Pantnagar Journal of Research

**(Formerly International Journal of Basic and
Applied Agricultural Research ISSN : 2349-8765)**

G.B. Pant University of Agriculture & Technology, Pantnagar

CONTENTS

Plumule colouration as a criterion to improve the efficiency of R1-nj marker based doubled haploid breeding in maize

PRABHAT SINGH, MUKESH KUMAR KARNWAL, SMRUTISHREE SAHOO, ARVIND CHAUHAN and NARENDRA KUMAR SINGH

Department of Genetics and Plant Breeding, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar- 263145 (U.S. Nagar, Uttarakhand)

ABSTRACT: The experiment was conducted using eight source populations comprised of F1s, F2s, landrace and composite genotype of both field corn and sweet corn and these genotypes were crossed as a female parent with CIMMYT bred tropicalized advance haploid inducer line possessing R1-nj colour marker gene. Based on the colour marker of endosperm as well as endosperm and embryo, the seeds were classified into contaminants, haploids and diploids. The haploid kernels were then categorized based on plumule coloration and no plumule colouration after *in vitro* germination. Plumule coloured seedlings were directly grown in the field by hypothesizing that these seedlings are similar to diploids but the confirmations were still needed. The remaining seedlings that did not have plumule colour were diploidized using colchicines (0.04%) after cutting shoot and root tips of the *in vitro* germinated seeds. The seedlings having colour on plumule exhibited differential segregation on kernels after selfing due to R1-nj background effect whereas seedlings without plumule colour showed significant haploid behaviour. The seedlings sorted from haploids after germination on the basis of plumule colour was highest in PSM-3 (22) followed by Landrace DL1 (19) whereas the misclassification percentage was highest of 25% in Landrace DL1 followed by PSM-3 (18%) and sweet corn F_1's (13%). The results therefore showed that second line of sorting using plumule colour in germinated seedlings has significant impact on reducing false doubled haploids and thereby increasing resource use efficiency in doubled haploid breeding program.

Key words: Contaminant, heploid, kemels, maize

In crops, doubled haploid (DH) programs seeks to produce entirely homozygous lines in a shorter duration of time than conventional breeding. In various crops, different DH development strategies are being used according to their efficiency and convenience of usage. In rice, for example, anther/pollen culture is the favored strategy, but in wheat, crossing with related species is the favored method such as maize × wheat hybridization and in barley gradual chromosomal elimination are very common doubled haploid development tools. In the case of maize, *in-vivo* haploid induction technique is preferred approach because of disadvantages of lower efficiency and high genotype specificity associated with *in-vitro* methods (Chaikam and Mahuku, 2012; Chaikam *et al.,* 2019). In case of *in-vivo* technique, haploid induction (crossing of source population with inducer genotypes), identification of putative haploids, chromosomal doubling, and creation of DH seeds are the steps in the development of DH in maize (Prasanna *et al.,* 2012; Chidzanga *et al.,* 2019). In the last two decades, the DH technique based on *in vivo* haploid induction (HI) has emerged as one of the most significant tools in maize breeding, displacing the conventional approach of recurrent selfing. In fact, haploid plants are induced naturally in maize fields however induction rate is very low of around 0.01 per cent (Chase,

1949). The discovery of maize lines Stock 6 and W23 (Coe and Sarkar, 1964), as well as subsequent improved haploid-inducing lines including ZSM, KMS, and MHI (Chalyk, 1994), has made significant strides in integration of DH technique in maize breeding. DH success rate of 8-20 per cent has been achieved in many maize breeding programmes using *in vivo* tool. When utilized as pollinators, maize haploid inducer effect results into the formation of haploid embryo seeds at an average rate of 8% due to hetero-fertilization and unsuccessful egg-sperm cell fusion (Tian *et al.,* 2018). With improving higher haploid induction rate as a major objective, the other factor for discussion should be to reduce the DH line production costs and improving chromosome doubling, there are less genetic research on spontaneous chromosome doubling (Chaikam *et al.,* 2018), and more efficient methodologies for selecting haploid kernels based on R1-nj expression on the basis of visual screening of seeds (Battistelli *et al.,* 2013) are being utilized. Despite the successful development of maize haploid inducers in the tropical regions (Chaikam and Mahuku, 2012, Couto *et al.,* 2019), data on DH mechanism is still lacking in tropical and subtropical regions especially in African and South-East Asian countries such as India, Bangladesh, Nepal and Pakistan. Although, after the development of tropically

