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Development and genetics of maize doubled haploid lines

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Abbreviations: *DH-line: Doubled haploid line, OPVs: open pollinated varieties, HIR: haploid induction rate: R1-Navajo (R1-nj) dominant anthocyanin color marker.*

Abstract

The present study was carried out to develop doubled haploid lines using in-vivo maternal haploid induction technique. The study was aimed at the reliability of haploid seed identification through the *R1-nj* visual colored marker, identification of spontaneous doubling in tropical germplasm, optimization of colchicine percentage and seedling cutting methods for artificial chromosome doubling and revealing the genetics of newly developed DH-lines. Two tropical haploid inducer lines with 4-6% HIR were used as male parents and crossed with a single hybrid FH-949 as female donor parent. The *R1-nj* visual colored marker was used to sort haploid from diploid kernels. To check the spontaneous chromosome doubling rate these haploid kernels were grown in the field. In the laboratory, different colchicine percentages and seedling cutting treatments were used to double these maternal haploids. The data for seedling survival and DHo seed formation was collected and generalized linear model GLM was used to interpret treatment results. SAS 9.2 was used to estimate confidence intervals for the binomial proportion having zero frequencies. Out of twenty doubled haploid lines developed, five were crossed with three OPVs in line × tester mating design. The data was collected and evaluated for combining ability and type of gene action for the yield and its related traits. The *R1-nj* dominant anthocyanin marker was found to be 91 percent effective in identifying maternal haploid seeds at the dormant stage. The spontaneous doubling percentage of maternal haploids was recorded 0.8%. All the lines showed good general combining ability. The additive type of gene action was prevailing in most of the traits studied.

Introduction

In order to meet the global demand for maize, we need to double its production by 2050. Traditional maize inbred line development requires 6-10 seasons of self-breeding but with the help of doubled haploid technology we can achieve this in just two seasons of selfing (Hallauer *et al.*, 2010). Therefore, doubled haploid technology is a perfect approach to accelerate varietal development and achieve genetic gain (Prigge *et al.*, 2012).

The maternal haploid inducer based DH-technology in maize was for the first time suggested by Chase in 1952. The method is based on in-vivo haploid induction and involves three steps: (i) production of haploid seeds through induction cross; (ii) distinguishing haploid seeds from normal diploid crossing seeds; and (iii) doubling the chromosomes of haploid plants (*Prigge et al.*, 2011). Some maize genotypes, when used as a male/ female parent, induce certain percentage of haploids kernels in the F_1 cob. These genotypes are called maternal/ paternal haploid inducers. 'Stock6' was the first maize haploid inducer line reported with the haploid induction rate (HIR) of 2% (Coe, 1959), it is the ancestor of all current inducers, WS14 (Lashermes and Beckert, 1988); ZMS (Chalyk *et al.*, 1999); MHI (Eder and Chalyk, 2002); KEMS (Shatskaya et al., 1994), and RWS (Rober et al., 2005). However, all of these inducers are of temperate origin. International Maize and Wheat Improvement Center (CIMMYT) has developed tropically adopted haploid inducer (TAIL) with HIR of 8-10%. This in-vivo maternal haploid inducer mediated doubled haploid inbred line developmental technique is native to temperate maize growing areas of the world. Now CIMMYT is collaborating and introducing this inbred line developmental technique to the tropical maize growing areas (Prasanna *et al.*, 2014).

A crucial and laborious step in the in-vivo DH technology is distinguishing haploid from crossed seeds. For this purpose, inducer genotypes are commonly equipped with the dominant R1-nj marker gene that causes purple coloration of the aleurone and scutellum due to anthocyanin pigmentation. When a haploid inducer line is used as a male parent and crossed with an otherwise

desirable non-colored donor parent two kinds of seeds are produced, i) diploid seeds with both aleurone and scutellum colored and ii) haploid seeds with scutellum non-colored but aleurone colored (Nanda and Chase, 1966). Haploidy induces sterility and haploid plants produce abnormal gametes (Tang *et al.*, 2009). The duplication of haploid chromosomes is essential to achieve fertility and produce doubled haploid lines. Colchicine is the most widely used chromosome doubling agent. Colchicine binds to tubulin and disrupts mitosis by the inhabitation of chromosome migration and duplication of chromosomes result (Wan *et al.*, 1989). Spontaneous chromosome doubling is a natural process in maize which can be exploited to replace artificial chromosomal doubling through toxic chemicals. Spontaneous chromosome doubling may occur via somatic cell fusion, endoreduplication, endomitosis and possibly many other mechanisms (Jensen, 1974). This spontaneous doubling rate is germplasm-specific and can be as low as 0 to 10 percent (Chase, 1969; Kato, 2002).

The doubled haploid technology has provided many quantitative genetic and economic advantages over the traditional method of line development (Nei, 1963; Rober *et al.*, 2005; Geiger, 2009). The maximum additive variance is available in doubled haploid inbred lines and evaluation of putative hybrids is possible at the beginning of any selection program. The doubled haploid technology reduces the costs for the nursery and maintenance of breeding work. In 2011, Pioneer reportedly generated more inbred lines via doubled haploid technology than it has produced in the first 80 years of the breeding program (Prasanna *et al.*, 2014). The superiority of doubled haploid (DH) lines lies in shorter breeding cycles, exploitation of the full genetic variance in segregating materials from the very beginning of the breeding process and simplified logistics and plant variety protection (Melchinger *et al.*, 2014). The DH technology not only accelerates the line development but also helps in identification and use of elite breeding lines. The DH technology has enhanced "forward breeding" (Geiger and Gordillo, 2009; Prasanna *et al.*, 2012). Residual heterozygosity cannot mask line performance thus ensuring earlier variety protection. Selection pressure effectively eliminates deleterious recessive alleles from germplasm pools during the haploid phase therefore the agronomic performance of doubled haploid lines is always high. (Prigge *et al.*, 2012). CIMMYT has reported four thousand doubled haploid lines during 2010 (Mahuku *et al.*, 2010).

The study was aimed

- To check the reliability of haploid seed identification through the *R1-nj* visual colored marker.
- To identify spontaneous doubling in tropical germplasm.
- To optimize colchicine percentage and seedling cutting methods for artificial chromosome doubling of haploid seedlings.
- To develop doubled haploid lines and using of line × tester mating design to develop single cross hybrids.
- Testing of their combining ability and gene action, to identify better-performing parents for the future hybrid breeding program.

Materials and Methods

The present study was carried out in the experimental area, greenhouse and anther culture laboratory of the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan.

Induction crosses and haploid seeds identification

150 induction crosses were planned taking a single hybrid (FH-949) as female donor parent and two haploid inducer lines with (4-6%) haploid induction rate (HIR) as male inducer parent. The germplasm was collected from Maize Research Station, Ayub Agricultural Research Institute, Faisalabad. After induction crosses, F^1 cobs were harvested and haploid kernels with non-colored scutellum and colored aleurone were sorted (Prasanna *et al.*, 2012

Optimization of colchicine percentage for artificial chromosome doubling

Haploid seeds were germinated in the laboratory using completely randomized design (CRD) with three replications. After a few days of incubation in a growth chamber, seeds with 2 cm coleoptile and 3-5 cm radical length were treated with 0.5% DMSO (Dimethyl Sulfoxide) in 0.02, 0.04, 0.06% colchicine solution and distilled water for 12 hours (Prasanna *et al.*, 2012). A total of 120 seedlings i.e., 30 in each treatment and 10 in each replication were treated. The treated seedlings were immediately transplanted in disposable pots filled with peat moss and placed in a growth chamber. Later on, the established seedlings were transferred to the greenhouse in large pots. The fertile plants were selfed to produce DH_0 plants. The putative DH lines i.e., D_0 plants were selfed with care to produce D_1 seeds

- **Radical (2 cm)**. Only radical of the haploid seedlings

were cut 2 cm each without cutting the coleoptiles.

