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Full Length Article

In vivo Maternal Haploid Seed Production and Chromosome Doubling with different Anti-microtubular Agents in Maize

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Abstract

In maize breeding use of doubled haploids (DH) is very important as these are shortening the time for development of inbred lines. Inbred lines developed through doubled haploid technology are completely homozygous and homogeneous. These inbred lines could be used for heterosis breeding with increased selection efficacy, reduced efforts for line maintenance, reduced length of breeding cycle and convenient applicability of molecular marker assisted breeding. Haploid induction followed by chromosome doubling are practiced for doubled haploid production. Present research emphasized (i) evaluation of four different inducer lines for *in-vivo* maternal haploid induction and (ii) effectiveness of chromosome doubling with colchicine and pendimethalin herbicide treatments. Haploid induction was done by Stock-6 and three locally produced inducer lines; (I) Stock-6-BC4, (II) Stock-6×CMST×Ig-gene, (III) (Ig-gene×Stock-6×CMST) × (Stock-6-BC4), which were derivatives of Stock6, CMST and Ig-gene. Stock-6, Stock-6-BC4, Stock-6 × (CMST × Ig-gene) and (Ig-gene × Stock-6 × CMST) × (Stock-6-BC4) had 1.01, 2.38, 8.18 and 7.53% average induction rates, respectively. Maternal haploids were identified by using purple color marker gene (R1-nj). Haploids were subjected to colchicine (0.04% colchicine + 0.5% DMSO) and different pendimethalin treatments (0.66, 0.33, 0.165 and 0.0825 ppm) for doubled haploid induction through chromosome doubling. In colchicne treatment, 3.2% plants survived out of which 37.5% reached to maturity and only 50% of these were partially fertile. At 0.66 ppm pendimethalin treatment, 23.3% plants survived out of which 28.57% reached to maturity and only 16.66% plants were partially fertile. Whereas, 0.33, 0.165, 0.0825 ppm treatment showed survival rate of 26.66, 33.33 and 11.11%, respectively but plants showed 100% sterility with sectoral diploidization. Conclusively, local derivative inducer lines *viz*. Stock-6 × (CMST × Ig-gene) and (Ig-gene × Stock-6 × CMST) × (Stock-6-BC4) showed satisfactorily higher haploid induction rate. These inducer lines are recommended for their further exploitation in haploid induction. For doubled haploid production, 0.66 ppm pendimethalin treatment was more effective in terms of survival, maturity and fertility comparative to all other studied treatments. This indicates that pendimethalin herbicide (0.66 ppm) is an attractive and cost-effective alternative of colchicine.

Keywords: Inducer lines; Tropical maize; Seed; Colchicine; Herbicides; Pendimethalin

Introduction

Maize hybrid seed production is dependent on the inbred lines. Inbred lines are genetically pure, homozygous and homogeneous in nature, which are conventionally developed by repeated self-pollination for several generations followed by evaluation of combining ability performance. Conventional inbred line development is extensively time consuming process, whereas alternatively development of maize inbred lines using doubled haploid (DH) technology has become a very useful technology in breeding research (Rober *et al.,* 2005). DH technology significantly shortens the breeding cycle by rapid development of completely homozygous inbred lines in 2 to 3 generations whereas, conventional inbred line development process requires 6 to 8 generations for 99% homozygosity (Forster and Thomos, 2005; Chang and Coe, 2009; Geiger and Gordillo, 2009). DH technology in maize, requires less time, financial resources, labor (Greiger and Gordilla, 2009), enhances precision and selection efficiency, accelerated pyramiding of favorable alleles, completely fulfill the criteria of DUS (distinctness, uniformity, stability), requires least efforts for maintenance of lines, facilitates the undertaking of markers assisted introgression (Foster and Thomas, 2005), association mapping, molecular cytogenetics, functional genomics and genetic engineering (Foster *et al*., 2007).

