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Molecular Characterization of CMS Lines and Standardization of Hybrid Seed Production Technique in Chilli (*Capsicum annuum* L.)

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Abstract: The study has been initiated with an aim to utilize cytoplasmic male sterile (CMS) associated gene fragment marker to understand the marker flow in segregating population and nature of dominance of the marker. And further it was aimed to understand the best pollination time for the maximum fruit set and to economise the chilli hybrid seed production based on CMS lines. Hence, two CMS based high yielding hybrids, which were found to be much more potential than that of the non CMS based hybrids, have been identified. The CMS gene was dissected from one of the high yielding hybrid. The marker was successfully amplified in A line, F_1 and F_2 population with a polymerase chain reaction (PCR) product of 600 bp. The seedlings were transplanted at the ratio of 2:1, 2:2 and 3:2 sterile:fertile for natural pollination and 2:1 sterile:fertile for artificial pollination. The percent of fruit set was calculated without emasculation (CMS line) and pollination, and with emasculation and pollination, respectively. The maximum fruit set of 95.24% per plant was recorded when artificial crossing attempted between 10:00 am and 11:00 am using male sterile lines. While, 40% fruit set was observed in emasculation and pollination system. The maximum numbers of fruit set (351 and 75) were registered in JNA1 and ACA1 male sterile lines, respectively, thorough artificial pollination. However, the maximum numbers of fruit set (20.24 and 14.74) were recorded in JNA1 and ACA1, respectively, by natural pollination. Pollinating more number of flowers and fruit set success was recorded using male sterile lines than that of the bisexual plant in chilli.

Key words: Cytoplasmic male sterility, molecular marker, standardization, emasculation, pollination, chilli.

1. Introduction

In India, chilli are cultivated for dry-red and fresh-green fruits on 0.78 million ha with a total production of 1.5 million ton of dry fruits, 6,800 ton of fresh fruits and productivity of 1.93 ton/ha. The area of chilli cultivation in Karnataka was 0.09 million ha with a production of 0.112 million ton and productivity of 1.25 ton/ha [1]. The heterosis has been commercially exploited in several vegetable crops, but very few commercial hybrids are available in chilli. The greater extent of outcrossing and large number of viable seeds produced by crossed chilli fruit facilitate for development of commercial hybrids. The required goals of increasing productivity in the quickest possible time can be achieved only through heterosis breeding, which is feasible in this crop [2]. However, the cost of hybrid chilli seeds is quite high due to high labour cost. In the recent years, hybrid cultivars have become popular and many farmers are producing hybrid seeds of hot pepper based on nuclear male sterility [3]. In *Capsicum*, very few stable nuclear-cytoplasmic male sterile (CMS) lines are known, because male sterility expressions in CMS

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lines have been found to be temperature sensitive. Temperature alteration may induce a degree of variation in male sterility, ranging from complete to partial. MS-12, a monogenic, recessive genetic male sterile (GMS) line, has been developed and commercially exploited in India. This resulted in the adoption of hybrid seed production and hybrid production technologies by farmers [4]. Marker validation and utilization for screening CMS trait has been demonstrated [5]. SCAR130/140 and Rf locus associated marker (CRF-S870) were used in a multiplex polymerase chain reaction (PCR) protocol to facilitate efficient screening of cytoplasm types in peppers [6]. Male sterility systems are widely used to produce cost-effective hybrids and their seeds. In India, both nuclear GMS and CMS systems have been developed and characterized in chilli and are being used for the development of experimental crosses, potential hybrids and production of hybrid seeds. It is expected that use of male sterility system in chilli would facilitate to reduce the cost of producing hybrids seeds by 40% [7].

Exploitation of natural outcrossing could render commercial hybrid seed production technology economically viable through use of male sterile lines. Hence, concerted efforts are required to be made to identify the stable male sterile lines along with maintainer and restorer lines for exploitation of heterosis. Study has been initiated with CMS associated gene fragment marker to understand the marker flow in segregating population and nature of dominance of the marker. The CMS based hybrids have been developed and tested over a location and years. Two high yielding hybrids, namely JCH-42 (JNA1/BVC-42) and BCH-42 (ACA1/BVC-42), have been identified at University of Agricultural Sciences, Raichur, Karnataka, India. These hybrids are found to be 50% to 60% more potential than that of the non CMS based hybrids. But the hybrid seed production of CMS based hybrids is yet to be standardized. Hence, the research on standardization of hybrid seed production technology was undertaken to commercialize these hybrids.