adapted inducer lines (TAILs) by CIMMYT, Mexico and University of Hohenheim, Germany for haploid induction in maize grown in tropical regions (Prigge *et al.*, 2011) the way for integration of DH tool in line development has now become more easy however many issues including efficient dormant stage seed haploid identification remained unresolved. Another issue is the present of inhibitor to R1-nj marker in the background of many targeted population leading to misclassification of haploids seeds. Problem of inhibitor gene also limits the use of R1-nj marker system with much potential population possessing inhibitory property. This problem can be minimized by using NMR technique; however the cost is the major barrier which may hinder the usage of this technique. In the present investigation, a screening technique based on plumule colour of seedlings and sorting of these seedlings before chromosome doubling, apart from R1-nj marker based haploid seed sorting was experimented for more precise screening and increasing putative haploids frequency at initial stage.

MATERIALS AND METHODS

Generation of induction crosses: Eight source populations and the CIMMYT haploid inducer Tropicalized advance inducer line were materials used in the investigation. Targeted source populations were F_1, F_2s of field and sweet corn, maize landrace DL1, and composite PSM-3 from the Pantnagar maize breeding program. For the generation of Doubled haploid lines, the CIMMYT methodology outlined by Prasanna *et al.* (2012) was followed with subtle tweaks based on local conditions. In Rabi 2020, at the Crop Research Centre, G B Pant University of Agriculture and Technology, Pantnagar (29.0222° N, 79.4908° E), the source populations were planted in 3 m long rows. To achieve a final stand of 15

plants in each row, the row-to-row spacing was kept at 60 cm and the plant-to-plant spacing was kept at 20 cm. The number of plants per source population ranged from 100 to 150. Because TAIL is a shy pollen producer at Pantnagar due to the lack of acclimatization, a large sufficient population of TAILs was grown to ensure adequate pollen for the source populations. TAIL plants were planted at 7-day intervals to achieve flowering synchronicity with the donor populations. Individual plants in source populations were manually pollinated with TAIL's bulk pollen at flowering. Each source population's pollinated ears were harvested and sun-dried separately at physiological maturity.

Visual screening of kernels and raising of DH0 Population -
In Kharif-2020, the DH_0 populations of the eight induction crosses were raised using the CIMMYT procedure, which included (i) haploid seed categorization, (ii) seed germination, and (iii) colchicine treatment (0.04 per cent for 12 h) (iii) transplanting treated seedlings into cups (iv) transplanting plants into the field (v) removing false
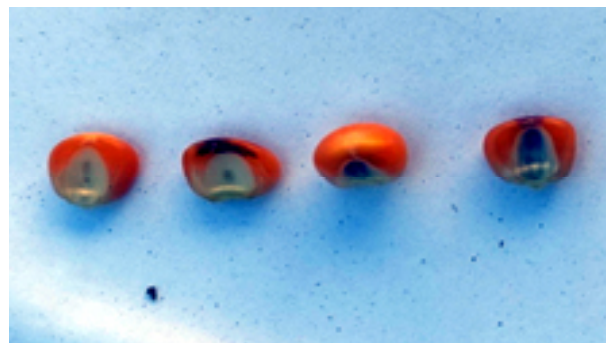