- **Coleoptile (1 cm)**. Seedlings were cut only from the tips 1cm each without cutting the radical.
- **Coleoptile (1 cm)** and radical (2 cm). Seedlings were cut from root tips about 2 cm and 1 cm from the shoot tip.
- **A control treatment without seedlings cutting**. A control treatment without cutting the coleoptile and radical was also performed.

Data analysis

The analysis of data for optimization of colchicine percentage in experiment number 2 and optimization of seedling cutting treatments in experiment number 3 were carried out using SAS 9.2 program.

The response we measured is a binomial distribution variable, "success" or "fail". We used generalized linear model (GLM) to interpret the results.

GLM = Logit(p) = log(p/(1-p)) = media + treatment + replicate when we use original measurements i.e., percentages, the model is:

p = exp(media + treatment + replicate)/(1+ exsp(media + treatment + replicate)) where, p is the percentage.

As in the data of seedling survival and death, there were certain observations that were zero. The problem with zeros is that standard error is huge and sometimes infinite. However, the tests are valid but we cannot compare a treatment with zero with another with some percentage. To do this, each treatment is considered independently and obtains a confidence interval for the percentage for each one. SAS 9.2 was used for estimating confidence intervals for the binomial proportion having zero frequencies (Xiaomin, H and P. A. Shwu-Jen Wu, 2009).

The comparison of the Spontaneous and artificial chromosome doubling and confirmation of DH lines uniformity

In the field study, spontaneous doubling percentage of maternal haploids was recorded 0.8%. The haploid seeds germination percentage was recorded 95.1% (Table, 3). The seedlings reached till first leaf stage was 90.7%. From this stage till the appearance of reproductive parts i.e., tasseling and silking this remained the same. After the flag leaf stage (Vf) we only score the plants that developed tassels i.e., (17.9%) (Table, 4). Although the plant survival percentage remains the same from tasseling till seed setting, but here survival is taken in term of success towards seed setting i.e. cob formation, silking and seed setting. All the plant survive till VF (flag leaf stage), succeed in producing tassels but mostly very short, unable to shed pollen and sterile. Most of the plants produced rudimentary cob like structures, with leaves wrapped around each other to form cobs. Few produced silks and developed seeds. Out of 1.3% plants that produced cobs only 2 plants succeed in developing seeds i.e., 0.8% (Table, 4).

The survival percentage of colchicines treated seedlings was recorded 71.6% after V1 stage. It is the stage when seedlings are treated with colchicines. With the gradual decrease till V4 (four leaf stage) the seedling survival percentage reaches at 23.3%, i.e. about 48.3% decrease from V1 to V4 stage. It is the time when seedling got well establish in big pot in the greenhouse. At two leaf stage V2 when the seedlings were shifted from growth chamber to the greenhouse the survival percentage decreased from 33.3% to 23.3% i.e., about 10% (Table, 4). All the plant survived till 'V4' reached till the tasseling stage (Rt) but only 18.3% produced cobs. Out of those only 6.7% developed seeds due to unsynchronized tassel and silk formation as tassel dried before the silk matured. Sectoral fertility in tassels was also noted. All the artificially colchicine treated plants that survived till the seed formation (Rs) stage were taller, with good tassel and cob size as compared to haploid plants grown in field for spontaneous doubling (Table, 1).

In the case of treatment with best response (colchicine 0.04%, root cutting 2cm and shoot cutting 1cm), after 100% germination, seedling survival at the beginning of first leaf stage (V1) was also 100%. It was the stage when the seedlings were treated with colchicines. After the treatment with colchicines at first leaf stage (V1) this percentage decreased till 43.3% which is about 56.7% decrease from the previous stage. Later from this stage till three to four leaf stage 40% survival was recorded i. e., 3.3% decrease from the previous stage. This decrease was due to seedling death from transplantation of seedling to the bigger pots in the greenhouse. All the plants that survived this stage developed seeds with successful tassel and cob formation i. e., 40% (Table, 4).

Combining ability analysis of newly developed DH-lines

The combining ability analysis showed highly significant ($P < 0.05$) results for genotypes, parents, interaction of parents and hybrids, parents and lines, for all the traits under study. The combining ability analysis showed non-significant results for replications, testers and line × testers, for all the traits under study. The non-significant differences in

tester mean square observed for most of the traits suggested that the testers used in the current study had comparable potential for the studied traits and they belonged to the same heterotic groups. The tester mean squares for the grain yield per plant showed significant differences. This was because only yield differences were considered during choosing the low yielding varieties as testers. The non-significant line × tester interaction for all the traits under study showed the crosses didn't perform better than the parents (Table. 6). It was found that all the lines showed good general combining ability, especially L_2, L_3 and L_4 for most of the yield enhancing traits (Table. 7). From testers, the tester T_4 possessed better general combining ability for most of the studied yield traits (Table. 7). The line L_1 is contributing toward increase in cob diameter. The line L_2 is contributing toward the increase in kernel rows per cob, number of grains per plant, grain yield per plant and 100 grain weight. The increase in kernel rows per cob seems the reason of increase in number of grain per plant and grain yield per plant. Mostly with increase in ability of kernel rows per cob the grain size decreases but here 100 grain weight is increasing with the increase in kernel rows per cob. Therefore, this line L_2 is very important in breeding point of view and can be use in future breeding programmes for positive contribution of this rare combination of increase in kernel rows per cob and 100 grain weight. The line L_3 is contributing toward more kernels per cob row, number of grains per plant, grain yield per plant and decrease in plant and cob height. The increase in kernels per cob row can be the reason for more number of grains per plant and grain yield. The significant contribution in increase of grain yield and decrease of plant and cob height gives this line a combination of two important characters vital in maize breeding point of view. The line L_4 is contributing toward increase in cob length, kernels per ear row, grain per plant, 100 grain weight and decrease in plant and cob height. The significant contribution ability of the line toward kernels per cob row and grain per plant appears because of increase in cob length but surprisingly in spite of all this there is no significant contribution toward increase in grain yield. A breeder can suggest a cross between the lines L_2 and L_4. The rare combination of increase in kernel rows per cob, 100 grain weight and the grain yield in the line L_2 and the significant contribution of the line L_4 toward increase in cob length, kernels per ear row, grain per plant, 100 grain weight and decrease in plant and cob height can produce a cross combination with full package of all these characteristics. The line L_5 is contributing toward increase in cob diameter. Both the lines L_1 and L_5 are showing ability toward significant increase in cob diameter but all other traits are either with non-significant GCA results (L_5) or with significant but contributing negatively in some characters (L_1). Here linkage may be playing some role. All these doubled haploid lines are developed from a single F_1 hybrid (FH-949). After looking at the variation present in all the above discussed results one can easily claim this doubled haploid technique as a breeder's tool of creating variation which is main concern of a breeding programme.

