Indian J. Genet., 80(3) 261-266 (2020)
DOI: 10.31742/IJGPB.80.3.4

Doubled haploid production in maize under sub-montane Himalayan conditions using *R1-nj*-based haploid inducer TAILP1

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(Received: January 2020; Revised: July 2020; Accepted: August 2020)

Abstract

The use of *in vivo* haploid induction system makes the doubled haploid (DH) technology easier to adopt for the conventional maize breeders. However, despite having played an important role in the initial developmental phases of DH technology, Indian maize research has yet to harvest its benefits. Haploid Inducer Lines (HILs) developed by CIMMYT are being widely used in maize breeding programmes in many countries including India. There, however, is no published information on the efficiency of DH line production using CIMMYT HILs in Indian maize breeding programmes. In the present study, the efficiency of DH production using CIMMYT's tropically adapted inducer line TAILP1 was investigated with eight source populations including two of sweet corn. The average haploid induction rate (HIR) of TAILP1 was 5.48% with a range of 2.01 to 10.03%. Efficiency of DH production ranged from 0.14 to 1.87% for different source populations with an average of 1.07%. The information generated will be useful for maize breeders intending to use DH technology for accelerated development of completely homozygous lines.

Key words: Maize, haploid inducer, doubled haploid, HIR, TAILs

Introduction

Doubled Haploid (DH) programmes in crops aim at obtaining completely homozygyous lines in a shorter period of time compared to conventional breeding. Different DH development methods are employed in different crops depending upon their efficiency and ease of use. For example, while anther/pollen culture is the popular method in rice, crossing with related species is the preferred method in wheat. In case of maize, due to lower efficiency and high genotype specificity of *in vitro* methods, the more efficient *in vivo* method is commonly used (Chaikam et al. 2012; Chaikam et al. 2019a). *In vivo* DH production in maize involves haploid induction, identification of putative haploids followed by chromosome doubling and generation of DH seed (Prasanna et al. 2012; Chidzanga et al. 2019).

In Europe, North America and China, *in vivo* DH technology has been adopted as a routine method by many commercial maize breeding programmes (Chaikam et al. 2019a; Molenaar and Melchinger 2014). Collaborated efforts by CIMMYT and the University of Hohenheim have helped in making the technology accessible to tropical breeding programmes in both public- and private-sector organizations (Chaikam et al. 2019a).

In India, major maize breeding companies have been known to be using DH lines in their maize programmes either sourced from their parent companies/associates overseas or developed in their Indian programmes. However, despite having made significant contributions in the initial developmental phases of the DH technology (Coe and Sarkar 1964; Sarkar and Coe 1966; Sarkar and Coe 1971; Aman and Sarkar 1978), Indian maize research has lagged behind in adopting DH technology and harvesting its benefits. Barring few sporadic reports on some elementary work on DH (Khulbe et al. 2019), there is hardly anything substantial as far as large-scale production of DH lines and release of DH-based hybrids is concerned probably due to the lack of knowledge and expertise in DH technology and limited resources and the basic infrastructure required for DH

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

programmes. Among the key factors determining success of a DH programme, efficiency of haploid induction, referred to as Haploid Induction Rate (HIR), is of critical importance. The haploid inducers developed by CIMMYT are accessible to public and private maize programmes. The first set of Tropically Adapted Inducer Lines (TAILs) developed by CIMMYT in collaboration with the University of Hohenheim (TAILs) has HIR of 6-13% (Chaikam et al. 2012). The second generation inducer lines released by CIMMYT in 2018 (CIM2GTAILs) are reported to have higher HIR (9-14%), besides being agronomically better (Chaikam et al. 2018; Chaikam et al. 2019a). These inducer lines carry anthocyanin marker on seed that enables detection of haploid seed in an induction cross. HIR of inducer lines varies with source populations as the trait is influenced by maternal genetic background (Prigge et al. 2011; Kebede et al. 2011; Nair et al. 2019).

Though a number of private seed sector companies in India are known to be using DH technology, there are no published reports on the HILs and chromosome doubling agents being used, and on the overall efficiency in their DH programmes. The present study, therefore, was undertaken with the objective of evaluating the HIR of CIMMYT haploid inducer line TAILP1 and the overall efficiency of the DH programme based on CIMMYT protocol (Prasanna et al. 2012) under sub-montane Himalayan conditions at ICAR-VPKAS, Almora.

Materials and methods

Generation of induction crosses

Eight source populations and CIMMYT haploid inducer TAILP1 were used in the study. The source populations comprised F_1 hybrids/F_2s of elite private and public bred maize varieties and in-house experimental hybrids belonging to early maturity group. CIMMYT protocol described by Prasanna et al. (2012) was followed for production of DH lines with minor modifications according to local conditions.