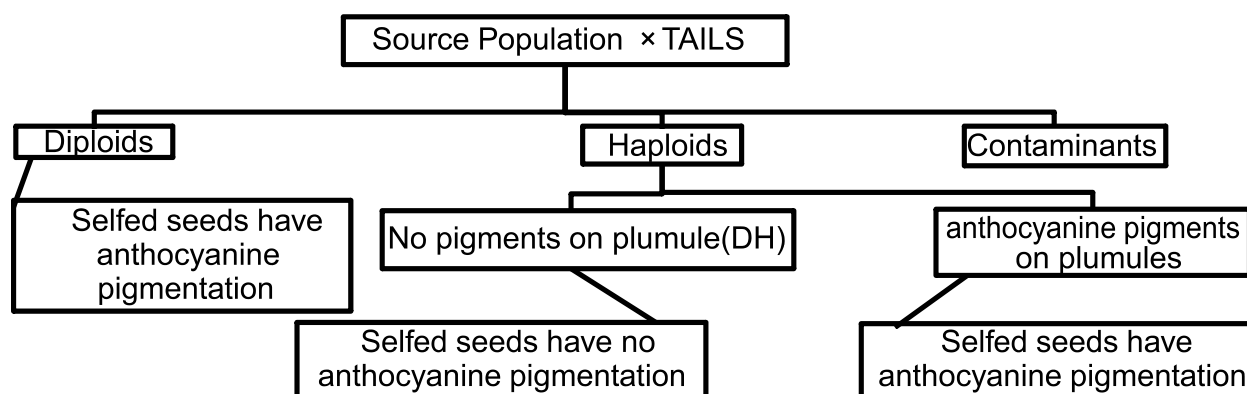**Fig.2: Categorization of haploids Diploid and contaminant kernels**

Fig.1: Flow diagram of doubled haploids and Diploid kernels segregation

positives (diploid plants) (vii) self-pollination of viable DH_0 plants and (vi) harvesting of DH_1 ears. At the initial categorization there were three different categories which are as follows: 1) Contaminants are the seeds having no color on kernels and are the result of open pollination. 2) Haploid kernels are identified on the basis of color only on the endosperm and the embryo is devoid of the color. 3) Diploid kernels can be grouped into further two categories the kernels with both embryo and endosperm color and the kernels with only embryo color . After categorization, the putative seedlings were grown in germination chamber having 85 percent relative humidity and 28^0C temperature by wrapping into germination paper. After germination the seedlings were further screened out on the basis of plumule color and without plumule color. Seedlings having plumule color were grown into the field separately with proper tagging, whereas the seedlings having no color were treated with colchicine and transferred to cups having coco-pit + Vermicompost + sandy soil (1:1:1) media at room temperature. After two leaf stage the plants were transferred to the field and further planted materials were screened out on the basis of color on leaves and associated traits and after the confirmation of Haploids selfing program were also carried out for the large number of doubled haploid populations development.

Haploid Induction Efficacy (HIE)-

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

Rate of haploids Induction - The percentage of putative haploid seeds (as determined by marker data) to the total seed set in a haploid induction cross is calculated as the haploid induction frequency, commonly known as the haploid induction rate (HIR). The Haploid Induction Rate

$$\text{Rate of haploids Induction} = \frac{\text{Haploids Seeds}}{\text{Total Seeds}} \times 100$$

(HIR) is the ratio of haploid seeds to the total seeds multiplied by 100.

Frequency of chromosome doubling: The frequency of chromosomal doubling is determined later to transplanting plantlets into the field, basically the deformity of haploids are seen in the field due to improper doubling of chromosomes.

$$\text{Per cent Chromosome Doubling} = \frac{\text{Number of plants survived after treatment}}{\text{Number of putative haploids identified and treated}} \times 100$$

Total Doubled haploid plants in field: After transplanting seedlings to the field the remaining plants survived into the field are generally termed as total DH plants into the field.

$$\text{Per cent survived DH Plants} = \frac{\text{Total seedlings transplanted in field}}{\text{Total seed potted in cups}} \times 100$$

Mis-classification percentage (MCP): Mis-classification percentage can be counted on the basis of initial screened haploids and after seedlings removal on the basis of plumule purple coloration at the time of colchicine treatment.

$$\text{MCP DH} = \frac{\text{Number of diploid seeds counted as haploids}}{\text{Number of putative haploids for chromosome doubling}} \times 100$$

Stomata count: For calculating the number of stomata per mm^2 of the leaf surface, the diameter of microscope was taken as the whole area and the r is taken 0.5mm standard length of the circle of microscope and the total stomata numbers were noted in the specified area, which can be denoted as follows:

$$\text{Area} = \pi r^2$$

RESULTS AND DISCUSSION

Haploid Induction Rate (HIR): The haploid induction rate for total 8 genotypes ranged from 6-11% at initial kernels screening. In PSM-3 genotype it was highest of 11% and the lowest haploid induction of 6% was noted in

Table 1: Percentage *per se* and relative percentage of haploid induction and germination of haploids