In maize, occurrence of haploid plants in nature was

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described by Chase (1949, 1952) and found that spontaneous induction rate of haploids was very low (1 out of 1000 seeds). Due to less occurrence of natural haploids it was impractical to manipulate them in maize breeding (Geiger, 2009). However, Coe (1959) developed haploid-inducing inbred source known as Stock 6. This haploid-inducer line had an induction rate of 2 to 3%. This development of haploid inducer line made it possible to exploit the haploids for inbred line development through doubled haploid technology. *In vivo* haploid induction results in maternal or paternal haploids. Genomes of the maternal haploids are solely derived from the seed parent plant (Rober *et al.,* 2005) and induction is caused by pollinating parent (Coe, 1959). The reverse pertains to paternal haploids, where the female line serves as the haploid inducer and the pollinator is the genome donor (Kermicle, 1969). For above mentioned system an efficient screening method is required for the separation of haploid from non-haploid seeds. This is done by incorporation of anthocyanin marker gene, R1-*nj* (Nanda and Chase, 1966; Greenblatt and Bock, 1967; Chase, 1969) in the inducer genetic backgrounds to facilitate easy screening of haploids at both seed as well as seedling stages and deriving new inducer lines having higher induction rate.

Haploid induction must be followed by chromosome doubling either spontaneous or induced to develop doubled haploid plants which actually are diploids (2n genome). Spontaneous chromosome doubling rate for male (2.8 to 46%) and female (25 to 94%) inflorescences are highly genotype specific (Liu and Song, 2000; Han *et al.,* 2006), very rare and their background mechanism is still unknown. Spontaneous chromosome doubling may occur via somatic cell fusion, endoreduplication, endomitosis and possibly many other mechanisms (Jensen, 1974) but due to low spontaneous doubling rates, artificial chromosome doubling methods are required for application of haploids in breeding programs (Rober *et al.,* 2005; Chang and Coe, 2009; Geiger, 2009).

Traditionally, seedlings are treated with 0.06 to 0.50% solution of colchicine (Eder and Chalyk, 2002; Han *et al.,* 2006) to boost chromosome doubling rates. Colchicine disrupts mitosis by binding with tubulin, thus inhibiting the alignment and separation of chromosomes (Wan *et al.,* 1991). However, treatment with colchicine is not always completely effective and sectoral diploidization of male and female inflorescence can occur. Moreover, colchicine is highly carcinogenic, requires careful handling and safe disposal. It has been reported that herbicides such as pronamid, amiprophos-methyl, trifluralin and oryzalin are also effective as mitotic inhibitors (Häntzschel and Weber, 2010). These are cost effective and less toxic than colchicine and also easier to handle with safe disposal.

Pendimethalin herbicide belongs to dinitroaniline class, which is used to control the broad leaf weeds and annual grasses in pre-emergence and post-emergence stages. Pendimethalin controls the weeds and grasses by preventing their cell elongation and cell division however, well known as anti-microtubular agent (Häntzschel and Weber, 2010). Due to having anti-microtubular activity, pendimethalin was supposed to possibly cause chromosome doubling. Verification of this supposition was also key objective in this research experiment.

CIMMYT Global maize program (GMP) initiated the optimization of DH technology for tropical and subtropical regions. Tropically adapted inducer lines (TAILs) with 8 to 10% haploid induction rate were developed by CIMMYT in collaboration with University of Hohenheim, Stuttgart, Germany. Whereas, in Pakistan not a single attempt was previously made for exploitation of DH technology in maize. *In vivo* haploid induction through inducer lines and chromosome doubling with colchicine or any other anti-microtubular agents were also not attempted in indigenous Pakistani maize germplasm. To fulfill the currently prevailing gaps in the hybrid seed availability in Pakistan, current experiment was planned with objectives to evaluate the efficacy of haploid induction rate of local four inducer lines and to evaluate the effectiveness of chromosome doubling agents.

Materials and Methods

This was a lab cum field experiment and was conducted at Maize Research Station, Ayub Agricultural Research Institute, Faisalabad. Experimental site has clay loamy soil with dry semi-arid agro-climatological conditions and located at 73.1^{o} longitude, 31.43^{o} latitude and 184.5 m elevation from sea level. Meteorological conditions of experimental site were given in Table 1.