For the plant height, the lines L_1 and L_5 were poor general combiners and L_3 and L_4 were good general combiners (Table. 7). For cob height, the line L_1 was a poor general combiner and L_2, L_3 and L_4 were good general combiner (Table. 7). The F_1 hybrids L_1×T3, L_5×$T_1$and L_5× T_2 exhibited poor specific combining ability (SCA) effects and L_1×T_2, L_2× T_3, L_3×T_1, L_4×T_1 and L_4× T_2 exhibited good specific combining ability effects for cob height (Table. 8). All the three testers exhibited non-significant GCA effects for the cob length (Table. 7). The crosses L_1×T_2, L_1×T_3, L_2× T_1, L_4×T_1, L_4× T_2, L_4× T_3 and L_5×T_2 exhibited good specific combining ability (SCA) effects for cob length (Table. 8). For cob diameter two lines L_1 and L_5 exhibited good GCA effects (Table. 7). The cross combinations L_1×T_2, L_1×T_3, L_5×T_1 and L_5× T_2 exhibited significant positive SCA effects for the cob diameter (Table. 8). The line L_2 possessed good GCA effects for kernel row per cob (Table. 7). The cross combinations L_2× T_1, L_2× T_2 and L_2× T_3 possessed good SCA effects for kernel row per cob (Table. 8). The lines L_2 and L_4 were found to be good general combiners for 100-grain weight (Table. 7). In case of 100-grain weight the cross combinations L_2× T_2, L_2× T_3 and L_4×T_1 exhibited significant positive SCA effects (Table. 8). The lines L_3, L_4, and L_5 exhibited good GCA effects for the kernels per cob row (Table. 7). The estimates of SCA effects were found good for the crosses L_2×T_3, L_4×T_1, L_4×T_2 and L_4×T_3 for the kernels per cob row (Table. 8). The lines L_2, L_3, and L_4 exhibited positive and highly significant GCA effects for grain per plant (Table. 7). The cross combinations L_2× T_1, L_2× T_2, L_2× T_3, L_3× T_3 and L_4× T_2 exhibited good SCA effects with the tendency to increase grain per plant (Table. 8). The lines L_2 and L_3 were good general combiners with a tendency to increase the grain yield per plant (Table. 7). The cross combinations L_2× T_3, L_2× T_2, L_3× T_3, L_2× T_1, and L_4× T_2 exhibited good specific combining ability (SCA) effects for the grain yield per plant (Table. 8). The greatest increase in the grain yield per plant among all different cross combinations was

shown by $L_2 \times T_3$ with significant reduction in plant height and cob height. The second highest increase in the grain yield per plant was shown by the cross combination $L_2 \times T_2$ with significant reduction in plant height and non-significant reduction in the cob height. The third highest increase in the grain yield per plant was shown by the cross combination $L_3 \times T_3$ with a non-significant increase in plant height and non-significant decrease in the cob height (Table. 8). From the genetic component analysis σ2gca/ σ2sca ratio was greater than unity for all the traits under study except for the trait 100 grain weight. This is an indication that additive genetic variance was more significant in the inheritance of most the traits except for 100 grain weight where non-additive genetic variance was more significant in the inheritance (Table 9).

Results

Induction crosses and haploid seeds identification

Out of 150 induction crosses, there were 750 haploid kernel/grains which were identified at dormant seed stage on the basis of visual observation i.e., presence or absence of embryo color (Table 1). In the field studies, out of 250 seeds 217 were haploid which was identified at vegetative and reproductive stages (Table 1). About 91 percent of the seeds identified as haploids at dormant seed stage were confirmed as haploids after growing and evaluating them in the field.

Optimization of colchicine percentage and seedling cutting methods for artificial chromosome doubling

Total 120 seedlings were used in four different colchicine concentration optimization treatments (Fig. 1). The results for the second 0.04% colchicine solution treatment gave promising results. Where with 95% confidence interval the true mean of the seedling population succeed in producing seeds lies between (0.2266 – 0.5940) while, (0.4060 – 0.7734) for the seedling failed in producing seeds (Table 2). Total 120 seedlings were used in four different cutting treatments (Fig. 1). For all the cutting treatments colchicine percentage remained the same i.e., 0.04%. Out of these only 4 seedlings with a percentage success of 3.3% successfully doubled and produced seeds. The results for the seedling cutting treatment with the cutting of coleoptile 1cm and radical 2cm gave better results. Here, with 95% confidence interval the true mean of the seedling population succeed in producing seeds lies between 0.0211 to 0.2653%, while, 0.7347 to 0.9789% for the seedling failed in producing seeds (Table 2).

Table 1 Haploid seed identification on the basis of visual observations

Identification method	No. of crosses made	No. of cobs harvested	No. of haploid seeds detected from each cob	Total no. of haploid kernels	
Visual observation (through R1-nj, color marker system)	150	150	4-6	750	
	Total seed	**No. of seed germinated**	**No. of diploids**	**No. of haploids**	
Field observation Vegetative + reproductive stages	250	239	22	217	
			Features	**Features**	
			Tall, hybrid looking plants with purple pigmentation in leaves, stems, roots and reproductive parts. Well developed tassel and silks. Cobs harvested with purple colour seeds.	Short statured	217
				Purple pigment	absent
				stripe leaves	217
				Without tassel formation	176
				Tassels with partially fertile florets	41
				Cobs formation	3
				Spontaneously doubled	2

The comparison of the Spontaneous and artificial chromosome doubling and confirmation of DH lines uniformity

In the field study, spontaneous doubling percentage of maternal haploids was recorded 0.8%. The haploid seeds germination percentage was recorded 95.1% (Table, 3).

Table 2 Confidence interval for maternal haploids under seedling cutting and colchicine treatments

Seedling cutting treatments	For category "0"		For category "1"		
	95% Lower C.I	95% Upper C.I	95% Lower C.I	95% Upper C.I	Success Proportion P
Coleptile 1cm	0.8278	0.9992	0.0008	0.1722	0.03
Radical 2cm	0.8843	1.0000	0.0000	0.1157	0.00
Coleptile 1cm Radical 2cm					
0.7347	0.9789	0.0211	0.2653	0.10	
Control	0.8843	1.0000	0.0000	0.1157	0.00
Colchicine percentage treatments					
0.02%	0.7793	0.9918	0.0082	0.2207	0.07
0.04%	0.2266	0.5940	0.4060	0.7734	0.40
0.06%	0.8843	1.0000	0.0000	0.1157	0.00
Control	0.8843	1.0000	0.0000	0.1157	0.00

- Category "0" stands for seedlings failed to produce seeds.
- Category "1" stands for seedlings successed in producing seeds.

The seedlings reached till first leaf stage was 90.7%. From this stage till the appearance of reproductive

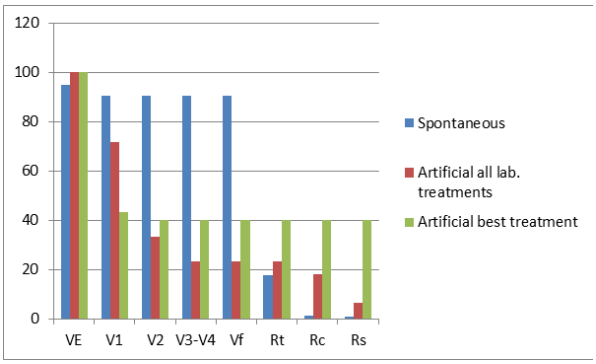

Fig: 1. The frequency of haploid seedling survival during different growth stages in the field and in the laboratory.

parts i.e., tasseling and silking this remained the same. After the flag leaf stage (Vf) we only score the plants that developed tassels i.e., (17.9%) (Table, 4). Although the plant survival percentage remains the same from tasseling till seed setting, but here survival is taken in term of success towards seed setting i.e. cob formation, silking and seed setting. All the plant survive till VF (flag leaf stage), succeed in producing tassels but mostly very short, unable to shed pollen and sterile. Most of the plants produced rudimentary cob like structures, with leaves wrapped around each other to form cobs. Few produced silks and developed seeds. Out of 1.3% plants that produced cobs only 2 plants succeed in developing seeds i.e., 0.8% (Table, 4).