2. Materials and Methods

The material for present study involved two stable male sterile lines, *viz.*, JNA1 and ACA1 along with their maintainers JNB1 and ACB1 and one promising strong restorer line BVC42. The best performing high yielding hybrid BCH-42 was selected for dissecting the CMS gene along with ACA1 used as male sterile line, ACB1 as maintainer line and BVC42 as a restorer. The F_1 hybrid was selfed to produce F_2 population. The leaf samples were collected separately from each plant and DNA isolation was done through cetyl trimethymammonium bromide (CTAB) method. Amplification was done using gene specific fragment through PCR. Electrophoresis was carried out and banding pattern was observed in sterile (A), maintainer (B) and restorer (R) lines and F_1 and F_2 population. CMS-associated gene fragment was amplified using primers P1: 5'-CCGGAATTCCAGCCTAGCTCGACCCAA-3' (EcoRI restriction site in bold) and reverse primer P2: 5'-CCCAAGCTTGCCTCCATCCTCCGTTAT-3' (HindIII restriction site in bold) designed according to Refs. [8, 9]. The 20 μL reaction system was: 1.5 μL of (25 ng/μL) DNA, 1 μL of 2.5 mM mixed dNTPs, 2 μL of 10 × Taq DNA polymerase buffer ($MgCl_2$), 0.4 μL of 10 μM forward and reverse primer, 0.3 μL of 5 U/μL Taq DNA polymerase and 13.4 μL of sterile water. The PCR program initially started with 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 57 °C for 45 s, 72 °C for 90 s and then 72 °C extension for 10 min, and finally 4 °C to terminate the reaction. This primer amplifies CMS associated gene fragment (ORF456).

The experiment for standardization of seed production technique was conducted at experimental field of University Campus with three replications using two male sterile lines, *viz*., JNA1 and ACA1, and one fertility restorer line BVC-42. The seedlings

were transplanted at the ratio of 3:2, 2:2 and 2:1 sterile:fertile for natural pollination. However, 2:1 sterile:fertile was the transplanting ratio for artificial pollination. Five plants were selected from each replication and each planting ratio using two sterile lines for natural pollination and subjected to statistical analysis as presented in Table 1. However, 15 plants of each sterile line were pollinated artificially. Fruit set of randomly selected three plants was pooled to get per plant fruit set in artificial pollination. The percentage of fruit set was calculated without emasculation (CMS line) and pollination, and with emasculation and pollination, respectively (Table 2). The recommended cultural practices were followed as per package of practices.

3. Results and Discussion

3.1 Identification of Molecular Markers for Cytoplasmic Male Sterile Line

The molecular markers were used to characterize the male sterile, maintainer and restorer lines. The samples were collected in seedling stage from all above A, B and R lines as well as F_1 and F_2 population of male sterility based hybrids. The male sterility restorer gene specific P_1 and P_2 molecular markers were identified to trace the male sterile line (Fig. 1). The banding pattern was observed at 600 bp in case of male sterile plants, like A_1, A_2, A_3, A_4 and A_5 as well as F_1 hybrid, *viz.*, F_11, F_12 and F_13, and the segregating plants F_21, F_22 and F_23. However, it was absent in sterility maintainer line B_1 and B_2 and fertility restorer line R_1 and R_2 plant and segregating plant F_24. The results are concordance with the findings of Kim et al. [9] and Liu et al. [10].

3.2 Standardization of CMS Based Hybrid Seed Production Technique

Two CMS lines, like JNA1 and ACB1, and one restorer line, i.e., BVC42 were used to standardize the hybrid seed production technique for CMS based hybrid seed production in chilli. The range of 250-451 crossed fruits/plant were possible to produce with mean