Maternal Haploid Inducer Lines

Inducer lines were imported by Maize Research Station, Ayub Agricultural Research Institute Faisalabad, Pakistan from Maize Genetic COOP stock center, Urbana State, United States of America. Exotic inducer lines were of temperate type; these inducer lines were bred with indigenous lines to develop locally adapted inducer lines. In present research four locally developed inducer lines were collected from Maize Research Station, Ayub Agricultural Research Institute, Faisalabad, Pakistan to produce maternal haploids. These inducer lines were:

(1) Stock-6 (exotic inducer),
(2) Stock-6-BC4 (indigenous inducer),
(3) Stock-6 × CMST × Ig gene (indigenous inducer),
(4) [(Ig gene × Stock-6 × CMST) × (Stock-6-BC4)] (indigenous inducer).

Maternal Haploid Induction

Inducer lines were tested to produce maternal haploid seeds in cob of source genotype in randomized complete block design with three replications. The inducer lines were used

as pollinating parent (male parent), while source genotype i.e., EV-77 was used as seed parent (female parent). EV-77 genotype was repeated in pairs with each inducer line in one block. Four pairs comprising of eight entries (EV-77 + Stock-6; EV-77 + Stock-6-BC4; EV-77 + Stock-6×CMST×Ig-gene; EV-77 + [(Ig-gene×Stock-6×CMST)×(Stock-6-BC4)]) were included in one block. This blocking pattern was repeated for three times under randomized complete block design. Identification of haploids was performed at dormant stage by means of the anthocyanin marker gene, R1-*nj* (Nanda and Chase, 1966; Greenblatt and Bock, 1967; Chase, 1969; Eder and Chalyk, 2002). Haploid and non-haploid kernels were discriminated with the help of R1 nj gene already present in inducer lines which induce the pigmentation in the seed. In Haploid seeds, R1 nj gene induce the pigmentation in aleurone layer only but not in scutellum. Non-haploid/hybrid kernels have the purple pigments in the aleurone layer of endosperm and scutellum of embryo (Nanda and Chase, 1966; Greenblatt and Bock, 1967; Chase, 1969). Induction rate of haploid inducer lines was calculated by following formula:

$$\text{Induction Rate (IR)} = \frac{Number\ of\ Haploid\ Kernels}{Total\ Harvested\ Kernels} \times 100$$

Chromosome Doubling

Chromosome doubling of haploid kernels was done with colchicine and different pendimethalin treatments after germination of haploid kernels. This part of the experiment was conducted under completely randomized design with three replications each. Chromosomal doubling work was splitted into following work areas; (I) seed germination in growth chamber or incubator, (II) isolated workbench for colchicine and pendimethalin treatment, (III) colchicine and pendimethalin waste storage and waste management chamber, (IV) acclimatization of doubled haploid seedlings from growth chamber to field.

Seed Germination and Establishment of Seedlings

Seeds were placed on disinfected germination paper. Germination papers were folded tightly into a bundle and tied with rubber bands at both ends. These bundles were kept vertically in a mesh bag that was placed in plastic containers with disinfectant solution. Plastic tubs having paper wrapped seed bundles were placed in incubation chamber at 25 to 28°C for 72 h. After incubation, plastic containers with seed bundles were removed from incubation chamber. Seedlings with root length of 3-5 cm and coleoptile length of about 2 cm were ideally selected for colchicine and pendimethalin treatments. Before treatment, root and shoot tissues were cut at about 2 cm and 1 cm from tip respectively. Cutting the root tips make handling of seedlings easy during transplantation and cutting of shoot tips enhance the exposure of Shoot Apical Meristem (SAM) to colchicine and pendimethalin.

Colchicine and Pendimethalin Treatments

Seedlings were treated with colchicine and pendimethalin (N-(1-ethylpropyl)-3, 4-dimethyl-2, 6-dinitrobenzenamine; effective concentration 33%) in specialized tanks under dark conditions that allowed workers to avoid direct contact with colchicine and pendimethalin. A solution with 0.04% colchicine and 0.5% DMSO was used for chromosomal doubling as per recommendations of Prasanna *et al.* (2012). Promkaew *et al.* (2010) recommended the pendimethalin dose of 0.02 to 0.05 mL/L at effective concentration of 33% for studying the chromosomal aberrations in mitotic cells of maize. However, in current study whole genome was the target so different higher levels of pendimethalin were used for optimization of effective concentration. Pendimethalin (33%) with four different levels i.e., 0.66 ppm, 0.33 ppm, 0.165 ppm and 0.0825 ppm were used for treating the seedlings. Volume of colchicine and pendimethalin solution required was estimated by estimating the volume of water required to completely immerse the seedlings. Seedlings were kept in colchicine tank for 12 h. Seedlings were washed at least three times after treatment with distilled water.

