

## Evaluation of Cytoplasmic Male Sterile lines and Restorer lines in *Moricandia* system for heterosis breeding in Indian Mustard [*Brassica juncea* (L.) Czern & Coss.]

### Abstract:

The present investigation was undertaken with the objectives of Cytoplasmic Male Sterility (CMS) assessment and study of pollen fertility restoration using five *Mori* based Cytoplasmic Male Sterile lines viz., Mori 'A' SKM 109, Mori 'A' SKM 125, Mori 'A' SKM 201, Mori 'A' SKM 219, Mori 'A' SKM 303 and eight diverse *Mori* based fertility restorer lines viz., Mori 'R' GM 2, Mori 'R' GM 3, Mori 'R' SKM 9033, Mori 'R' SKM 301, Mori 'R' Pusa Bahar, Mori 'R' Vardan, Mori 'R' Bio 902, Mori 'R' 1-14 to identify good fertility restorer line and stain ability of pollen grains of sterile and fertile lines to *Moricandia arvensis*, cytoplasmic background of converted cytoplasmic male sterile lines of Indian Mustard [*Brassica juncea* (L.) Czern & Coss.] by repeated backcrossing at the Main Castor-Mustard Research station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar-385506 Banaskantha. The pollen stain ability, plant fertility status and percentage siliquae set per plant was recorded for the morphological characterization and to confirm stability of true Cytoplasmic Male Sterile (CMS) line. The experimental material comprised of fifty-four genotypes consisting of five diverse CMS lines and eight Fertility Restorer lines were crossed in line X tester mating design and resultant forty hybrids along with their thirteen parents and standard check variety (Kranti) were evaluated in randomized block experimental design. The morphological characters were studied viz., pollen fertility, Number of siliquae set per plant, per cent siliquae set per plant in field as well as laboratory tests were conducted with 2 % Aceto-carmin to confirm pollen stain ability and purity of CMS (A-lines) for male sterility and pollen fertility restorability of R-lines. There were visual differences observed for the parents (male sterile lines and fertility restorer lines), all the F<sub>1</sub> crosses and standard check parent for pollen fertility. The male sterile lines exhibited (100%) pollen sterility and the pollen fertility restorer lines varied from 80.05 per cent (Mori 'R' Pusa Bahar) to 97.97 per cent (Mori 'R' SKM 9033).

**Key words:** *Brassica juncea*, Cytoplasmic Male Sterility, *Moricandia*, Restorers and pollen stainability, fertility restoration

### 1. Introduction

Mustard (*Brassica juncea* L.) is a major oilseed crop in India. It is largely self-pollinated, and pure-line cultivars are progeny of single self-fertilizing homozygous plant. Yield plateauing and the need to produce more has prompted interest in alternate strategies like heterosis breeding for shifting the yield frontiers beyond the level of current commercial cultivars. Cytoplasmic male sterility (CMS) coupled with fertility restoring systems has been used widely to facilitate pollination control and mass production of hybrid seeds in hermaphrodite crops (Banga and Banga, 2003). A stable CMS line of *B. juncea* was derived from the somatic hybrid *Moricandia arvensis* + *B. juncea* following repeated back crosses with *B. juncea*. The fertility restorer gene

from *M. arvensis* was introgressed into *B. juncea* through the homoeologous recombination (Prakash *et al.*, 1998). In order to deploy CMS systems in the development of commercial *brassica* hybrids, it is essential to sort-out effective restorer lines. Fertility restorers have been identified in the *Trachystoma* and *Moricandia* based CMS lines of *B. juncea* (Prakash and Kirti, 1997). Exploitable level of standard heterosis depends on an effective male sterility and fertility system which is the most important prerequisites for the development of commercially viable hybrids. Restoration ability in CMS line is an important factor for the exploitation of hybrid in the breeding programme (Ashutosh *et al.*, 2014). Heterosis has been extensively explored and utilized for boosting various quality traits in *Brassica* because of an effective and economic pollination control system for production of F<sub>1</sub> hybrid seeds on a large scale. In the present study, Cytoplasmic male sterility assessment and fertility restoration ability were estimated for the hybrids development using L x T cross experiment.