Sl.	Genotypes	Diploids		haploids	contaminants	Total seeds	Percent haploid induction	Germinated haploid seeds	Percent Germinated haploid seeds
		Whole seed colored	Only embryo colored						
1	PSM-3 × TAIL-1	1157	14	147	52	1370	11%	147	100%
2	Landrace DL1 × TAIL-1	844	28	95	37	1004	9%	94	99%
3	(LM-13 × CAL-147) × TAIL-1	1289	19	111	65	1484	7%	109	98%
4	CAL-147 × TAIL-1	954	9	84	79	1126	7%	83	99%
5	(LM-13 × CAL-147) F2 × TAIL-1	1353	57	130	82	1622	8%	126	97%
6	(sweet corn-8051) F1 × TAIL-1	1677	25	117	55	1874	6%	110	94%
7	(sweet corn-8001)F1 × TAIL-1	1857	10	128	22	2017	6%	123	96%
8	(sweet corn-8052) F1 × TAIL-1	1590	5	116	37	1748	7%	111	96%

Table 2: Survival ability and Percentage of DH plants at different working steps of the DH production process

S. No.	Genotypes	Haploid Seeds		True haploids	True haploid %	mis-classification %	After chromosomal doubling survived DH	After chromosomal doubling survived DH%	After transplanting survived DH	After transplanting survived DH%	After transplanting survived purple seedlings
		Colored plumule	No plumule coloration								
1.	PSM-3 × TAIL-1	22	125	125	9%	18%	108	86%	82	76%	20
2.	Landrace DL1 × TAIL-1	19	76	76	8%	25%	59	78%	56	95%	12
3.	(LM-13 × CAL-147) × TAIL-1	9	102	102	8%	9%	91	89%	68	75%	6
4.	CAL-147 × TAIL-1	8	125	125	7%	6%	108	86%	95	88%	9
5.	(LM-13 × CAL-147) F2 × TAIL-1	11	119	119	8%	8%	97	75%	84	87%	10
6.	(sweet corn-8051) F1 × TAIL-1	13	104	104	6%	11%	87	74%	55	63%	13
7.	(sweet corn-8001)F1 × TAIL-1	17	111	111	6%	13%	59	46%	47	80%	17
8.	(sweet corn-8052) F1 × TAIL-1	16	100	100	7%	14%	65	56%	32	49%	12

Table 3: After transplanting differential screening of haploids- diploids and final DH₁ cobs recovery

S. No.	Genotypes		Features								
			PPL	Stripe leaves	Tassel formation	Stomata counts	HKC	DT	TCWK	CWK	Total DH cobs
1.	PSM-3 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	20	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	75	07	78	04
2.	Landrace DL1 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	12	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	50	06	54	02
3.	(LM-13 × CAL-147) × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	06	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	59	09	65	03
4.	CAL-147 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	09	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	90	05	92	03
5.	(LM-13 × CAL-147) F2 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	10	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	84	00	84	00
6.	(sweet corn-8051) F1 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	13	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	36	19	45	10
7.	(sweet corn-8001) F1 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	17	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	40	07	40	07
8.	(sweet corn-8052) F1 × TAIL-1	PCS(Diploid)	No	Yes	All fertile	Relatively higher	PCK	All Normal	12	NA	NA
		NPS(DH)	Yes	No	Not all fertile	Low	NPCK	23	09	27	05

Abbreviations- PCS- plumule colored seedlings, NPS- No plumule colored seedlings, PPL- Purple pigments on leaves, HKC- Harvested kernel color, NPCK- No Purple coloured kernels, PCK- Purple colored kernels, HLP-Hybrid looking plants, TCWK - Total cobs with kernels, CWK-Cob without kernels, DT- Deformed Tassels.

sweet corn- 8051 F1 hybrid and sweet corn- 8001 F1 hybrids. The observations indicate that that average induction rate is lower in sweet corn hybrids as compared to other corn genotypes (Table-1).

Haploid germination percentage: The overall percent haploid germination was significantly higher and ranged from 94-100 per cent in germination chambers. The highest germination percentage was of PSM-3 haploid seed whereas the minimum percentage was of sweet corn-8051 F_1 hybrid which was 94 percent. All the three sweet corn hybrid genotypes showed comparatively lower germination percentage with respect to normal corn.