Seedling Transplanting from Plant Growth Chamber to Field

Seedlings taken out of treatment tank and immediately transplanted into Styrofoam trays containing Promix (peat moss). Seedlings were handled very carefully as these become brittle after colchicine and pendimethalin treatments. Seedlings with long hypocotyls are more susceptible to damage. Seedlings were maintained in Styrofoam trays for 2 weeks in plant growth chamber where temperature, light intensity and photoperiod were maintained at 28–30°C, 84 µmol/m^2/s and 16/8 h, respectively.

As a first irrigation, only water was used whereas, with second irrigation, NPK (13-40-13) and micronutrients were applied. To prevent fungal attacks, the fungicide was also applied on every third day. A systemic insecticide was applied once a week before transplanting. After two weeks well-established seedlings were transplanted in the field.

Results

Maternal Haploid Induction

Inducers lines, Stock-6, Stock-6-BC4, Stock-6 × CMST × Ig gene, and [(Ig gene × Stock-6 × CMST) × (Stock-6-BC4)] were crossed with EV-77 and resultantly produced kernels were collected separately for each inducer line. Haploid and diploid kernels were isolated on gene based phenotypic color markers (R1 nj). Total number of kernels produced, total number of haploid kernels and haploid induction rate were treated as separate traits and these were subjected to analysis of variance with inducer lines as

Table 1: Meteorological conditions of the experimental site (2014)

Trial	Month	Minimum Temperature (°C)	Maximum Temperature (°C)	Average Temperature (°C)	Humidity (%)	Precipitation (mm)
Haploid Induction trial	February	6	22	14	69	6
	March	13	28	18	63	7
	April	18	39	24	58	7
	May	17	42	30	50	3
Hardening of doubled haploid seedlings	August	27	37	32	72	15
	September	23	34	28	80	5
	October	17	34	24	72	0
	November	11	27	17	76	1

Table 2: Mean squares for different traits associated with haploid induction performance of inducer lines

SOV	DF	Total number of kernels	Haploid kernels	Haploid Induction Rate
Rep	2	47	0.091	1.01
Genotypes	3	158546**	608.59**	1247.99**
Residuals	390	4480	14.46	14.37

**: highly significant at 0.05% level of significance

source of variation. Mean squares for total number of kernels, total number of haploid kernels and haploid induction rate were significantly different for subjected four different inducer lines at 0.05% level of significance (Table 2). Performance of inducer lines was separately elaborated in crosses with EV-77 genotype.

Stock-6 × CMST × Ig-gene was locally produced from Stock-6, CMST and Ig-gene. Number of total kernels produced were ranged from 8 to 240, number of haploid kernels were ranged from 0 to 18, whereas haploid induction rate was ranged from 0 to 17%. The average induction rate for all of 33 crosses were 8.18%, whereas highest 18 kernels were haploid out of total 110 kernels on an individual cob with induction rate of 16.36%. Minimum haploid induction rate was 3%, in which 4 haploid kernels were identified from total of 140 kernels. Minimum haploid induction rates for all inducer lines were discussed exceptional to 0.00. Average of this inducer line for all of 33 crosses with EV-77 showed that total number of kernels and haploid kernels were 85.66 and 7.35, respectively (Table 3; Fig. 1, 2).

Stock6-BC4 inducer line has average haploid induction rate of 2.38%. Out of 33 crosses the maximum haploid induction rate was 7% (2 haploid kernels out of total 30 kernels). The minimum induction rate observed was 1% in which 2 haploid kernels were identified from total of 200 kernels. Nine haploid kernels were highest value for individual cob in this case. However, on an average of all 33 crosses, this inducer line produced 118.82 kernels out of which only 2.74 were haploid (Table 3; Fig. 1, 2).