## 2. Material and Methods

Five cytoplasmic male sterile (CMS) lines of *Moricandia* viz., Mori 'A' SKM 109, Mori 'A' SKM 125, Mori 'A' SKM 201, Mori 'A' SKM 219, Mori 'A' SKM 303 were crossed with eight diverse fertility restorer lines viz., Mori 'R' GM 2, Mori 'R' GM 3, Mori 'R' SKM 9033, Mori 'R' SKM 301, Mori 'R' Pusa Bahar, Mori 'R' Vardan, Mori 'R' Bio 902, Mori 'R' 1-14 to identify good restore line to *Moricandia arvensis* cytoplasmic background at Main Castor–Mustard Research Station, S. D. Agricultural University, S. K. Nagar, Banaskantha, Gujarat during *Rabi* 2013-2014. To assess the pollen viability, a few matured buds and their pollens were collected from the five selected plants during anthesis period. These pollen grains were placed on clean slide and one or two drops of 2 per cent aceto-carmin were poured on the pollen. Then a coverslip was put on the pollens and lightly pressed to removed air bubbles as well as excess solution of aceto-carmin. The stained and non-stained pollen were counted under the microscope at 40 X magnification to know their fertility and sterility, respectively. The percentages of pollen fertility of F<sub>1</sub>'s were used as an index of fertility restoring ability of different pollen parents. Pollen fertility per cent of F<sub>1</sub>'s in the crosses involving five CMS lines is given in Table 1. This study was investigated by visual observation of freshly opened and fully developed flowers for the presence (male fertile) or absence (male sterile) of the pollen in CMS lines, restorer lines and their crosses. Pollen grains collected from mature flower bud of five randomly selected plant from each row of male sterile line, fertility restorer lines and their F<sub>1</sub> plants were dusted on glass slide, a drop of 2 per cent aceto-carmin stain was added and pollen staining ability was examined for authentication of stability in male sterility character of CMS lines, restoration ability of restorer lines and fertility restoration in their crosses (F<sub>1</sub>'s) as per method proposed by Alexander, 1969. The experimental materials were also subjected to selfing of one inflorescence of each F<sub>1</sub> plant and their parental lines to examine ability or inability of the plant to produce selfed seeds for field confirmation. (Table 2)

Visual observation for presence or absence of the pollen by dusting and crushing of the mature flower bud about to open in morning was also recorded for presence or absence of yellow pollen

dust. The presence of yellow pollen dust was indicated as fertile and absence of yellow pollen dust as sterile indicating plant fertility status. (Table 1)

### 3. Results and Discussion

There were visual differences observed for the parents (male sterile line and male fertile line), all the F<sub>1</sub> crosses and standard checks for the pollen fertility are presented (Table 1). The male sterile lines (P1 to P5) in Table 1 exhibited (100%) pollen sterility with 2 per cent aceto-carmine stain. The pollen fertility in fertility restorer lines (P6 to P13) in Table 1 varied from 80.05 per cent (Mori 'R' Pusa Bahar) to 97.97 per cent (Mori 'R' SKM 9033). The F<sub>1</sub> generation of all the crosses exhibited fertility restoration with pollen fertility except F<sub>1</sub> crosses of all male sterile lines with Mori 'R' GM 3 restorer line (0%) ranging from 65.15 (Mori 'A' SKM 109 x Mori 'R' Pusa Bahar) to 88.51 per cent (Mori 'A' SKM 219 x Mori 'R' SKM 301) over the standard check Kranti 91.22 per cent (Table 1).