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Table 3 Comparison of the spontaneous and artificial chromosome doubling.

No.	Conditions	Spontaneous doubling in the field	Artificial doubling in laboratory	
			Results of all treatments	Result of best treatment
1	**Germination**	95.1% was recorded	100% because of optimum growth conditions	100% because of optimum
2	**Critical stage**	Cob development (Rc)	After colchicines treatment till transfer to the green house (V1-V4)	After first leaf stage (V1) i.e., after colchicines treatment
3	**Seed setting**	0.8%	6.7%	40%

Note. Critical stage is one during which most negative response of a treatment is observed and any improvement in the response will affect the results positively.

Table 4 Haploid seedling survival in the field and in the laboratory

Conditions		No. of seeds	Ve	V1	V2	V3-V4	Vf	Rt	Rc	Rs
Spontaneous		**228**	**217**	**217**	**217**	**217**	**217**	**41**	**3**	**2**
		Percentage	95.1	90.7	90.7	90.7	90.7	17.9	1.3	0.8
Artificial	**All lab. treatments**	240	240	172	80	56	56	56	44	16
		Percentage	100	71.6	33.3	23.3	23.3	23.3	18.3	6.7
	Best treatment	30	30	13	12	12	12	12	12	12
		Percentage	100	43.3	40	40	40	40	40	40

Note. (Total 250 haploid seeds were sown in the field but in above table we have considered 228 i.e., after deduction of 22 diploid plants)
Ve (seed germination, start of vegetative stage),V1 (vegetative stage with first leaf),V2 (vegetative stage with two leaves), V3 and V4 (vegetative stage with three and four leaves),Vf (appearance of flag leaf), Rt (reproductive stage with tasseling), Rc (reproductive stage with cob appearance), Rs (seed formation).

that survived till the seed formation (Rs) stage were taller, with good tassel and cob size as compared to haploid plants grown in field for spontaneous doubling (Table, 1).

In the case of treatment with best response (colchicine 0.04%, root cutting 2cm and shoot cutting 1cm), after 100% germination, seedling survival at the beginning of first leaf stage (V1) was also 100%. It was the stage when the seedlings were treated with colchicines. After the treatment with colchicines at first leaf stage (V1) this percentage decreased till 43.3% which is about 56.7% decrease from the previous stage. Later from this stage till three to four leaf stage 40% survival was recorded i. e., 3.3% decrease from the previous stage. This decrease was due to seedling death from transplantation of seedling to the bigger pots in the greenhouse. All the plants that survived this stage developed seeds with successful tassel and cob formation i. e., 40% (Table, 4).

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Table 5. Genetic components for grain yield and its related traits

Parameter		S.O.V	S.S.	VAR (G)	VAR (P)	VAR (E)	SE
Plant Height (cm)	1	TREAT.	23332.792	350.442	353.527	3.085	0.009
		ERROR	407.211	GCV	PCV	ECV	
		TOTAL	23762.879	12.181	12.235	1.143	
		G.M.	153.683				
Cob Height (cm)	2	TREAT.	10369.806	154.438	157.118	2.681	0.013
		ERROR	353.844	GCV	PCV	ECV	
		TOTAL	10752.266	18.623	18.784	2.454	
		G.M.	66.730				
Cob Length (cm)	3	TREAT.	411.704	6.125	6.238	0.113	0.067
		ERROR	14.903	GCV	PCV	ECV	
		TOTAL	427.584	19.861	20.043	2.697	
		G.M.	12.461				
Cob Diameter (cm)	4	TREAT.	19.287	0.274	0.292	0.018	0.313
		ERROR	2.366	GCV	PCV	ECV	
		TOTAL	21.813	11.469	11.837	2.932	
		G.M.	4.567				
Kernels Rows Per ear	5	TREAT.	101.217	1.291	1.534	0.243	0.140
		ERROR	32.087	GCV	PCV	ECV	
		TOTAL	134.551	8.031	8.755	3.486	
		G.M.	14.145				
Kernels per ear row	6	TREAT.	5601.884	83.301	84.877	1.576	0.018
		ERROR	208.029	GCV	PCV	ECV	
		TOTAL	5817.217	30.916	31.207	4.252	
		G.M.	29.522				
Grains per plant	7	TREAT.	1680765.072	25370.7	25466.1	95.481	0.001
		ERROR	12603.536	GCV	PCV	ECV	
		TOTAL	1693695.072	40.680	40.756	2.496	
		G.M.	391.551				
100 grain weight (g)	8	TREAT.	603.995	7.423	9.151	1.728	0.058
		ERROR	228.148	GCV	PCV	ECV	
		TOTAL	834.429	8.961	9.950	4.324	
		G.M.	30.404				
Grain yield per plant (g)	9	TREAT.	203181.053	3040.454	3078.501	38.046	0.003
		ERROR	5022.138	GCV	PCV	ECV	
		TOTAL	208257.359	45.773	46.059	5.120	
		G.M.	120.464				

the line L_2 and the significant contribution of the line L_4 toward increase in cob length, kernels per ear row, grain per plant, 100 grain weight and decrease in plant and cob height can produce a cross combination with full package of all these characteristics. The line L_5 is contributing toward increase in cob diameter. Both the lines L_1 and L_5 are showing ability toward significant increase in cob diameter but all other traits are either with non-significant GCA results (L_5) or with significant but contributing negatively in some characters (L_1). Here linkage may be playing some role. All these doubled haploid lines are developed from a single F_1 hybrid (FH-949). After looking at the variation present in all the above discussed results one can easily claim

Table 6. Mean square of genotypes, parents, interaction of the parents and hybrids, crosses, lines, testers and line × testers for grain yield and yield contributing traits of maize DH-lines

SOV	DF	Plant height	Kernel rows per cob	Kernels per cob row	Grains per plant	Grain yield per plant	Cob length	Cob height	Cob diameter	100 grain weight
Replication	2	1.2ns	0.8ns	0.8ns	0.6ns	0.2ns	1.4ns	1.7ns	1.4ns	0.2ns
Genotypes	22	114.5**	6.3**	53.8**	266.7**	80.9**	55.2**	58.6**	16.3**	5.2**
Parents	7	267.2**	10.1**	133.9**	684.0**	222.8**	140.3**	143.5**	38.4**	13.4**
Int.(Parent.Hyb)	1	380.8**	26.7**	110.7**	611.1**	73.4**	157.1**	92.7**	0.2 ns	0.5ns
Crosses	14	19.2**	2.9**	9.7**	33.4**	10.5**	5.4**	13.6**	6.3**	1.5ns
Lines	4	58.2**	9.3**	33.1**	116.4**	28.5**	17.2**	43.1**	20.9**	2.0ns
Testers	2	0.3ns	0.3ns	0.6ns	0.7 ns	9.7**	0.9ns	3.1ns	0.6ns	1.7ns
Line × Tester	8	1.1ns	0.4ns	0.3ns	0. ns	1.8ns	0.65ns	1.7ns	0.52ns	1.3ns
Error	44									
Grand	68									

this doubled haploid technique as a breeder's tool of creating variation which is main concern of a breeding programme.