(Ig-gene × Stock-6 × CMST) × (Stock6-BC4) was locally produced from stock-6-BC4, Stock-6, CMST and Ig-gene. Maximum induction rate observed was 20% (7 haploid kernels out of total 35 kernels). The minimum induction rate was 2%, in which only 1 haploid kernel was recognized out of 45 kernels. Highest number of haploid kernels were 17 for an individual cob which showed the haploid induction rate of 15%. Based on average of all 33 crosses, this haploid inducer line produced 3.75 haploid

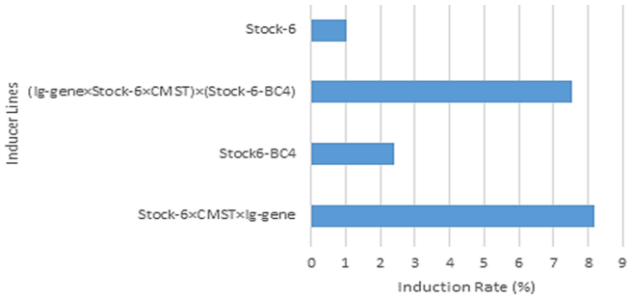

Fig. 1: Average haploid induction rate of four different inducer lines with EV-77 genotype

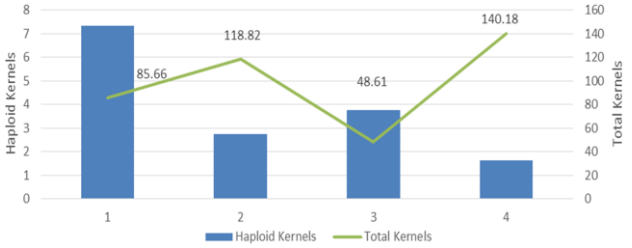

Fig. 2: Average total number of kernels and haploid kernels produced by four different inducer lines. Inducer line 1 is Stock-6 × CMST × Ig-gene, 2 is Stock6-BC4, 3 is (Ig-gene × Stock-6 × CMST) × (Stock6-BC4) and 4 is Stock-6

kernels out of total 48.61 kernels and average haploid induction rate was 7.53% (Table 3; Fig. 1, 2).

Stock-6 was crossed with EV-77 for making 33 crosses which showed maximum induction rate was 2% (4 haploid kernels out of 259 total kernels). The minimum haploid induction rate was 0.43% (1 haploid kernel out of total 232 kernels). This inducer line produced highest number of total kernels but lowest number of haploid kernels which were only 1.62 average haploid kernels.

Table 3: Performance of crosses of four different haploid inducer lines with seed producing parent EV-77

Cross	Stock-6×CMST×Ig-gene			Stock6-BC4			(Ig-gene×Stock-6×CMST)×(Stock-6-BC4)			Stock-6		
	Haploid Kernels	No of kernels	IR	Haploid Kernels	No of kernels	IR	Haploid Kernels	No of kernels	IR	Haploid Kernels	No of kernels	IR
1	0	21	0	1	100	1	4	24	17	2	137	1
2	4	140	3	1	100	1	6	56	11	0	40	0
3	2	18	11	1	20	5	4	148	3	2	150	1
4	0	8	0	3	100	3	0	20	0	2	210	1
5	0	8	0	5	150	3	1	8	13	1	130	1
6	11	82	13	2	50	4	1	14	7	0	30	0
7	2	18	11	2	50	4	2	12	17	1	60	2
8	2	20	10	0	10	0	0	10	0	2	140	1
9	4	24	17	0	10	0	0	23	0	1	50	2
10	4	27	15	3	230	1	3	34	9	0	20	0
11	18	140	13	5	220	2	9	80	11	5	250	2
12	13	140	9	2	90	2	2	25	8	2	120	2
13	11	240	5	4	80	5	1	45	2	1	192	1
14	5	90	6	0	11	0	0	25	0	2	231	1
15	4	60	7	1	120	1	1	12	8	0	20	0
16	2	22	9	9	210	4	7	35	20	1	109	1
17	0	10	0	3	100	3	0	14	0	1	60	2
18	8	102	8	4	180	2	0	14	0	1	130	1
19	11	90	12	2	100	2	3	50	6	2	120	2
20	2	24	8	6	222	3	6	64	9	2	130	2
21	16	130	12	3	150	2	1	13	8	2	190	1
22	15	150	10	1	150	1	3	18	17	4	259	2
23	15	160	9	2	133	2	1	40	3	1	87	1
24	16	120	13	1	89	1	1	44	2	3	224	1
25	8	130	6	1	77	1	6	100	6	1	123	1
26	2	19	11	3	180	2	12	90	13	2	209	1
27	18	110	16	2	200	1	17	113	15	5	260	2
28	16	140	11	9	250	4	2	75	3	2	238	1
29	0	30	0	5	200	3	4	55	7	0	10	0
30	8	220	4	3	120	3	11	211	5	1	232	0.43
31	2	50	4	2	30	7	5	42	12	2	208	1
32	7	80	9	4	200	2	5	55	9	3	249	1
33	15	200	8	1	20	5	3	35	9	0	20	0