The source populations were planted in 3 m long rows in *kharif* 2018 at ICAR-VPKAS, Experimental Farm, Hawalbagh (1250 m amsl, latitude 29°362 N, longitude 79°402 E). The row-to-row spacing was kept at 60 cm and plant-to-plant spacing was maintained at 25 cm so as to have a final stand of 12 plants in each row. Number of plants per source population ranged from 100 to 200 depending upon the DH lines targeted to be obtained from each population. *R1-nj*-based TAILP1 (EC805127), developed at CIMMYT in collaboration with University of Hohenheim and provided by ICAR-Indian Institute of Maize Research, Ludhiana, was used as the male parent.

Since TAILP1 is a shy pollen producer at Hawalbagh location, a sufficiently large population (20 rows of 3 m long each) of TAILP1 was raised to have sufficient pollen for pollinating the source populations. Staggered planting of TAILP1 at 7 days interval was done to ensure synchrony in flowering with the source populations. At flowering, individual plants in source populations were pollinated manually with bulk pollen of TAILPI. The pollinated ears from each source population were harvested separately at physiological maturity and sun-dried.

Raising of D_0 populations

The D_0 populations of the eight induction crosses were raised in *kharif* 2019 following standard steps of CIMMYT protocol that comprised (i) haploid seed classification (ii) seed germination and colchicine treatment (0.04%, 12 h) (iii) transfer of treated seedlings to cups/protrays (iv) transfer of plants to field (v) removal of false positives (diploid plants) (vi) self-pollination of fertile D_0 plants, and (vii) harvesting of D_1 ears.

Haploid Induction Rate (HIR)

The HIR was calculated as:

No. of putative haploid seeds/total seeds in the induction cross x 100 whereas total no. of seeds determined as wt. of total seeds in the induction cross x 1000/1000-seed wt* (*1000-seed was averaged over three samples).

The relative and *per se* percentage at different working steps of the process were calculated as per the procedure followed by Couto et al. (2019):

100 x Total seeds transferred to cups after colchicine treatment/Total haploid seeds; 100 x Total seedlings transplanted in field/total seed potted in cups and 100 x Total plants in field/total seedlings transplanted in field.

Total haploid plants in field after removing false positives (diploid plants) were determined as 100 x Total haploid plants in field/total plants in field. Self-pollination in D_0 plants were calculated as 100 x Total plants selfed/total haploid plants while total D_1 ears harvested were found out as 100 x Total plants with seed set/total plants selfed.

Per se percentage at each working step was worked out as per the formula: No. of plants at a working step/Total seeds in the induction cross x 100.

Mis-classification percentage (MCP) was worked out as: 100 x Total diploid plants/total plants in field while efficiency of DH method was calculated as 100 x Total D_1 ears harvested/ Total seeds in the induction cross.

Results

The results presented in Table 1 are summarized as follows:

Haploid Induction Rate (HIR): The average haploid induction rate (HIR) of TAILP1 was 5.48% with a range from 2.01% in Golden Super Sweet to 10.03% in MCT-3. Average HIR for both the sweet corn source populations (Golden Super Sweet and Sugar 75 F_2) was lower compared to normal corn source populations.

Transfer to cups: The average relative percentage of seedlings transferred to cups after germination and colchicine treatment was 89.9 with a range of 82.7 to 94.1, whereas the *per se* percentage was 4.93.

Transfer to field: The relative percentage ranged from 53.8 to 96.6 with an average of 75.0. Average *per se* percentage at this step was 3.7.

Total plants in field: Survival in the field ranged from 49.2 to 85.1 per cent with an average of 66.1 per cent. The average *per se* survival percentage was 2.44 per cent.

Total haploid plants in field: The relative percentage of total haploid plants in the field ranged from 94.5 to 99.06 with an average of 98.1. The *per se* average percentage was 2.40.

Self-pollinated D_0 plants: The relative percentage of self-pollinated D_0 plants ranged from 36.2 (Sugar 75 F_2) to 73.3 (Pusa Vivek QPM9 Improved) with an average of 60.9. The average *per se* percentage at this step was 1.46.

Total D_1 ears harvested: Relative percentage of harvested D_1 ears ranged from 41.2 in Sugar 75 F_2 to 90.8 in VMH 43 F_2 with an average of 73.7. The *per se* percentage of harvested D_1 ears was 1.07.

Mis-classification percentage (MCP)

The percentage of mis-classified plants in the field was small. The average relative percentage was 1.93 with a range of 0.94 (MTC-2) to 5.48 (MTC-1).

Comparison of loss of population occurring at different working steps (Table 2) indicated that maximum loss was due to lower frequency of fertile plants with both ear and tassel (39.1%, Step 4-5) followed by lower field survival (33.9%, Step 2-3), lower frequency of plants with seed set (26.6%, Step 5-6) and toxic effect of colchicine (25%, Step 1-2).