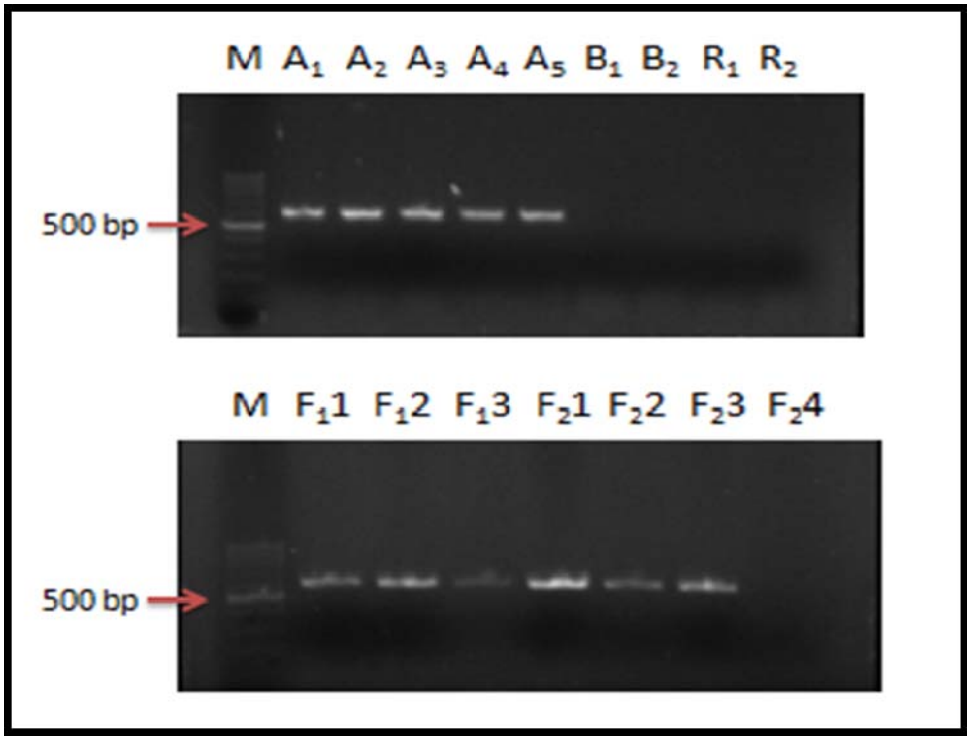

Fig. 1 Identification of sterility maintainer gene specific primers P_1 and P_2.
M: ladder or marker. The banding pattern was observed at 600 bp for male sterile plants A_1-A_5, F_11-F_13 and segregating plants F_21-F_23 which were governed by the male sterile gene.

of 351 fruits/plant by using male sterile line JNA1. However, 56-95 fruits/plant with a mean of 75 crossed fruits/plant were produced by male sterile line ACA1 under artificial pollination. While, range of 15.33-17.00, 24.00-27.00 and 15.33-16.33 and 23.66-24.00, 15.00-19.00 and 21.00-24.00 crossed fruits were produced by JNA1 and ACA1 using 2:1, 2:2 and 3:2 planting ratio (sterile:fertile), respectively, under natural pollination (Table 1).

3.3 Production of Hybrid Seed Using Male Sterile Line and Emasculation and Pollination

Flowers were pollinated without emasculation and with emasculation using CMS lines and bisexual plants, respectively, to calculate percentage of success of fruit set. No significant differences were observed in case of all above ratio for fruit set in natural pollination. The number of fruit set was different in different male sterile lines. The maximum fruit set of 95.24% per plant was recorded when crossing attempted between 10:00 am to 11:00 am without emasculation and pollination using CMS lines (Table 2). However, fruit set was possible to extend up to 40% in case of emasculation and pollination using bisexual flower. The finding was concordance with the finding of Kivadasannavar et al. [11, 12]. They recorded significantly higher fruit set (53.63%) when pollination attempted at 9:00 am to 12:00 noon on one day after emasculation. The rate of success of fruit set (85% to 95%) was increased between 10:00 am and 11:00 am at the maximum and minimum temperature range of 32.8 °C to 20 °C, respectively. It has been noticed that during cloudy weather (January 19 to January 23, 2016), the maximum of 73.68% fruit set was achieved without emasculation and pollination using CMS lines at the maximum temperature below 30 °C and the minimum temperature above 20 °C. However, only 22.50% fruit set was noticed with emasculation and pollination using bisexual flowers during afternoon hours (14:30 pm) as temperature falls down. Desirable percentage of fruit set was observed between the temperature range of the maximum 32-35 °C and the minimum 20-22 °C. Crossing was also affected during pick period of flowering. It was possible to pollinate 98.75 fruits/20 min (296.25 fruits/h) by using male sterile line with the maximum percentage of success of 94.89%. However, only 42.18 fruits/20 min (126.54 fruits/h) were emasculated and pollinated by using bisexual flowers with the maximum percentage of success of 40% (Table 3).