Note: Number of haploid kernels, total number of kernels produced, and haploid induction rate of each inducer line is mentioned separately for every individual cross

Haploid induction rate for Stock-6 was 1.01% based on average of all 33 crosses (Table 3; Fig. 1, 2).

Chromosome Doubling with Colchicine and Pendimethalin Treatments

Haploid kernels were sown and germinated seedlings were subjected to colchicine and different pendimethalin treatments for chromosome doubling. After treatment, seedlings were grown further with regular provision of all management practices. Seedlings were acclimatized from well-established growth chamber environment to field conditions through gradual hardening. During acclimatization process, data for number of survived plants, number of matured plants, number of sterile plants and number of fertile plants were counted. Data for these traits were subjected to analysis of variance for all of five treatments (colchicine, pendimethalin 0.66 ppm, pendimethalin 0.33 ppm, pendimethalin 0.165 ppm, pendimethalin 0.082 ppm) as source of variation. Number of matured plants were not significantly different across the treatments. Results showed that there were significant differences among effects of different treatments (Table 4). Doubled haploid plants were identified based on vigorous seedling and larger leaves which were used as screenable markers for chromosome doubling.

Total 500 haploid seedlings were treated with colchicine of 0.04% concentration, out of which 16 seedlings survived, whereas only 6 seedlings remained alive up to maturity. Out of total 6 matured plants, 3 plants were sterile and other 3 were fertile (Table 4). Proportionally in colchicine treatment, 3.2% plants survived. Out of total survived plants only 37.5% plants reached at maturity stage. It was observed that 50% of the total survived plants were fertile and 50% sterile (Fig. 3).

Pendimethalin treatment levels were 0.66 ppm, 0.33 ppm, 0.165 ppm and 0.0 82 ppm. Total 90 haploid maize seedlings were treated with 0.66 ppm pendimethalin solution, out of which 21 seedlings survived and only 6 seedlings reached to maturity. Out of total 6 mature plants, 5 plants were sterile and remaining 1 was fertile (Table 4). Proportionally in this treatment, 23.3% plants survived after

Table 4: Mean squares for treatments of anti-microtubular agents for chromosome doubling and treatment mean comparison

SOV	DF	Survived Plants	Matured Plants	Sterile Plants	Fertile Plants
Treatments	4	108.60**	0.0068ns	5.100**	5.100**
Residual	10	1.00	1.00	1.00	0.400
Tukey's HSD Treatment Mean Comparison					
Treatment	Conc.	Survived Plants	Matured Plants	Sterile Plants	Fertile Plants
Colchicine	0.04%	16.00 (D)	6.00 (A)	3.00 (B)	3.00 (A)
	0.66 ppm	21.00 (C)	6.00 (A)	5.00 (AB)	1.00 (B)
Pendimethalin	0.33 ppm	24.00 (B)	6.00 (A)	6.00 (A)	0.00 (B)
	0.165 ppm	30.00 (A)	6.00 (A)	6.00 (A)	0.00 (B)
	0.0825 ppm	30.00 (A)	6.00 (A)	6.00 (A)	0.00 (B)
Critical Value for Comparison		2.687	2.687	2.69	1.70

* = significant at 5%, ** = significant at 1%, ns = non-significant

treatment and among total survived plants only 28.57% reached to maturity. However, 16.66% of total survived plants were fertile and 83.33% sterile (Fig. 3).