Discussion

The mean HIR of TAILP1 (5.48%) and range (2.01-10.03%) obtained in the present investigation falls in the range reported earlier for the TAILs (6-12%, Chaikam et al. 2012; 6.17-8.48%, 4 to 10%, Nair et al. 2019; 0.13-15.49%, Couto et al. 2019). The variation in HIR among the source populations may be attributed to the maternal genotypes. Influence of maternal genotype on the HIR has been reported by many workers (Röber et al. 2005; Prigge et al. 2011; Eder and Chalyk 2002; Kebede et al. 2011; Nair et al. 2019). Some studies also indicated the influence of environment on HIR which has also been recorded by several workers earlier (Röber et al. 2005; Kebede et al. 2011; Fuente De La et al. 2018; Aman and Sarkar 1978).

HIR is computed on the basis of proportion of putative haploid seed or plants, and the identification of haploid seed is based on *R1-nj* expression in the seed of induction cross. Poor intensity of the *R1-nj* marker expression can result in high rates of misclassification (Röber et al. 2005; Melchinger et al. 2014; Prigge et al. 2011). Compared to earlier reports on misclassification percentage (MCP) (1.46-37.34%, Kebede et al. 2011; 10-30%, Chaikam and Mahuku 2012), MCP in the present investigation was low (0.94-5.48%). The lower MCP may be attributed to full expression on *R1-nj* marker that allowed haploid classification with high accuracy in the induction crosses of all the eight source populations. Lower MCP may also result if high stringency is practiced in haploid classification. This however, may cause loss of some true haploid seed due to their misclassification as diploid seed. The reasons for lower HIR in the two sweet corn source populations are a matter of further scientific investigation as the chances of seed mis-classification were least since *R1-nj* marker expression was complete. Moreover, detection of selfed and out-crossed seed is easy in sweet corn because of its wrinkled seed, which becomes normal due to xenia effect when pollinated with normal maize pollen.

The Table 2 indicated that maximum loss of population was due to lower frequency of normal

Table 1. Percentage *per se* and relative percentage at different working steps of the DH production process

S.No.	Source populations	Total induction cross (IC) ears	Seeds/ ear	Total seed (TS)	Total putative haploid seed (THS)		Self/out-crossed inhibited		Aborted seed (TDS)		Total diploid seed		Total Transferred to Cups (TTC)			Total Transferred to Field (TTF)		
					No.	% of TS	No.	% of TS	No.	% of TS	No.	% of TS	No.	% of THS	% of TS	No.	% of TTC	% of TS
1	Pusa Vivek QPM 9 Imp.	108	132	14295	1146	8.02	331	2.32	252	1.76	12566	87.90	1018	88.83	7.12	718	70.53	5.02
2	VMH 27 F_2	133	153	20361	915	4.49	388	191	37	0.18	19021	93.42	857	93.66	4.21	733	85.53	360
3	VMH 43 F_2	104	151	15725	619	3.94	200	1.27	362	2.30	14544	92.49	562	90.79	3.57	436	77.58	2.77
4	Sugar 75 F_2	46	107	4938	102	2.07	106	2.15	147	2.98	4583	92.81	87	85.29	1.76	84	96.55	1.70
5	Golden Super Sweet	127	91	11530	232	2.01	1327	11.51	26	0.23	9945	86.25	207	89.22	1.80	160	77.29	1.39
6	MTC-1	54	161	8679	272	3.13	33	0.38	53	0.38	8341	96.11	225	82.72	2.59	121	53.78	1.39
7	MTC-2	98	119	11672	1110	9.51	193	1.65	144	1.23	10225	87.60	1045	94.14	8.95	780	74.64	6.68
8	MTC-3	83	101	8367	839	10.03	203	2.43	159	1.90	7166	85.65	707	84.27	8.45	500	70.72	5.98
	Total	**753**	**1015**	**95567**	**5235**	**5.48**	**2781**	**2.91**	**1160**	**1.21**	**86391**	**90.40**	**4708**	**89.93**	**4.93**	**3532**	**75.02**	**3.70**

Table 1 contd.....