These varying results were further confirmed by ability to produce seed set upon self-fertilization. The number of siliqua set and per cent siliqua set upon self-pollination was obtained similarly 0 per cent in all CMS lines indicating stable male sterility, in fertility restorer lines 79.01 (Mori 'R' Pusa Bahar) to 92.22 (Mori 'R' SKM 9033) revealing associated results with pollen stain ability. It ranges from 0 to 86.26 per cent for F<sub>1</sub> crosses of five male sterile lines with Mori 'R' GM 3 restorer line and Mori 'A' SKM 219 x Mori 'R' SKM 301 F<sub>1</sub> cross respectively over the standard check Kranti 90.34 % per cent (Table 2).

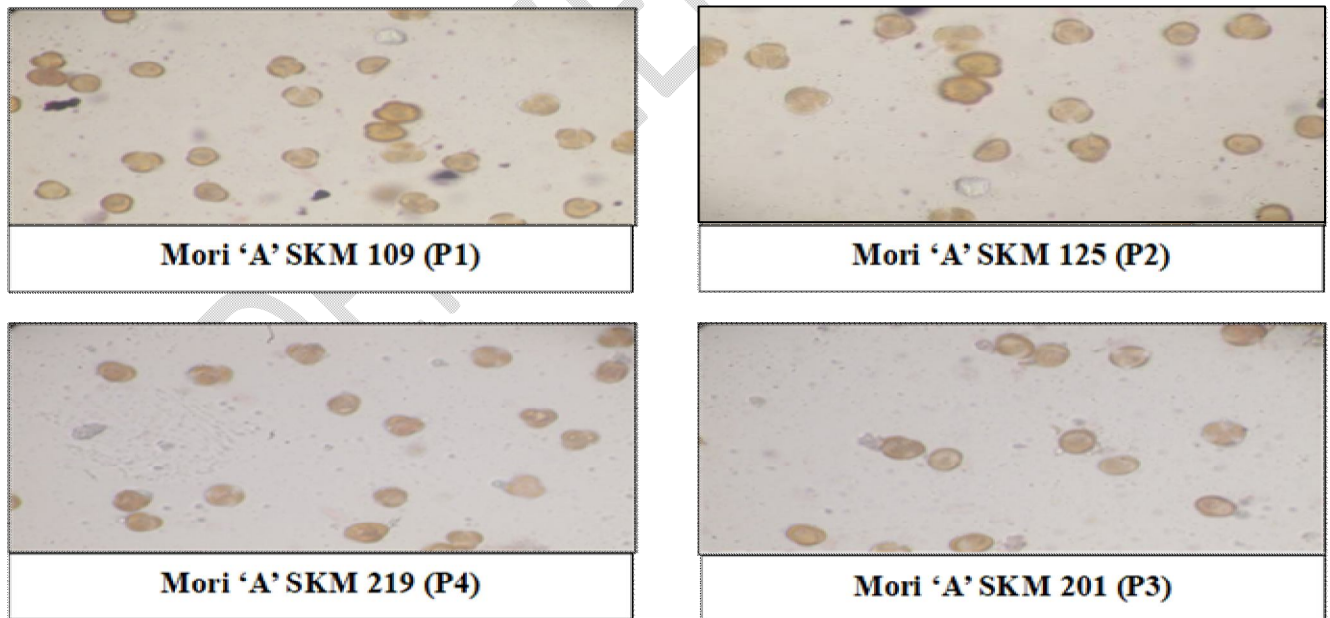
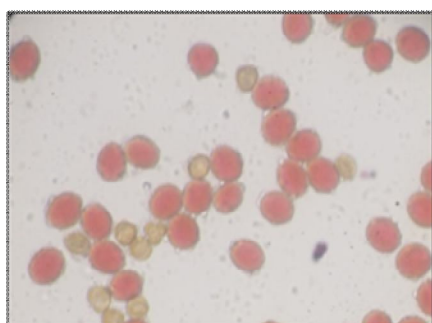
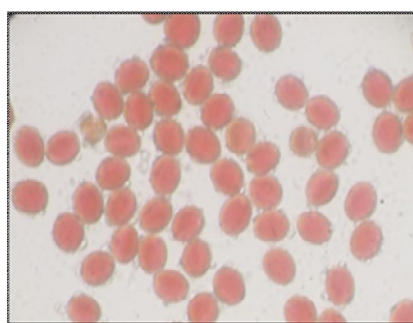


