c
Mean	32.13 A	21.75 B	33.41 A	23.04 B	27.58
	b. Number of fruits plant^{-1}				
CMS4611A	75.20 A[a]	37.90 B	89.56 A	35.66 B	59.58 gh[b]
CMS4614A	69.00 A	25.66 B	76.50 A	40.01 B	52.79 hi
CMS46113A	108.25 A	44.67 B	115.28 A	51.50 B	79.93 cd
CMS4622A	99.50 A	53.00 BC	91.66 AB	36.50 C	70.16 ef
CMS4623A	88.00 A	43.87 B	106.33 A	35.20 B	68.35 efg
CMS4624A	133.00 A	39.50 B	136.50 A	27.66 B	84.16 c
CMS4626A	99.00 A	29.50 B	110.66 A	23.50 B	65.66 fg
CMS4627A	91.50 A	37.66 B	119.82 A	54.66 B	75.91 cde
CMS46213A	157.50 A	55.25 B	148.33 A	48.50 B	102.39 b
CMS46214A	151.50 A	49.50 B	154.50 A	25.50 B	95.25 b
CMS463D2A	147.25 A	58.89 C	130.67 AB	69.33 BC	101.53 b
CMS463D13A	159.50 A	72.44 B	167.80 A	73.28 B	118.25 a
CMS463D14A	133.33 A	67.25 B	142.50 A	49.50 B	98.14 b
CMS463L3A	66.50 A	58.33 A	75.82 A	62.50 A	65.78 fg
CMS463L5A	56.62 A	52.77 A	53.66 A	40.22 A	50.82 hi
CMS463L9A	43.62 A	57.19 A	40.50 A	46.70 A	47.00 i
CMS463L11A	107.00 A	59.30 B	83.75 AB	45.80 B	73.96 def
Mean	105.07 A	49.57 B	108.46 A	45.06 C	77.04
	c. Number of seed fruit^{-1}				
CMS4611A	35.70 A[a]	24.60 A	31.90 A	20.90 A	28.27 f[b]
CMS4614A	31.70 A	21.30 A	37.40 A	23.28 A	28.42 f
CMS46113A	41.30 A	32.70 A	40.50 A	34.20 A	37.17 e
CMS4622A	39.30 A	20.70 B	41.70 A	17.40 B	29.77 f
CMS4623A	47.78 AB	26.95 B	51.40 AB	66.90 A	48.26 c
CMS4624A	30.70 A	21.60 A	28.60 A	29.10 A	27.50 f
CMS4626A	47.90 A	28.40 B	40.50 AB	32.50 AB	37.32 e
CMS4627A	66.90 A	42.60 A	58.70 A	60.30 A	57.12 b
CMS46213A	45.10 A	40.60 A	49.20 A	33.60 A	42.12 d
CMS46214A	45.80 A	34.30 A	50.30 A	41.60 A	43.00 d
CMS463D2A	50.90 B	38.28 C	55.60 B	63.10 A	51.97 c
CMS463D13A	79.80 A	47.20 A	76.30 A	68.65 A	67.99 a
CMS463D14A	65.90 A	38.50 A	69.50 A	53.90 A	56.95 b
CMS463L3A	35.90 A	25.70 A	28.10 A	29.80 A	29.87 f
CMS463L5A	40.30 AB	21.29 B	47.20 A	31.70 AB	35.12 e
CMS463L9A	36.40 AB	35.65 B	38.90 AB	59.40 A	42.58 d
CMS463L11A	27.40 B	28.10 B	30.50 B	58.82 A	36.20 e
Mean	45.22 A	31.08 C	45.66 A	42.65 B	41.16

† Values are presented as mean.

[a] Between environments means of a genotype with same upper case letter are not significantly different at $p < 0.01$ according to the Duncan's multiple range test.

[b] Across environments pooled means with same lower case letter are not significantly different at $p < 0.01$ according to the Duncan's multiple range test.

Table 7. Performance of CMS lines of chilli pepper for important fruit traits under open pollination conditions