For the plant height, the lines L_1 and L_5 were poor general combiners and L_3 and L_4 were good general combiners (Table. 7). For cob height, the line L_1 was a poor general combiner and L_2, L_3 and L_4 were good general combiner (Table. 7). The F_1 hybrids $L_1 \times T_3$, $L_5 \times T_1$and $L_5 \times T_2$ exhibited poor specific combining ability (SCA) effects and $L_1 \times T_2$, $L_2 \times T_3$, $L_3 \times T_1$, $L_4 \times T_1$ and $L_4 \times T_2$ exhibited good specific combining ability effects for cob height (Table. 8). All the three testers exhibited non-significant GCA effects for the cob length (Table. 7). The crosses $L_1 \times T_2$, $L_1 \times T_3$, $L_2 \times T_1$, $L_4 \times T_1$, $L_4 \times T_2$, $L_4 \times T_3$ and $L_5 \times T_2$ exhibited good specific combining ability (SCA) effects for cob length (Table. 8). For cob diameter two lines L_1 and L_5 exhibited good GCA effects (Table. 7). The cross combinations $L_1 \times T_2$, $L_1 \times T_3$, $L_5 \times T_1$ and $L_5 \times T_2$ exhibited significant positive SCA effects for the cob diameter (Table. 8). The line L_2 possessed good GCA effects for kernel row per cob (Table. 7). The cross combinations $L_2 \times T_1$, $L_2 \times T_2$ and $L_2 \times T_3$ possessed good SCA effects for kernel row per cob (Table. 8). The lines L_2 and L_4 were found to be good general combiners for 100-grain weight (Table. 7). In case of 100-grain weight the cross combinations $L_2 \times T_2$, $L_2 \times T_3$ and $L_4 \times T_1$ exhibited significant positive SCA effects (Table. 8). The lines L_3, L_4, and L_5 exhibited good GCA effects for the kernels per cob row (Table. 7). The estimates of SCA effects were found good for the crosses $L_2 \times T_3$, $L_4 \times T_1$, $L_4 \times T_2$ and $L_4 \times T_3$ for the kernels per cob row (Table. 8). The lines L_2, L_3, and L_4 exhibited positive and highly significant GCA effects for grain per plant (Table. 7). The cross combinations $L_2 \times T_1$, $L_2 \times T_2$, $L_2 \times T_3$, $L_3 \times T_3$ and $L_4 \times T_2$ exhibited good SCA effects with the tendency to increase grain per plant (Table. 8). The lines L_2 and L_3 were good general combiners with a tendency to increase the grain yield per plant (Table. 7). The cross combinations $L_2 \times T_3$, $L_2 \times T_2$, $L_3 \times T_3$, $L_2 \times T_1$, and $L_4 \times T_2$ exhibited good specific combining ability (SCA) effects for the grain yield per plant (Table. 8). The greatest increase in the grain yield per plant among all different cross combinations was shown by $L_2 \times T_3$ with significant reduction in plant height and cob height. The second highest increase in the grain yield per plant was shown by the cross combination

Table 7. Estimates of general combining ability of the maize DH-lines and testers for grain yield and its related traits

SOV	Plant height	Kernel rows per cob	Kernel per cob row	Grains per plant	Grain yield per plant	Cob length	Cob height	Cob diameter	100 grain weight
Line1	5.4**	0.1 ns	-6.6**	-99.6**	-28.0**	0.2 ns	10.6**	0.3**	-0.8 ns
Line2	-1.5 ns	1.5**	-1.1 ns	36.6**	19.4**	-0.5**	-3.2**	-0.1 ns	1.2*
Line3	-6.0**	-0.5**	3.2**	56.9**	16.1**	-0.7**	-5.4**	-0.5**	-0.8 ns
Line4	-8.9**	-0.5**	3.6**	15.8**	-3.6 ns	1.3**	-1.7**	-0.2 ns	1.1*
Line5	10.9**	-0.5**	0.9 ns	-9.7**	-3.9 ns	-0.4**	-0.3ns	0.4**	-0.8 ns
Tester1	-0.5 ns	0.001 ns	-0.2 ns	0.5 ns	-7.7**	-0.2ns	-1.4**	0.004 ns	-0.7 ns
Tester2	0.3ns	0.1 ns	-0.3 ns	-4.1 ns	-1.6 ns	0.2 ns	1.2 ns	-0.05 ns	-0.2ns
Tester3	0.2 ns	-0.1 ns	-0.5 ns	3.5 ns	9.3**	-0.002ns	0.2ns	0.04 ns	0.9ns

Table 8. Estimates of specific combining ability of the DH-lines and testers for grain yield and its related traits

SOV	Plant height	Kernel rows per ear	Kernels per ear row	Grains per plant	Grain yield per plant	Cob length	Cob height	Cob diameter	100 grain weight
$L_1 \times T_1$	1.2 ns	0.0 ns	-0.0ns	3.7 ns	3.2ns	-0.4 ns	1.6 ns	0.1 ns	-0.2 ns
$L_1 \times T_2$	8.7**	-0.7**	-5.6**	-143.1**	-43.9**	1.3**	-14.6**	0.3**	-1.3 ns
$L_1 \times T_3$	9.9**	0	-9.2**	-154.8**	-27.7**	1.0**	17.6**	0.8**	1.4 ns
$L_2 \times T_1$	8.2**	2.0 **	-5.6**	18.7**	15.7**	1.4**	-0.7ns	0.1 ns	-2.3 ns
$L_2 \times T_2$	-14.3**	2.0 **	-1.6 ns	37.8**	29.2**	-0.2 ns	-1.8ns	-0.5**	2.9 **
$L_2 \times T_3$	-4.9**	1.2 **	6.8**	149.6**	65.9**	-0.9**	-14.1**	-0.3**	5.1**
$L_3 \times T_1$	-6.8**	-2.1 **	4.6 ns	24.8**	-4.9ns	-0.5ns	-7.5**	-0.4**	-2.5 ns
$L_3 \times T_2$	2.1ns	-0.1 ns	-0.4 ns	0.8ns	-2.0ns	-0.03ns	0.1ns	0.2 ns	-0.4 ns
$L_3 \times T_3$	2.0 ns	-0.1 ns	0.9 ns	47.9**	28.2**	-2.1**	-1.9 ns	-0.3**	-0.4 ns
$L_4 \times T_1$	-20.8**	-0.1 ns	2.6**	28.4**	-15.6**	1.5**	-4.0**	-0.5**	3.1**
$L_4 \times T_2$	-14.7**	-0.5 ns	9.2**	111.7**	17.2**	1.3**	-9.1**	-0.7**	0.3 ns
$L_4 \times T_3$	-6.8**	-1.9 **	5.0**	-23.1**	-17.0**	1.6**	-0.8 ns	-0.0 ns	-1.1 ns
$L_5 \times T_1$	15.7**	0.1 ns	-1.9 ns	-69.4**	-27.4**	0.02ns	3.9**	0.8**	-0.5 ns
$L_5 \times T_2$	20.0**	0.1 ns	-3.6**	-31.6**	-10.1 ns	1.5**	3.2**	0.5**	-2.7**
$L_5 \times T_3$	0.4**	0. ns	-0.9ns	-1.5ns	-10.6 ns	0.1ns	-1.2 ns	0.0 ns	-1.3 ns

$L_2 \times T_2$ with significant reduction in plant height and non-significant reduction in the cob height. The third highest increase in the grain yield per plant was shown by the cross combination $L_3 \times T_3$ with a non-significant increase in plant height and non-significant decrease in the cob height (Table. 8). From the genetic component analysis σ2gca/ σ2sca ratio was greater than unity for all the traits under study except for the trait 100 grain weight. This is an indication that additive genetic variance was more significant in the inheritance of most the traits except for 100 grain weight where non-additive genetic variance was more significant in the inheritance (Table 9).