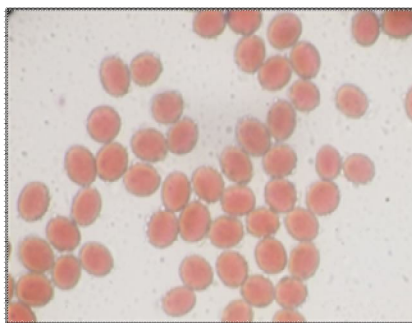
Plate 1: Unstained pollen grain showing male sterile lines



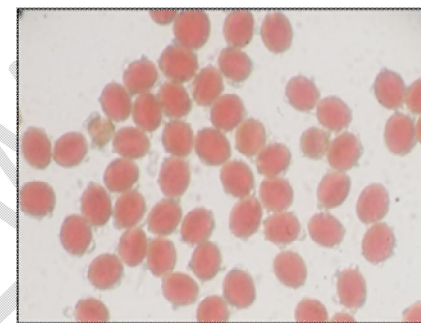
**Mori 'R' GM 2 (P6)**



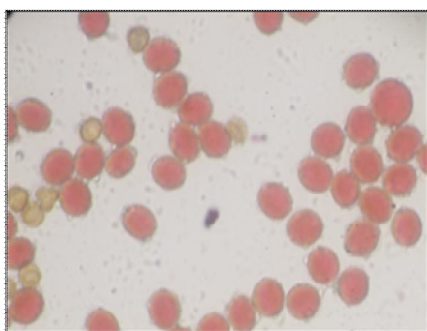
**Mori 'R' GM 3 (P7)**



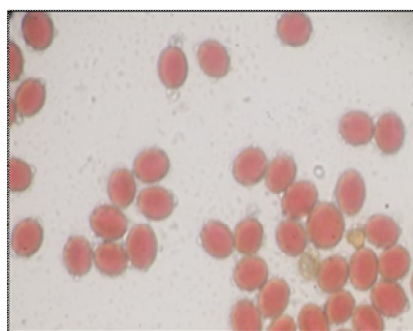
**Mori 'R' SKM 9033 (P8)**



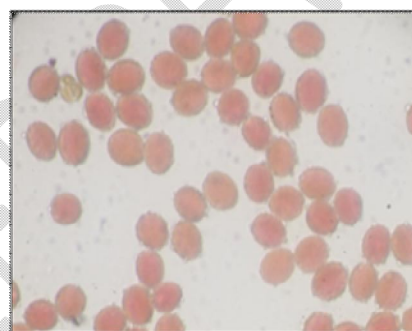
**Mori 'R' SKM 301 (P9)**



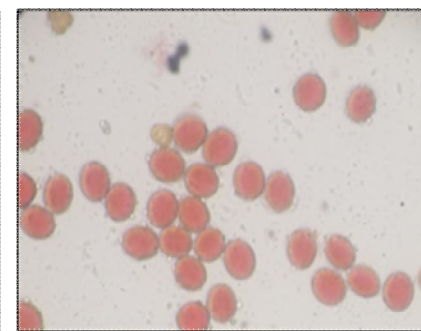
**Mori 'R' PusaBahar (P10)**



**Mori 'R' Vardan (P11)**



**Mori 'R' Bio 902 (P12)**



**Mori 'R' 1-14 (P13)**

**Plate 2: Pollen stain ability of eight restorer lines**

UNDER



Plate 3a: Single  
anther of male fertile



Plate 3b: Single  
anther of male sterile

**Table 1: Pollen fertility status of CMS lines, Restorer lines, their Hybrids and Standard check (Kranti) in mustard**

Sr. No.	Genotype	R I		R II		R III		Total		Total pollen observed	Plant Fertility	Pollen Fertility (%)
		Sterile	Fertile	Sterile	Fertile	Sterile	Fertile	sterile	Fertile			
P1	Mori 'A' SKM 109	55	0	78	0	96	0	229	0	229	S	0.00
P2	Mori 'A' SKM 125	88	0	67	0	72	0	227	0	227	S	0.00
P3	Mori 'A' SKM 201	80	0	70	0	93	0	243	0	243	S	0.00
P4	Mori 'A' SKM 219	90	0	97	0	53	0	240	0	240	S	0.00
P5	Mori 'A' SKM 303	106	0	83	0	74	0	263	0	263	S	0.00
P6	Mori 'R' GM2	12	57	29	142	5	46	46	245	291	F	84.19
P7	Mori 'R' GM3	3	30	1	39	2	85	6	154	160	F	96.25
P8	Mori 'R' SKM 9033	1	74	2	56	1	63	4	193	197	F	97.97
P9	Mori 'R' SKM 301	7	140	8	176	12	150	27	466	493	F	94.52
P10	Mori 'R' PusaBahar	29	63	20	128	27	114	76	305	381	F	80.05
P11	Mori 'R' Vardan	28	187	5	100	16	123	49	410	459	F	89.32
P12	Mori 'R' Bio-902	2	53	7	110	8	85	17	248	265	F	93.58
P13	Mori 'R' 1-14	13	91	28	148	15	143	56	382	438	F	87.21
14	Mori 'A' SKM 109 x Mori 'R' GM 2	70	172	71	147	52	146	193	465	658	F	70.67
15	Mori 'A' SKM 109 x Mori 'R' GM 3	100	0	99	0	64	0	263	0	263	S	0.00
16	Mori 'A' SKM 109 x Mori 'R' SKM 9033	45	122	33	133	40	118	118	373	491	F	75.97
17	Mori 'A' SKM 109 x Mori 'R' SKM 301	42	121	53	161	45	131	140	413	553	F	74.68