Table 1 Fruit set using artificial and natural pollination per plant using 2:1, 2:2 and 3:2 of sterile and fertile ratio under field condition.

Plant No.	Artificial pollination		Natural pollination					
	Sterile line (unreplicated)		Sterile line with different female:male planting ratio (replicated)					
	JNA1	ACA1	JNA1 2:1	ACA1 2:1	JNA1 2:2	ACA1 2:2	JNA1 3:2	ACA1 3:2
1	250	95	17.00	24.00	16.00	25.33	15.00	21.00
2	312	66	15.66	24.66	16.00	24.00	19.00	22.00
3	402	93	15.33	25.66	16.33	23.66	15.33	24.00
4	451	56	16.66	27.00	15.33	25.33	18.66	24.00
5	338	64	17.00	26.33	16.00	24.66	16.00	21.00
Mean	351	75	16.33	25.53	15.93	24.60	16.80	22.40
CV	-	-	20.24	14.74	17.13	9.90	16.21	13.80
CD	-	-	NS	NS	NS	NS	NS	NS

NS: not significant.

Unreplicated means trial was without replication using five sterile plants for each sterile line, but in case of natural pollination replicated trial was conducted using different ratio of sterile and fertile plants.

Table 2 Artificial pollination using CMS line and emasculation and pollination technique.

Date	Time	Crossing using CMS				Crossing with emasculation and pollination				Temperature	
		Success	Fail	Total	% success	Success	Fail	Total	% success	Max	Min
10/11/2015	10:00-11:00	35	5	40	87.50	15	25	40	37.50	32.80	21.60
11/11/2015	10:00-11:00	38	2	40	95.00	16	24	40	40.00	30.50	20.60
12/11/2015	10:00-11:00	34	6	40	85.00	14	26	40	35.00	32.30	20.00
10/12/2015	10:00-11:00	22	20	42	52.38	8	38	40	20.00	31.80	20.80
12/12/2015	10:00-11:00	26	15	41	63.41	7	33	40	17.50	32.60	20.30
19/01/2016	14:30-15:15	22	18	40	55.00	6	34	40	15.00	31.00	20.90
19/01/2016	15:15-16:00	20	16	36	55.56	6	34	40	15.00	31.00	20.90
20/01/2016	14:30-15:15	14	5	19	73.68	9	31	40	22.50	24.80	20.80
20/01/2016	03:15-04:15	15	10	25	60.00	7	33	40	17.50	24.80	20.80
21/01/2016	14:00-15:00	10	12	22	45.45	6	34	40	15.00	29.70	18.40
22/01/2016	14:15-15:00	12	15	27	44.44	5	35	40	12.50	29.70	19.90
23/01/2016	14:00-15:00	06	36	42	14.29	3	37	40	7.50	30.50	18.50
10/02/2016	10:00-11:00	24	6	30	80.00	10	30	40	25.00	35.10	21.00
13/02/2016	10:00-11:00	27	9	36	67.00	6	34	40	15.00	35.20	22.10
14/02/2016	10:00-11:00	24	10	34	70.59	9	31	40	22.50	35.40	20.90
15/02/2016	10:00-11:00	20	1	21	95.24	12	28	40	30.50	34.40	22.00
01/03/2016	10:00-11:00	10	10	20	50.00	7	33	40	17.00	35.10	23.70
04/03/2016	10:00-11:00	09	11	20	45.00	6	34	40	15.00	35.80	23.60

Table 3 Number of crosses attempted by skilled worker using CMS lines and emasculation and pollination technique during pick flowering time.

Days	Crossing using CMS				Crossing with emasculation and pollination			
	Time (min)	Crosses attempted	Success	% success	Time (min)	Crosses attempted	Success	% success
1st	20	100.00	94.00	94.00	20	35.00	14.00	40.00
2nd	20	105.00	99.00	94.28	20	40.00	12.00	30.00
3rd	20	106.00	100.00	94.33	20	38.00	15.00	39.47
4th	20	110.00	105.00	95.45	20	45.00	15.00	33.33
5th	20	95.00	90.00	94.73	20	50.00	18.00	36.00
6th	20	98.00	93.00	94.89	20	39.00	15.00	38.46
7th	20	85.00	80.00	94.11	20	45.00	18.00	40.00
8th	20	102.00	95.00	93.13	20	48.00	19.00	39.58
9th	20	99.00	92.00	92.92	20	34.00	13.00	38.23
10th	20	97.00	92.00	94.84	20	38.00	15.00	39.47
11th	20	89.00	75.00	84.26	20	52.00	20.00	38.46
Mean		98.75	92.27	84.82		42.18	15.81	37.54

4. Conclusions

From the present study, it has been concluded that male sterile lines can be easily identified to create the variability by using molecular markers to fasten the breeding work for the development of CMS based hybrids. Fruit set was too high using male sterile lines with artificial pollination between 10:00 am and 11:00 am. Hence, it is recommended to attempt the artificial pollination to achieve commercial hybrid seed production using male sterile line.