Discussion

The maternal haploid induction and identification depend upon the presence of R1-Navajo (R1-nj) dominant anthocyanin color marker. In the inducer line, the purple color appears in both outermost layers of the maize endosperm and embryo while the female source populations used in the induction cross do not possess any anthocyanin coloration in these layers. In this way, R1-nj dominant color marker system helps in the differentiation of haploid kernels from the diploid kernels. Different factors like environment and genetic background of inducer and female parent etc affect the expression of the R1-nj color marker (Chase, 1952; Rober et al., 2005; Prigge et al., 2011). But in this case, only a single dented hybrid was used as a source material and induction crosses were made in the same season of a single year. The contribution of the two different inducer lines to the color expression of the F_1 seeds was almost similar. Therefore, the expression of the R_1-nj color marker in the F_1 seed was almost the same. The source or donor material with dominant anthocyanin inhibitor genes such as C1-I, C2-Idf, and In1-D can make the R1-nj color marker system ineffective (Coe, 1949). But in the case of our induction cross, the expression of color was fairly good with no sign of anthocyanin inhibitor genes (Fig, 2).

Table 9. General combining ability variance (σ2gca), specific combining ability variance (σ2sca), σ2gca/ σ2sca, gene action, additive variance (A) and dominance variance (D) for the grain yield and its related traits

	Plant height	Kernel rows per ear	Kernel per ear row	Grains per plant	Grain yield per plant	Cob length	Cob height	Cob diameter	100 grain weight
σ2gca	5.7	0.6	1.5	322.8	34.1	0.055	3.3	0.011	0.05
σ2sca	0.4	0.2	1.1	82.5	25.7	0.04	1.8	0.009	0.5
σ2gca/ σ2sca	16.1	4.0	1.4	3.9	1.3	1.4	1.8	1.2	0.01
Type of gene action	Additive	Additive	Additive	Additive	Additive	Additive	Additive	Additive	dominance
A	22.8	0.25	6.02	1291.2	136.3	0.22	13.07	0.043	0.19
D	1.407	0.06	4.32	329.8	102.9	0.16	7.28	0.034	1.99

Figs: 2. Two maternal haploid inducer lines used as male parent; hybrid FH-949 used as female donor parent; seeds with purple scutellum and aleuron layer are diploid and with purple colored aleuron and non-colored scutellum are haploid

Characteristics of haploid and diploid kernels

- **Diploid**- Embryo and endosperm with purple outer layers
- **Haploid**- Only endosperm with purple outer layer and non-colored embryo

Characteristics of haploid and diploid plants in the field

- **Haploids** – They possess narrow leaves. These narrow leaves are often with white stripes. They have high sterility in both tassel and ear inflorescences. These tassels and ears occasionally show sectoral fertility (Chase, 1969). The roots, stems, leaves or any part of the haploid plants lack purple pigmentation.
- **Diploids/ Hybrids** – These plants are with highly fertile tassel and ear inflorescences. They show hybrid vigor and lack sectoral sterility in both tassel and ear inflorescences (Kato, 2002). The purple pigmentation appears in any part of the plants.
- **Spontaneously doubled haploids** – These plants are with highly fertile tassel and ear inflorescences but they lack hybrid vigor (Kato, 2002), and lack of purple pigmentation in the plant tissue.

Colchicine and seedling cutting methods affected haploid seedling in different ways. Most of the seedlings died after transplanting to bigger pots. Remaining seedlings which reached till tassel and cob formation stage exhibited sectoral sterility in most of their tassels. Some plants developed cobs when tassels got dried. There were also 3-5 multicobs instead of a single cob with insect attack. Some plants were very small with yellow and thick multistem. Selfing was not possible because of unsynchronized tassel and cob formation. Therefore, there was no seed formation in the cobs. There were some seedlings which grew into vigorous plants with good tassel size, synchronized cobs, timely silking and good seed set. For the genome duplication of maternal haploid plants, an efficiently reliable method is required which must be cost-effective (Wan et al., 1991). For the germplasm where spontaneous chromosomal duplication is non-existing or very low, the artificial duplication is necessary (Hansen and Andersen, 1996). This is done through the utilization of colchicine chemical. It is a yellow colored poisonous alkaloid obtained from a plant. The chemical influences cell division of a plant and inhibits mitosis (Hantzschel and Weber, 2010). A part from colchicine many world known breeding seed companies use their patented less hazardous artificial chromosome duplication treatments (Geiger and Gordillo, 2009). Before the 1990s, colchicine reputation was not desirable as a doubling agent because of its genotype specific nature. It was reported less effective for plant spindle protein (Eigsti and Dustin, 1955). Its certain concentrations were considered toxic for plant seedlings (Jensen, 1974). But still, it is the predominant artificial chromosome doubling agent of choice in corn. The root and shoot tissues are cut at a certain length during colchicine treatment. The root tips trimming helps in the comfortable handling of the

delicate seedlings during the colchicine treatment and seedlings transplantation while, trimming the shoot tips enhances the chances of chromosomal doubling by the exposing apical meristem to colchicine chemical treatment (Vanous, 2011).

The pollen fertility in haploid maize plants normally falls between 2.8 to 46% (Liu and Song, 2000; Wei and Chen, 2006; Han et al., 2006). This normal anthesis fertility rate is highly germplasm-specific. Spontaneous chromosome doubling of the haploid plant in case of the female inflorescence and seed development is reported to range from 25 to 94 percent, through different investigations (Chalyk et al., 1994; Liu and Song, 2000; Han et al., 2006). Many mechanisms are reported in spontaneous chromosome doubling in haploid maize plants, including somatic cell fusion, endoreduplication, endomitosis, etc. (Jensen, 1974; Testillano et al., 2004). Protoplasts of sterile haploid maize plant cells fuse to form fertile diploid cells. The process of somatic cell fusion starts with cell wall digestion. The cell walls of two neighboring somatic cells get digested with cellulase enzyme. The two different protoplasts and nuclei fuse with each other. After their fusion again hormones are released and cell wall reappears around the two fused cells, which now becomes a single cell. This single cell is with doubled chromosome number and is now diploid. This process of endoreduplication/ endomitosis occurs in the absence or decline in the process of mitosis (Scanlon and Takaes, 2009). Maize sporophytes with gametic chromosome number were observed by many scientists. In normal progeny, they observed a frequency of one out of one thousand to two thousand in the field (Randolph, 1932, 1940). These haploids were not used for doubled haploid development instead, only preferred for genetic and breeding studies (Blakeslee and Belling, 1924; East, 1930). The first study of commercial maize hybrid development, utilizing these spontaneous monoploids was initiated at the Iowa state college (1947 to 1953). It also started at Illinois from 1954 to 1966 (Chase, 1969). Stadler in 1940 for the first time reported doubled haploid maize. Chase (1947, 1951) provided comprehensive information to use maize spontaneous haploids in the development of homozygous inbred lines. For a breeding program this haploid induction rate of 0.1% was very low (Rober et al., 2005).

The physical appearance, as well as differences in the percentage of seed formation between maternal haploid plants grown in the field for spontaneous doubling and raised in the laboratory after artificial doubling agent colchicine treatment, was a clear indication that the doubled haploid was not the early doubled haploids (Penghao et al., 2014). Their field performance of seed setting and physical appearance were not as good as the laboratory performance after treatment with the artificial doubling agents. It means the colchicine treatment improves the doubling rate and development of the doubled haploids. There must be some genetic reasons promoting chromosomal doubling spontaneously as well as through doubling agent (Prasanna et al., 2012). In the field experiment, the spontaneous doubling of these maternal haploids seemed the process of "endomitosis". This process begins around 10 to 14 days after pollination (Kowles and Phillips, 1985).