18	Mori 'A' SKM 109 x Mori 'R' Pusa Bahar	76	129	30	75	69	123	175	327	502	F	65.14
19	Mori 'A' SKM 109 x Mori 'R' Vardan	48	120	58	132	62	128	168	380	548	F	69.34
20	Mori 'A' SKM 109 x Mori 'R' Bio 902	53	100	36	108	42	110	131	318	449	F	70.82
21	Mori 'A' SKM 109 x Mori 'R' 1-14	87	193	37	150	42	154	166	497	663	F	74.96
22	Mori 'A' SKM 125 x Mori 'R' GM 2	33	116	48	105	53	130	134	351	485	F	72.37

Sr. No.	Genotype	R I		R II		R III		Total		Total pollen observed	Plant Fertility	Pollen Fertility (%)
		Sterile	Fertile	Sterile	Fertile	Sterile	Fertile	sterile	Fertile			
23	Mori 'A' SKM 125 x Mori 'R' GM3	121	0	84	0	49	0	254	0	254	S	0.00
24	Mori 'A' SKM 125 x Mori 'R' SKM 9033	56	115	34	117	48	140	138	372	510	F	72.94
25	Mori 'A' SKM 125 x Mori 'R' SKM 301	45	135	30	144	55	162	130	441	571	F	77.23
26	Mori 'A' SKM 125 x Mori 'R' Pusa Bahar	56	122	48	128	84	153	188	403	591	F	68.19
27	Mori 'A' SKM 125 x Mori 'R' Vardan	70	194	81	161	75	154	226	509	735	F	69.25
28	Mori 'A' SKM 125 x Mori 'R' Bio 902	40	96	33	95	45	94	118	285	403	F	70.72
29	Mori 'A' SKM 125 x Mori 'R' 1-14	63	180	34	152	70	197	167	529	696	F	76.01
30	Mori 'A' SKM 201 x Mori 'R' GM 2	43	142	50	115	53	140	146	397	543	F	73.11
31	Mori 'A' SKM 201 x Mori 'R' GM 3	86	0	74	0	46	0	206	0	206	S	0.00
32	Mori 'A' SKM 201 x Mori 'R' SKM 9033	39	105	40	89	41	105	120	299	419	F	71.36
33	Mori 'A' SKM 201 x Mori 'R' SKM 301	31	103	58	102	47	118	136	323	459	F	70.37
34	Mori 'A' SKM 201 x Mori 'R' Pusa Bahar	80	157	42	128	48	120	170	405	575	F	70.43
35	Mori 'A' SKM 201 x Mori 'R' Vardan	63	100	58	153	49	108	170	361	531	F	67.98
36	Mori 'A' SKM 201 x Mori 'R' Bio 902	55	119	29	107	36	112	120	338	458	F	73.80
37	Mori 'A' SKM 201 x Mori 'R' 1-14	46	103	18	86	44	115	108	304	412	F	73.79
38	Mori 'A' SKM 219 x Mori 'R' GM 2	31	114	62	168	38	110	131	392	523	F	74.95
39	Mori 'A' SKM 219 x Mori 'R' GM 3	53	0	88	0	64	0	205	0	205	S	0.00
40	Mori 'A' SKM 219 x Mori 'R' SKM 9033	27	168	20	123	35	154	82	445	527	F	84.44