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References

[1] Geetha, R., and Selvarani, K. 2017. "Study of Chilli Production and Export from India." *IJARIIE* 3 (2): 205-10.

[2] Joshi, S., and Singh, B. 1980. "A Note on Hybrid Vigour in Sweet Pepper (*Capsicum annuum* L.)." *Haryana Journal of Horticulture Science* 9: 90-2.

[3] Dash, S. S., Kumar, S., and Singh, J. N. 2001. "Cytomorphological Characterization of a Nuclear Male Sterile Line of Chilli Pepper (*Capsicum annuum* L.)." *Cytologia* 66 (4): 365-71.

[4] Dhaliwal, M. S. 2010. "Exploitation of Male Sterility System." In *Advances in Chilli Research,* edited by Kumar, R., Rai, A. B., Rai, M., and Singh, H. P. New Delhi, India: Studium Press Pvt. Ltd., 133-44.

[5] Kumar, A. M., Reddy, K. N., Sreevathsa, R., Ganeshan, G., and Udayakumar, M. 2009. "Towards Crop Improvement in Bell Pepper (*Capsicum annuum* L.): Transgenics (uid A::hpt II) by a Tissue-Culture-Independent *Agrobacterium* Mediated in Planta Approach." *Scientia Horticulturae* 119 (4): 362-70.

[6] Lin, S., Shieh, H., Teoh, Y., and Kumar, S. 2016. "Markers for Cytoplasmic Male Sterility (CMS) Traits in Chili Peppers (*Capsicum annuum* L.): Part I, Multiplex PCR and Validation." SABRAO *Journal of Breeding and Genetics* 48 (4): 465-73.

[7] Lin, S. W., Chou, Y. Y., Shieh, H. C., Ebert, A. W., Kumar, S., Mavlyanova, R., Rouamba, A., Tenkouano, A., Afari-Sefa, V., and Gniffke, P. A. 2013. "Pepper (*Capsicum* spp.) Germplasm Dissemination by AVRDC (the World Vegetable Center): An Overview and Introspection." *Chronica Horticulturae* 53 (3): 21-7.

[8] Deng, M. H., Wen, J. F., Huo, J. L., Zhu, H. S., Wang, P., Dai, X. Z., Zhang, Z. Q., Zhou, H., and Zou, X. X. 2012. "Molecular Characterization and Prokaryotic Expression of Orf507 Sterility-Associated Gene in Chilli Pepper (*Capsicum annum* L.) Cytoplasmic Male Sterility." *Pak. J. Bot.* 44 (5): 1497-502.

[9] Kim, D. H., Jeong, G. K., and Kim, B. D. 2007. "Isolation and Characterization of the Cytoplasmic Male Sterility Associated Orf456 Gene of Chili Pepper (*Capsicum annuum* L.)." *Plant Molecular Biology* 63 (4): 519-32.

[10] Liu., C., Ma, N., Wang, P. Y., Fu, N., and Shen, H. L. 2013. "Transcriptome Sequencing and De Novo Analysis of a Cytoplasmic Male Sterile Line and Its Near-Isogenic Restorer Line in Chili Pepper (*Capsicum annuum* L.)." *PLoS ONE* 8(6): e65209.

[11] Kivadasannavar, P. 2008. "Standardization of Hybrid Seed Production Techniques in Chilli (*Capsicum annuum* L)." Ph.D. thesis, Department of Seed Science and Technology, Dharwad University of Agricultural Sciences, India.

[12] Kivadasannavar, P., Deshpande, B. S., Vyakaranahal, V. K., Mohankumar, H. D., Biradar, D. P., and Nadaf, H. L. 2009. "Studies on Emasculation and Pollination in Hybrid Seed Production of Chilli (*Capsicum annuum* L.)." *Karnataka Journal of Agricultural Sciences* 22 (2): 301-5.