There can be many reasons for seedling death in the laboratory. The toxic effects of colchicine can be a vital reason. The genetic background of the source material and the procedure followed for the colchicine application also influence the seedling death rate in the laboratory (Prasanna et al., 2012). In the field low development of tassels and cobs in the haploid plants was because of the sterility prevailing in haploid plants. At CIMMYT 85–90% haploid kernels germinate. Only 40–80% of colchicine treated seedlings grow till maturity. Among these 10–30% diploids are reported. The remaining true haploids, 0–40% produce male and female inflorescences and functional pollen and silks to achieve successful pollination (Prasanna et al., 2012).

The shorter plant is advantageous in the case of lodging resistance. Therefore, the crosses which showed the tendency to decrease plant height are of breeders concern. The existence of both positive and negative SCA effects in maize crosses has been also reported by (Alamnie et al., 2003; Vacaro et al., 2002; Dhliwayo et al., 2009). The mid to low bearing cob height is a desirable trait in maize hybrids to avoid stem lodging. For cob height, both negative and positive significant specific combining ability effects were observed among the crosses. The crosses with negative and non-significant estimates of SCA effect could be selected for their specific combining ability to be use in maize improvement program (Abrha et al., 2013). The ear length is a major yield component and is directly proportional to grains per cob. Longer the ear length, higher will be the grain yield. A Similar genotypic difference for cob length was reported by different researchers (Sofi and Rathor, 2006; Narro et al., 2003). The increase in ear length is of utmost importance in the improvement of maize yield. The positive general combining ability effect is desired for a number of rows per cob as it is the most important yield component that directly contributes to increased grain yield (Asif and Iqbal, 2007; Stuber et al., 1992). The significant value of additive components and the non-significant values of dominance components of kernel rows per

ear indicated stability in the additive variance of this trait. It can thus be improved through simple selection procedures (Jagtap, 1986). The trait was controlled by the additive type of gene action. Therefore, the selection of this trait would be fruitful and reliable in early segregating generations (Tabbasum, 1993). The significant value of additive components for kernel per ear row indicated stability in the additive variance of this trait; it can thus be improved through selection procedures in the next generation. The higher additive gene action for grains per plant suggested that the selection on the basis of grains per plant may be helpful to improve grain yield per plant. Larger the grains per plant higher will be the grain yield per plant. For grain yield, both negative and positive significant SCA effects were observed among the crosses. The variance due to general combining ability (34.1) was greater than variance due to specific combining ability (25.7) which verified the additive effect (136.3) which is greater than dominance effect (102.9) therefore, the additive type of gene action is controlling the character. The majority of yield-related traits are controlled by additive genes. The additive genes are more important than dominant genes for higher grain production (Ali et al., 2010). General combining ability is attributed to the additive type of gene effects, while specific combining ability is attributed to the non-additive type of gene actions. The non-additive type of gene actions is not reliably fixable whereas, additive type of gene actions or complementary type epistatic gene interactions are reliably fixable (Xiang, 2007; Iqbal et al., 2007). The additive type of gene action is prevailing in doubled haploid inbred lines and they can be directly used in the breeding program for exploitation of the traits under consideration. Similar results are presented by many previous researchers (Yadav et al., 2002; Rafique et al., 2004; Seanski et al., 2005; Akbar et al., 2006; Ali et al., 2010; Abdel et al., 2014; Sudika et al., 2015).

Conclusions

The present study shows that the R1-nj visual colored marker system is effective to sort haploid from diploid kernels in tropical germplasm. The R1-nj dominant anthocyanin marker was found to be 91 percent effective in identifying maternal haploid seeds at the dormant stage. We found that the spontaneous chromosomal duplication is present in tropical maize germplasms and it can be exploited to simplify this technique. The artificial doubling percentage of maternal haploids in the laboratory was recorded as 6.7%. The results show that the additive type of gene action is prevailing in these doubled haploid lines and these DH-lines are with good general combining ability. The present study suggested that these DH-lines can directly be utilized for varietal and hybrid development

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References

Abdel M, Sultan MS, Sadek SE, Shalof MS, 2014. Estimation of heterosis and genetic parameters for yield and yield components in maize using the diallel cross method. Asian J Crop Sci. 6:101-111.

Abrha SW, Zeleke HZ, Gissa DW, 2013. Line x tester analysis of maize inbred lines for grain yield and yield related traits. Asian J Plant Sci Res. 3:12-19.

Akbar M, Shabbir M, Amer H, Sarwar M, 2006. Evaluation of maize three way crosses through genetic variability, broad sense heritability, character association and path analysis. J. Agric Res. 46: 39-45.

Alamnie A, Nayakar Y, Wali MC, 2003. Combining ability, heterosis and per se performance of height characters in maize. Karnataka J Agric Sci. 16:131-133.

Ali AW, Hasan KA, Samir A, 2010. Genetic variances, heritability, correlation, and path coefficient analysis in yellow maize crosses (Zea mays L.). Agric Bio J N Amer. 1:630-637.

Ali Q, Ahsan M, Ali F, Aslam M, Saleem M, 2013. Heritability, heterosis and heterobeltiosis studies for morphological traits of maize (Zea mays L.) seedlings. Advancements Life Sci. 1:52-63.

Anonymous, 2014. Proc. 12th Int. AMC Conf., 30 October, Bangkok.

Asif M, Iqbal FA, Nehvi SA, Zahoor A, 2007. Combining ability analysis for yield and related traits in maize (Zea mays L.). Int. J. Plant Breeding Genet. 1:101-105.

Blakeslee AF, Belling J, 1924. Chromosomal mutations in the Jimson weed, Datura stramonium. J. Heredity. 15:195-206.

Chalyk S, Rotarenco A, 1999. Using maternal haploid plants in recurrent selection in maize. Maize Genet Coop Newsl. 73:56-57.

Chalyk ST, Bylich VG, Chebotar OD, 1994. Transgressive segregation in the progeny of a cross between two inducers of maize maternal haploids. Maize Genet Coop Newsl. p.68:47.