Sr. No.	Genotype	R I		R II		R III		Total		Total pollen observed	Plant Fertility	Pollen Fertility (%)
		Sterile	Fertile	Sterile	Fertile	Sterile	Fertile	sterile	Fertile			
41	Mori 'A' SKM 219 x Mori 'R' SKM 301	6	219	24	106	24	91	54	416	470	F	88.51
42	Mori 'A' SKM 219 x Mori 'R' Pusa Bahar	52	176	36	109	43	104	131	389	520	F	74.81
43	Mori 'A' SKM 219 x Mori 'R' Vardan	63	150	32	96	43	98	138	344	482	F	71.37
44	Mori 'A' SKM 219 x Mori 'R' Bio 902	40	112	30	108	42	101	112	321	433	F	74.13
45	Mori 'A' SKM 219 x Mori 'R' 1-14	38	121	49	114	41	127	128	362	490	F	73.88
46	Mori 'A' SKM 303 x Mori 'R' GM 2	51	127	23	68	35	125	109	320	429	F	74.59
47	Mori 'A' SKM 303 x Mori 'R' GM 3	47	0	54	0	44	0	145	0	145	S	0.00
48	Mori 'A' SKM 303 x Mori 'R' SKM 9033	39	99	45	93	44	94	128	286	414	F	69.08
49	Mori 'A' SKM 303 x Mori 'R' SKM 301	96	223	46	140	48	155	190	518	708	F	73.16
50	Mori 'A' SKM 303 x Mori 'R' Pusa Bahar	61	161	48	99	39	95	148	355	503	F	70.58
51	Mori 'A' SKM 303 x Mori 'R' Vardan	38	99	62	163	37	100	137	362	499	F	72.55
52	Mori 'A' SKM 303 x Mori 'R' Bio 902	62	170	59	157	60	169	181	496	677	F	73.26
53	Mori 'A' SKM 303 x Mori 'R' 1-14	41	155	31	149	38	147	110	451	561	F	80.39
54	Standard Check- Kranti	11	91	7	127	15	125	33	343	376	F	91.22

S: Sterile, F: Fertile

**Table 2: Number of Siliqua set and per cent Siliqua set upon selfing in CMS lines, Restorer lines, their hybrids and standard check (Kranti) in Mustard**