CMS lines	Fruit weight (g)†	Fruit length (cm)	Fruit width (mm)	Pericarp thickness (mm)
CMS4611A	3.66 gh	4.28 g	9.43 e–h	0.78 efg
CMS4614A	3.07 h	5.80 ef	10.17 c–f	0.72 fg
CMS46113A	2.81 h	4.98 efg	8.62 fgh	0.90 cde
CMS4622A	4.77 d–g	6.11 cde	10.27 b–f	0.74 efg
CMS4623A	5.07 c–g	5.92 def	11.52 a–d	0.62 g
CMS4624A	5.26 c–f	7.00 bcd	11.89 abc	0.80 ef
CMS4626A	5.86 bcd	7.29 ab	10.71 b–e	1.01 bcd
CMS4627A	4.11 e–h	5.58 ef	8.53 fgh	0.80 ef
CMS46213A	5.37 cde	7.75 ab	11.34 a–d	1.04 abc
CMS46214A	5.21 c–f	7.10 bc	10.60 b–e	1.12 ab
CMS463D2A	7.13 ab	7.92 ab	12.91 a	1.09 ab
CMS463D13A	7.19 ab	8.29 a	13.01 a	1.19 a
CMS463D14A	7.48 a	7.93 ab	11.96 abc	1.06 abc
CMS463L3A	5.80 bcd	5.16 efg	10.05 d–g	1.01 bcd
CMS463L5A	6.38 abc	5.02 efg	7.88 h	0.99 bcd
CMS463L9A	5.87 bcd	5.83 ef	12.08 ab	0.87 def
CMS463L11A	3.85 fgh	4.82 fg	8.35 gh	0.90 cde

† Values are presented as mean.

Values in columns with same letter are not significantly different at $p < 0.01$ according to the Duncan's multiple range test.

The results of pollen viability, pollen release score, and fruit and seed setting under the caged conditions were consistent, comparable and complemented each other. Out of the 17 CMS lines screened, the low temperature influenced the performance of seven lines. The G × E interaction effects indicated the differential response of these lines to the variation in temperature. Ten lines namely 'CMS4611A', 'CMS4614A', 'CMS4622A', 'CMS4624A', 'CMS4626A', 'CMS46213A', 'CMS463D2A', 'CMS463D13A', 'CMS463D14A', and 'CMS463L5A' expressed 100% pollen sterility across the environments. Male sterility of these lines was further confirmed by their inability to set fruit and seed under the caged conditions. Their inability to set fruit under the caged conditions is attributed to the lack of viable pollen. Since the four environments represented the varied temperature conditions ranging from 24.1°C/12.1°C to 41.1°C/28.5°C day/night, the 10 lines were regarded as completely male sterile and temperature stable.

Four CMS lines namely 'CMS4623A', 'CMS4627A', 'CMS463L9A', and 'CMS463L11A' were partially male fertile under both the low and the high temperature regimes. However, the level of fertility was higher under the low temperature than under the high temperature regime. Under low temperature, the periodic observations on pollen viability revealed that these lines produced some viable pollen during initial few weeks of screening when the corresponding day/night temperature was relatively low (<26°C/14°C day/night). As the temperature approached 32°C/23°C day/night, the lines showed complete male sterility. Interestingly, under the high temperature regime also, these lines expressed partial fertility during the initial stages of screening. It is inferred that the partial fertility of these lines was conditioned by the modifier genes. Activity of these modifier

genes slowed down with rise in temperature, and stopped completely at advanced stage of plant growth. Therefore, the partially fertile CMS lines are not suitable in 2-line (A and C) hybrid development by manipulating male fertility/sterility through the temperature interventions. Such a system was reported by Kim *et al.* (2013) and Shifriss (1997) where the thermo-sensitive CMS is maintained under low temperature (13°C night) and the hybrid seed is produced under the high temperature (>15°C night), thus eliminating the need of a B-line to maintain the CMS line.

The results further suggested that it might be possible to stabilize the unstable CMS lines with few additional cycles of backcrossing and selection. This is substantiated by the fact that two lines 'CMS46214A', and 'CMS463L3A' which were partially male sterile during the first year of evaluation were completely male sterile in the second year, expectedly due to one additional cycle of selection and backcrossing. It is speculated that the unstable CMS lines reacted to the temperature in first year due to heterozygous condition of the sterility modifier genes.

Apart from stability of male sterility, CMS lines should possess the normal female fertility, high seed yield and desirable fruit traits. Fruit length, fruit weight, fruit width and pericarp thickness are the commercially important fruit traits in chilli. The female parent with a higher yield potential ensures higher hybrid seed yield (Gniffke *et al.* 2009). Number of fruit plant^{-1} and number of seed fruit^{-1} provide a good index of seed yield. A strong association has been established between line *per se* and hybrid performance (Ertiro *et al.* 2013).

The 10 CMS lines identified as completely male sterile and temperature stable have been assessed for their use in hybrid development. Morphologically, sterility of these lines was not associated with any flower and fruit deformity. The fruit setting ability (18.09–42.22%), number of fruits plant^{-1} (47.00–118.25) and number of seed fruit^{-1} (27.50–67.99) under the open pollination conditions were comparable with the earlier reports (Kaur *et al.* 2016, Singh *et al.* 2014), indicating their normal female fertility. The lines possessed varied but commercially acceptable fruit traits and provide a wider choice of a female parent for the development of commercial chilli hybrids. Their potential to tolerate temperature variations would ensure genetic purity of the F_1 seed. The CMS transferred into the diverse genetic backgrounds will be useful to breed commercial chilli hybrids for different purposes.

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