Sorting on the basis of plumule color: The seedlings sorted from haploid seedlings on the basis of plumule color was highest in Landrace DL1 land race (19) whereas it was lowest in CAL-147 inbred line (08). Higher plumule coloration seedlings rate in Landrace DL1 denotes the hindrance of expression of R1-nj color marker in embryonic region of kernels (Table-2).

Mis-classification percentage (MCP): The per cent misclassification at the time of visual screening was highest in Landrace DL1 landrace(25%) which is directly related to purple colored seedlings obtained after germination, the second most misclassification percentage holding genotype was PSM-3 with (18%) whereas the lowest misclassification per cent was in CAL-147 genotype having only 6% misclassification percentage which directly refers that the inbred based haploids are far more

easier to identify at visual kernel screening (Table 2).

Transfer to cups: After colchicine treatment the transfer of seedlings to the pots plays an important role in total seedlings survival because the plant faces dual stress condition at this step leading to different level of seedling mortality. Sweet corn-8001 F1 and sweet corn-8052 F_1 hybrids have exhibited highest seedling mortality percentage of 46% and 58% of survival only whereas, LM-13 × CAL-147 have exhibited 89% of survival rate after chromosomal doubling. The results therefore indicate that the vigor effects of population of normal maize on haploid survival.

Transfer to field: After field transfer the maximum mortality was exhibited by sweet corn-8052 F_1 hybrid which is 49%, whereas, the maximum survival percentage was of Landrace DL1 genotype (95%), the range of all the eight genotypes survival percentage varied from 49-95%.

Total haploid plants in field: A comparison of population loss at various working steps (Table 2) revealed that the greatest loss was caused by a lower frequency of fertile plants with both ear and tassel which was a cumulative effect of lower chromosomal doubling which led to higher percent of tassel and silk deformity.

Screening of Haploid-Diploid plants in the field: The screening results of purple colored seedlings and normal seedlings without color was based on stripe leave trait, Purple pigments on leaves, Hybrid looking plants, Stomata
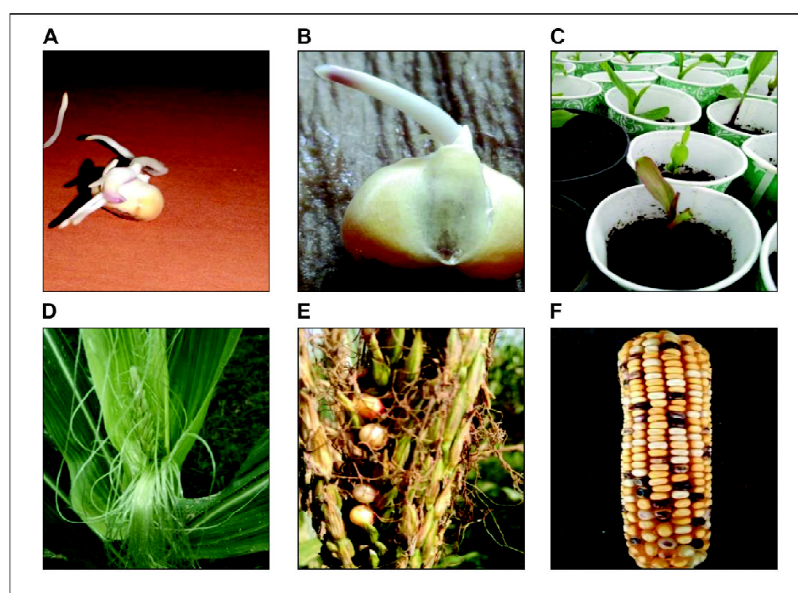
A&B: Seedlings are sorted initially before colchicine treatment on the basis of plumule color.

C: Seedlings having plumule color also have purple pigmentation on leaves.

D&E: Haploid plants having no chromosomal doubling by colchicine shows deformity during anthesis.

F: Plumule coloured seedlings sorted before chromosomal doubling are having differential segregation after selfing on their kernels.

Fig. 3: Differential pattern of expression of haploids and Diploids *in-vitro* and *in-vivo* conditions

counts, Tassel deformity and Harvested kernel color(Table 3). All the mentioned trait showed significant difference between Doubled haploid and Diploid (Plumule coloured) plants.