Sr. No.	Genotype / Entry	R I		R II		R III		Total Flower bud kept	Total siliquae set	Per cent siliquae Set
		Flower bud kept	Siliquae Set	Flower bud kept	Siliquae Set	Flower bud kept	Siliquae Set			
1	Mori 'A' SKM 109	58	0	51	0	49	0	158	0	0.00
2	Mori 'A' SKM 125	62	0	55	0	50	0	167	0	0.00
3	Mori 'A' SKM 201	54	0	52	0	57	0	163	0	0.00
4	Mori 'A' SKM 219	56	0	43	0	49	0	148	0	0.00
5	Mori 'A' SKM 303	50	0	51	0	48	0	149	0	0.00
6	Mori 'R'GM 2	50	42	41	34	52	41	143	117	81.82
7	Mori 'R'GM 3	60	55	50	43	33	31	143	129	90.21
8	Mori 'R' SKM 9033	54	47	53	50	60	57	167	154	92.22
9	Mori 'R' SKM 301	53	44	55	49	52	47	160	140	87.50
10	Mori 'R' Pusa Bahar	61	48	69	56	51	39	181	143	79.01
11	Mori 'R' Vardan	56	46	51	44	58	49	165	139	84.24
12	Mori 'R' Bio 902	52	44	50	43	45	40	147	127	86.39
13	Mori 'R' 1-14	46	38	48	39	52	44	146	121	82.88
14	Mori 'A' SKM 109 x Mori 'R'GM 2	50	34	52	35	49	33	151	102	67.55
15	Mori 'A' SKM 109 x Mori 'R'GM 3	47	0	40	0	49	0	136	0	0.00
16	Mori 'A' SKM 109 x Mori 'R' SKM 9033	46	33	49	38	43	30	138	101	73.19
17	Mori 'A' SKM 109 x Mori 'R' SKM 301	48	34	58	40	50	39	156	113	72.44
18	Mori 'A' SKM 109 x Mori 'R' Pusa Bahar	51	32	47	33	47	26	145	91	62.76
19	Mori 'A' SKM 109 x Mori 'R' Vardan	39	26	42	27	39	28	120	81	67.50
20	Mori 'A' SKM 109 x Mori 'R' Bio 902	44	29	50	34	50	35	144	98	68.06
21	Mori 'A' SKM 109 x Mori 'R' 1-14	51	39	50	35	43	31	144	105	72.92
22	Mori 'A' SKM 125 x Mori 'R' GM 2	50	37	42	29	50	34	142	100	70.42
23	Mori 'A' SKM 125 x Mori 'R' GM3	50	0	51	0	51	0	152	0	0.00
24	Mori 'A' SKM 125 x Mori 'R' SKM 9033	58	39	44	32	46	33	148	104	70.27
25	Mori 'A' SKM 125 x Mori 'R' SKM 301	45	37	55	34	47	38	147	109	74.15
26	Mori 'A' SKM 125 x Mori 'R' Pusa Bahar	50	33	53	33	36	26	139	92	66.19