S.No.	Source population	Total plants in field (TPF)			Total diploid plants in Field (TDPF)			Total haploid plants in Field (THPF)			Total seif-pollinated D Plants (TSPP)			Total harvested D ears (THDE)		
		No.	% of TF	% of TS	No.	% of TPF	% of TS	No.	% of TPF	% of TS	No.	% of THP	% of TS	No.	% of TPP	% of TS
1	Pusa Vivek QPM Improved	463	64.48	3.24	6	1.30	0.04	457	98.70	3.20	335	73.30	2.34	267	79.7	1.87
2	VMH 27 F_2	624	85.13	2.71	13	2.08	0.06	611	97.92	2.65	348	56.96	1.51	250	71.8	1.08
3	VMH43 F_2	246	33.42	1.56	8	3.25	0.05	238	96.75	1.51	130	54.62	0.85	118	90.8	0.75
4	Sugar 75 F_2	49	58.33	0.99	2	4.08	0.04	47	95.92	0.95	17	36.17	0.34	7	41.2	0. t4
5	Golden Super Sweet	103	64.38	0.89		0.97	0.01	102	99.03	0.88	57	55.88	0.49	37	64.9	0.32
6	MTC-1	73	60.33	0.84	4	5.48	0.05	69	94.52	0.80	50	72.46	0.58	37	74.0	0.43
7	MTC-2	532	68.21	4.56	5	0.94	0.04	527	99.06	4.52	295	55.98	2.53	183	62.0	1.57
8	MTC-3	246	49.20	2.94	6	2.44	0.07	240	97.56	2.87	164	68.33	1.96	126	76.8	1.51
	Total	**2336**	**66.14**	**2.44**	**45**	**1.93**	**0.05**	**2291**	**98.07**	**2.40**	**1396**	**60.9**	**1.46**	**1025**	**73.4**	**1.07**

Table 2. Loss of population at different working steps starting with haploid seeds

Working step	Total putative haploid seed (THS)	Total transferred to cups (TTC)	Total transferred to field (TTF)	Total plants in field (TPF)	Total haploid plants in field (THPF)	Total self-pollinated D_0 plants (TSPP)	Total harvested D_1 ears (THDE)
Total population	5235	4708	3532	2336	2291	1396	1025
% Loss	10.1	25.0	33.9	1.9	39.1	26.6	

(bearing both ear and tassel) fertile plants (39.1%) followed by lower field survival (33.9%), lower frequency of plants with seed set (26.6%) and loss after transfer to cups post-colchicine treatment (25%). Loss during potted-cup stage is attributable mainly to the toxic effect of colchicine and fungal/bacterial infection at initial and later stages. Good agronomic management also improves fertility and seed set in self-pollinated plants. Kleiber et al. (2012) observed almost five-fold higher fertile haploids under greenhouse (0-70%) than field conditions (0-20%) and suggested that shading nets, drip-irrigation, and effective plant protection measures such as insecticide application to the developing ear after self-pollination may contribute to increase proportion of fertile haploids under field conditions.

A comparison of efficiency of the DH production process in the present study with that inferred from earlier works (Erdal et al. 2019; Couto et al. 2019) indicated that the efficiency of DH production in the present study was significantly higher (1.07%). The higher efficiency in the present study resulted from higher field survival, lower mis-classification percentage, higher proportion of fertile plants and higher number of plants with seed set. Higher field survival is attributable to good agronomic management and favourable climatic conditions at Almora. The proportion of diploid plants (mis-classification percentage) was very low as all the source populations used in the study exhibited complete *R1-nj* expression allowing precision in identification of haploid seed. Good agronomic management and proper and adequate pollination is likely to have contributed to higher proportion of seed set in D_1 ears. However, as regards higher proportion of fertile haploid plants in the present study, genotypic differences also exist among different maize germplasm for spontaneous fertility restoration in haploids (Kleiber et al. 2012; Ma et al. 2018; Chaikam et al. 2019b). Spontaneous fertility restoration is caused by spontaneous doubling of the chromosome complement and results in haploid plants producing pollen and seed without being specially treated so as to stimulate doubling of the chromosome complement (Chase 1949), and may range from 0 to 70% (Kleiber et al. 2012; Chaikam et al. 2019b). A part of the variation observed for haploid fertility may, therefore, be attributed to genetic differences among the source populations studied.

In the present study, the number of DH lines obtained per induction cross ear was 1.36 and the number of DH lines obtained per plant of the source population (assuming loss of about 15% plants due to non-germination or post-germination mortality) worked out at 1.18, which broadly translates to one DH line per plant of the source population.

Authors' contribution

Conceptualization of research (RKK, AP); Designing of the experiments (RKK, AP); Contribution of experimental materials (RKK, AP); Execution of field/ lab experiments and data collection (RKK, GSB, MCP, VP, RK, NCM); Analysis of data and interpretation (RKK, AP); Preparation of manuscript (RKK, AP, LK).

Declaration

The authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the financial support received from ICAR-National Agricultural Science Fund, New Delhi and ICAR-VPKAS, Almora. The authors thank ICAR-Indian Institute of Maize Research, Ludhiana for providing *R1-nj*-based tropical haploid inducer TAILP1 used in this study.

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