Self-pollinated DH$_0$ plants: At the time of anthesis, a higher number supposed Doubled haploid plants have showed variable deformity traits, denoting the lesser efficacy of colchicine treatment. The maximum number of deformed plants has appeared in CAL-147 genotype after chromosomal doubling treatment and the most effective chromosomal doubling have been shown in all the three sweet corn hybrids because the minimum deformed tassels have been formed in these genotypes. After selfing of the Double haploid plants the maximum recover of DH Cobs were also contributed by sweet corn-8051 F1 and sweet corn-8001 F1 hybrids followed by other genotypes. The plumule coloured seedlings on the other hand have no Tassel deformity as well as after selfing and Cobs maturity seeds was segregated whereas doubled haploid plants selfed DH1 seeds are having no color on their seed endosperm or embryo.

The initial haploid induction rate of Tropicalized advance haploid inducer line on the different genotypes have ranged between (6-11 per cent), similar results have been also published by different authors on the TAILs' haploid induction previously reported range (6-12 per cent Chaikam and Mahuku 2012; 6.17-8.48 per cent, 4 to 10 per cent Nair et al., 2019; 0.13-15.49 per cent, Couto et al. 2019). The induction rate on differential source population have also been reported by many authors (Röber et al., 2005; Prigge et al., 2011; Eder and Chalyk, 2002; Kebede et al., 2011; Nair et al., 2019) which clearly denotes that the initial rate of haploid induction was also dependent on seed size and kernel embryonic as well as endospermic structural integrity. The further categorization of seedlings on the basis of plumule colour was although not done yet the previous researchers have noticed the variable expression patterns of R1-nj dominant purple colour marker. The data on environmental effects of haploid induction and field transfer survival is also available as per the various scientific findings (Fuente De La et al., 2018; Aman and Sarkar, 1978) denoting that mortality of the seedlings could be minimized by choosing winter season nursery for chromosomal doubling.

The seedling stage screening of haploid- diploid seedlings based on plumule color have resulted into the deviation of actual data of haploid induction percentage and propounds the higher mis-classification percent of 6-25%, which was due to poor intensity of R1-nj phenotypic marker expression. The same findings have been discussed by Melchinger et al., 2011 and Prigge et al., 2011. The lower rate of mis-classification percentage in the field corn maize hybrids and lines can be justified by full expression of R1-nj phenotypic marker on the kernels resulting into less number of individuals with plumule colouration at the seedling stage (Rotarenco et al., 2010; Li et al.,2009).

Kleiber et al. (2012) observed that field condition have higher mortality than green house conditions. They had noted five folds more efficient results with growing of seedlings totally from seedling stage to whole plant in the green house conditions. True haploids were the plants with normal stalk color. The effect of colchicine treatment on seed set was investigated in this group of haploid plants. The confirmed doubled haploids were those plants that showed seed set without purple marks on seeds. The majority of these plants produced no tassels, sterile tassels, or partially fertile tassels, and less viable pollen indicating that chromosomal doubling did not occur correctly. As a result, no seed was placed. Silk was either defective or appeared late in some of the plants, hindering pollen and silk receptivity from being synchronized.

CONCLUSION

We present quantitative data on the reliability of an integrated system for identifying haploid progenies at the early seedling stage before colchicine treatment even after sorting of seeds into different groups on the basis of embryo and endosperm coloration R1-nj phenotypic marker. The findings of DH breeding with tropical maize germplasm are more useful with respect to Indian context where the diverse populations are not fully being utilized for heterosis breeding due to linkage drags of variable genes, but the introduction of doubled haploidy in maize unravels the genome into its expressed form resulting into elimination of negative alleles. The major problem still resides with the technology is agronomic performance under variable agro climatic conditions as well as low expression of the R1-nj system, which has been documented by several researchers, might be the cause of false doubled haploids being incorrectly classified as putative haploids, which can be easily identified on the basis of plumule colour and can be initially eliminated without any cost. Automation of haploids identification process by incorporating multiple markers in a single Inducer system will enable high throughput and full proof haploid identification thereby significantly reducing the cost incurred in Doubled haploids development. Thus, there are numerous opportunities still remaining for further refining the DH line production process and improving its efficiency.

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