Sr. No.	Genotype / Entry	R I		R II		R III		Total Flower bud kept	Total siliquae set	Per cent siliquae Set
		Flower bud kept	Siliquae Set	Flower bud kept	Siliquae Set	Flower bud kept	Siliquae Set			
27	Mori 'A' SKM 125 x Mori 'R' Vardan	43	27	64	44	50	35	157	106	67.52
28	Mori 'A' SKM 125 x Mori 'R' Bio 902	57	38	54	39	50	34	161	111	68.94
29	Mori 'A' SKM 125 x Mori 'R' 1-14	60	48	61	40	50	37	171	125	73.10
30	Mori 'A' SKM 201 x Mori 'R' GM 2	48	35	48	34	50	36	146	105	71.92
31	Mori 'A' SKM 201 x Mori 'R' GM 3	51	0	42	0	50	0	143	0	0.00
32	Mori 'A' SKM 201 x Mori 'R' SKM 9033	47	28	45	32	41	30	133	90	67.67
33	Mori 'A' SKM 201 x Mori 'R' SKM 301	48	26	61	47	45	33	154	106	68.83
34	Mori 'A' SKM 201 x Mori 'R' Pusa Bahar	46	33	60	40	51	36	157	109	69.43
35	Mori 'A' SKM 201 x Mori 'R' Vardan	54	35	54	36	42	30	150	101	67.33
36	Mori 'A' SKM 201 x Mori 'R' Bio 902	42	28	42	32	50	35	134	95	70.90
37	Mori 'A' SKM 201 x Mori 'R' 1-14	41	26	45	37	44	32	130	95	73.08
38	Mori 'A' SKM 219 x Mori 'R' GM 2	50	38	42	31	48	33	140	102	72.86
39	Mori 'A' SKM 219 x Mori 'R' GM 3	52	0	49	0	35	0	136	0	0.00
40	Mori 'A' SKM 219 x Mori 'R' SKM 9033	44	34	59	52	39	32	142	118	83.10
41	Mori 'A' SKM 219 x Mori 'R' SKM 301	54	46	36	31	41	36	131	113	86.26
42	Mori 'A' SKM 219 x Mori 'R' Pusa Bahar	49	37	50	35	50	38	149	110	73.83
43	Mori 'A' SKM 219 x Mori 'R' Vardan	49	33	48	37	49	31	146	101	69.18
44	Mori 'A' SKM 219 x Mori 'R' Bio 902	48	34	54	36	50	41	152	111	73.03
45	Mori 'A' SKM 219 x Mori 'R' 1-14	46	32	51	31	41	37	138	100	72.46
46	Mori 'A' SKM 303 x Mori 'R' GM 2	55	33	55	43	46	36	156	112	71.79
47	Mori 'A' SKM 303 x Mori 'R' GM 3	50	0	50	0	51	0	151	0	0.00
48	Mori 'A' SKM 303 x Mori 'R' SKM 9033	44	28	51	33	35	25	130	86	66.15
49	Mori 'A' SKM 303 x Mori 'R' SKM 301	51	35	44	31	43	30	138	96	69.57
50	Mori 'A' SKM 303 x Mori 'R' Pusa Bahar	47	30	53	36	52	33	152	99	65.13
51	Mori 'A' SKM 303 x Mori 'R' Vardan	61	45	56	42	36	25	153	112	73.20
52	Mori 'A' SKM 303 x Mori 'R' Bio 902	48	29	49	37	41	31	138	97	70.29
53	Mori 'A' SKM 303 x Mori 'R' 1-14	44	37	55	36	50	44	149	117	78.52
54	Standard Check – Kranti	60	54	57	52	59	53	176	159	90.34

The F<sub>1</sub> plants of cross between (*Mori* based) male sterile lines and (*Mori* based) fertility restorer lines were male fertile except F<sub>1</sub> crosses of all CMS lines with *Mori* 'R' GM 3 restorer line. The results were also supported by pollen stain ability in the F<sub>1</sub> which was about 65.15 per cent to 88.51 per cent in *M. arvensis* based CMS.

Thus, it is evident that there were striking differences between the male sterile, male fertile lines and resulting hybrids for pollen fertility as well as for number of siliquae set and per cent siliquae set upon selfing. The visual observation for presence or absence of yellow pollen dust of individual plants in F<sub>1</sub> for fertility restoration evinced that all the F<sub>1</sub> crosses of five male sterile lines with all male fertile lines (Restorer) were male fertile except with *Mori* 'R' GM 3 restorer line which indicated absence of yellow pollen dust. It suggested that the male fertile line *Mori* 'R' GM 3 lacks the fertility restorer gene (*Rf*) and has no ability to restore fertility upon crossing with cytoplasmic male sterile line possessing *Moricandia* background. The results are supported by Bhat *et al.*, (2005), Kirti *et al.*, (1997), Ashutosh *et al.*, (2014), Bhat *et al.*, (2008)

#### 4. Conclusion

A major hurdle to developing commercial heterotic hybrids of crop plants is the non-availability of a reliable pollination control system, particularly for self-pollinated crops. Among the available systems, a combination of male sterility induced by cytoplasmic genes and restoration of fertility by nuclear genes is considered to be the most efficient.

Exploitable level of standard heterosis depends on an effective male sterility and fertility system which is the most important prerequisites for the development of commercially viable hybrids. Restoration ability in CMS line is an important factor for the exploitation of hybrid in the breeding programme. Heterosis has been extensively explored and utilized for boosting various quality traits in *Brassica* because of an effective and economic pollination control system for production of F<sub>1</sub> hybrid seeds on a large scale Ashutosh *et al.*, (2014).

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