Chase S, 1969. Monoploids and monoploid derivatives in maize (Zea mays L.). Botanical Review. 35:117-167.
Chase SS, 1952. Monoploids in maize. Iowa State College Press, Ames, Iowa, pp.389-399.
Chase SS, 1947. Techniques for isolating monoploid maize plants. Amer J Bot. 34:582.
Chase SS, 1951. Production of homozygous diploids of maize from monoploids. Agron J. 44:263-267.
Coe EH, Sarkar KR. 1964. The detection of haploids in maize. J Heredity. 55:231–233.
Coe E.H, 1949. Anthocyanin genetics. In: M Freeling, V. Walbot (eds.), The maize handbook. Springer Verlag, New York, pp.279-281.
Coe EH, 1959. A line of maize with high haploid frequency. Amer Naturalist. 93:381-382.
Dhliwayo T, Pixley K, Menkir A, Warburton M, 2009. Combining ability, genetic distances and heterosis among elite CIMMYT and IITA tropical maize inbred lines. Crop Sci. 49:1201-1210.
East EM, 1930. The production of homozygotes through induced parthenogenesis. Science. 72:148-149.
Eder J, Chalyk ST, 2002. In vivo haploid induction in maize. Theor Appl Genet. 104:703-708.
Eigsti OJ, Dustin P, 1955. Colchicine. In: Agriculture, Medicine, Biology and Chemistry. Iowa State College Press. Ames, Iowa.
Geiger H, 2009. Doubled haploids in hybrid maize breeding. Maydica. 54:485-499.
Geiger HH, Gordillo GA, 2009. Doubled haploids in hybrid maize breeding. Maydica. 54:485-499.
Hallauer AR, Carena MJ, Miranda FJB, 2010. Quantitative Genetics in Maize Breeding. Springer Science Business Media, New York.
Han X, Tang Q, Cao M, Rong T, 2006. Study on identifying methods of maize haploids induced by Stock 6. J Maize Sci. 14:64-66.
Hansen NJP, Andersen SB, 1996. In vitro chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in Brassica napus microspore culture. Euphytica. 88:159-164.
Hantzschel KR, Weber G, 2010. Blockage of mitosis in maize root tips using colchicines alternatives. Protoplasma. 241:99-104.
Iqbal AM, Nehvi FA, Wani SA, Qadir R, Dar AZ, 2007. Combining ability analysis for yield and yield related traits in Maize (Zea mays L.). Int J Plant Breeding Genet. 1:101-105.
Jagtap DR, 1986. Combining ability in upland cotton. Indian J Agric Sci. 12:833-840.
Jensen CJ, 1974. Chromosome doubling techniques in haploids. p.153-190. In: Kasha KJ (eds.), Haploids in Higher Plants: Advances and Potential. Univ. Guelph, Guelph.
Kato A, 2002. Chromosome doubling of haploid maize seedlings using nitrous oxide gas at the flower primordial stage. Plant Breed. 121:370-77.
Kempthorne O, 1957. An introduction to genetic statistics. John Wiley, New York.
Kowles RV, Phillips RL, 1985. DNA amplification patterns in maize endosperm nuclei during kernel development. PNAS. 82:7010-7014.
Lashermes P, Beckert M, 1988. Genetic control of maternal haploidy in maize (Zea mays L.) and selection of haploid inducing lines. Theor Appl Genet. 76:405-410.
Liu Z, Song TM, 2000. Fertility spontaneously restoring of inflorescence and chromosome doubling by chemical treatment in maize haploid. Acta Agron. 26:947-952.
Mahuku G, Martinez L, Prigge L, 2010. Progress in doubled haploid production at CIMMYT. Presentation held at the Drought Tolerant Maize for Africa (DTMA). Annual Project Meeting, 13-18 September, Nairobi, Kenya.
Melchinger AE, Schipprack W, Utz HF, Mirdita V. 2014. In vivo haploid induction in maize: Identification of haploid seeds by their oil content. Crop Sci. 54:1497-1504.
Nanda DK, Chase SS, 1966. An embryo marker for detecting monoploids of maize (Zea mays L.). Crop Sci. 6:213-215.
Narro L, Pandey S, Crossa J, Leon CD, Salazar F, 2003. Using line × tester interaction for the formation of yellow maize synthetics tolerance to acid soils. Crop Sci. 43:1717-1728.
Nei M, 1963. The efficiency of haploid methods of plant breeding. Heredity. 18:95-100.
Parsana BM, 2014. Maize Research for Development Scenario: Opportunities and Challenges for Asia. Proc. 12th Int. AMC Conf., 30 Oct., Bangkok.
Penghao W, Jiaojiao R, Liang L, Shaojiang C, 2014. Early spontaneous diploidization of maternal maize haploids generated by in vivo haploid induction. Euphytica. 200:127-138.
Prasanna BM, Chaikam V, Mahuku G, 2012. Double haploid technology in maize: Theory and practice. CIMMYT Manual.
Prigge V, Melchinger AE, 2011. Production of haploids and doubled haploids in maize. In: VM LoyolaVargas, Ochoa Alejo N (eds.), Plant cell culture protocols. Humana Press Springer Verlag, Totowa, New Jersey.
Prigge V, Xu X, Babu R, 2012b. New insight into

the genetics of in vivo induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics. 190:781-793.
Rafique M, Amer H, Tariq M, Alvi AW, 2004. Heritability and interrelationships among grain yield and yield components in maize (Zea mays L.). Int J Agric Bio. 6:1113-1114.
Randolph LF, 1932. The chromosomes of haploid maize with special reference to the double nature of the univalent chromosomes in the early meiotic prophase. Science. 75:566-567.
Randolph LF, 1940. Note on haploid frequencies. Maize Genet Coop Newslett. 14:23-24.
Rober FK, Gordillo GA, Geiger HH, 2005. In vivo haploid induction in maize performance of new inducers and significance of doubled haploid lines in hybrid breeding. Maydica. 50:275-283.
SAS, 2002. SAS Institute, Inc., SAS users' guide. Version 9.0th ed.Cary, NC.27513, USA. Cary. NC.
Scanlon MJ, Takaes EM, 2009. Kernel biology. J L. pp.121-143.
Seanski MT, Zivanovic T, Todorovic G, 2005. Components of genetic variability and heritability of the number of rows per ear in silage maize. Biotech Animal Husbandary. 21:109-121.
Shatskaya OA, Zabirova ER, Shcherbak VS, Chumak MV, 1994. Mass induction of maternal haploids. Maize Genet Coop Newslett. pp.68:51.
Sofi P, Rather AG, 2006. Genetic Analysis of Yield Traits in Local and CIMMYT Inbred Line Crosses Using Line × Tester Analysis in Maize (Zea mays L.). Asian J Plant Sci. 5:1039-1042.
Stuber CW, Lincoln SE, Wolff DW, Helentjaris T, Lander ES, 1992. Identification of genetic factors contributing to heterosis in a hybrid from two elite maize inbred lines using molecular markers. Genetics. 132:823-839.
Sudika I, Wayan NB, Andy S, 2015. Estimation of genetics variance components from composite and hybrid maize (Zea mays L.) hybridization. Int J Plant Res. 5:107-112.
Tabassum MI, Saleem M. 1993. Study of gene action for economic characters in maize. Pak J Agric Sci. pp.3:2.
Tang QL, Feng YC, Rong TZ, 2009. Study on haploid inducing and its meiotic abnormality in maize. Agric Sci. 8:1159-1165.
Testillano PS, Mogensen HL, Dumas C, Risueno MC, Matthys-Rochon E, 2004. Spontaneous chromosome doubling results from nuclear fusion during in vitro maize induced microspore embryogenesis. Chromosoma. 112:342-349.
Vacaro E, Barbosa JF, Pegoraro DG, Conceicao LDH. 2002. Combining ability of twelve maize populations. Pesquisa Agropecuária Brasileira. 37:67-72.
Vanous AE, 2011. Optimization of doubled haploid production in maize (Zea mays L.). Diss., Graduate Theses. 11974. http://lib.dr.iastate.edu/etd/11974
Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm MJ, 1991. The use of antimicrotubule herbicides for the production of doubled haploid plants from anther derived maize callus. Theor Appl Genet. 81:205-211.
Wan Y, Petolino J, Widholm J, 1989. Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. Theor Appl Genet. 77:889-892.
Wei JJ, Chen MX, 2006. Primary study on the natural fertility of maize haploids. J Maize Sci. 14: 24-26.
Xiang, Zhen S, Huo, Zhang X, 2007. Analysis on combining ability and its inheritance of stem quality traits in maize. J Maize Sci. pp.2:1.
Xiaomin H, Shwu-Jen PA, Wu, 2009. Confidence Intervals for the Binomial Proportion with Zero Frequency. Biostatistical Consultant, Austin, ICON Clinical Research, North Wales, Paper SP10 www.pharmasug.org/download/papers/SP10.pdf.
Yadav TP, Singh RD, Bhat JS, 2002. Genetic analysis of varietal crosses of maize (Zea mays L.). New Botanist. 29:131-140.