Total 90 haploid seedlings were treated with 0.33 ppm of pendimethalin solution, out of which 24 seedlings survived and 6 seedlings reached at maturity. All of the mature plants were sterile and no one was fertile or partially fertile (Table 4). Only 26.66% plants survived after treatment. Out of total survived plants only 25% plants reached at maturity but all of these were reproductively sterile (Fig. 3).

Total 90 haploid seedlings were treated with 0.165 ppm of pendimethalin herbicide solution, only 30 seedlings survived and 6 seedlings approached to maturity (Table 4). All the plants which survived were sterile. Only 33.33% plants survived after treatment and among survived plants only 20% plants reached to maturity but all of these matured plants were reproductively sterile (Fig. 3). Only 30 seedlings survived out of 90 haploid seedlings treated with 0.0825 ppm pendimethalin solution whereas, among treated seedlings, 6 plants attained the maturity but no one was fertile (Table 4; Fig. 3).

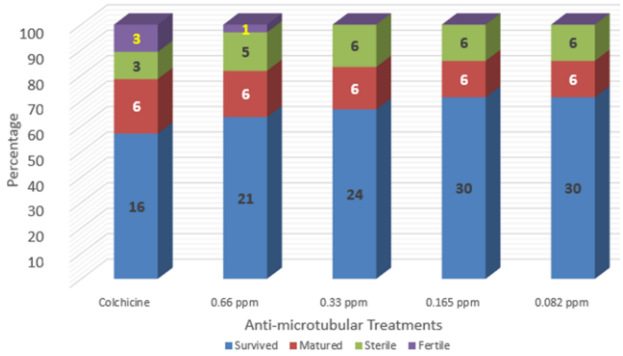

Fig. 3: Proportion of survived, matured, sterile and fertile plants following colchicine and different pendimethalin herbicide treatments of young haploid maize seedlings

Discussion

In current study, *in vivo* haploid induction was preferred over *in vitro* haploid induction due to dependence on well-equipped labs, highly skilled manpower and non-responsiveness of maize genotypes in later case (Prasanna *et al.*, 2012). Maize is moderately sensitive to salt and other abiotic stresses (Farooq *et al.*, 2009, 2015). However, developing and planting hybrids with tolerance against abiotic stress may help harvesting good yield even under less than optimum conditions. Doubled haploid (DH) production techniques based on *in vivo* haploid induction was well flourished in last decade typically in temperate regions (Prigge and Melchinger, 2012). Tropical regions across the world especially in Asia were lagging in adoption of DH technology due to lack of knowledge about DH technology and its integration in breeding, non-availability of tropical haploid inducer lines (Kebede *et al.*, 2011; Prasanna *et al.*, 2012). Present research targeted the performance of four inducer lines, which were locally developed for Pakistan by Maize Research Station, Ayub Agricultural Research Institute, Faisalabad-Pakistan from exotic temperate inducer lines i.e., Stock-6, CMST, and Ig-gene (Unpublished). Among these targeted inducer lines highest average haploid induction rate was of Stock-6 × CMST × Ig-gene (8.18%), whereas others have induction rate less than 8.00%. Research activities under CIMMYT Global Maize Program (CIMMYT-GMP) in collaboration with University of Hohenheim, Germany developed Tropically Adapted Inducer Lines (TAILs) with average haploid induction rate of 8–10%. So locally developed inducer line (Stock-6 × CMST × Ig-gene) is comparable with TAILs for rate of haploid induction. Stock-6 is founder line for haploid induction which was developed by Coe (1959) who observed 2–3% haploid induction rate. Haploid induction rate of inducer lines ranged from 2.86% to 16.36 % however, 4 out of 140 kernels were haploid in former case and 18 out of 110 were haploid in later case. A famous inducer line RWS (cross between KEMS and WS14) showed consistently 8.1% induction rate which is also comparable with (Stock-6 × CMST × Ig-gene) (Rober *et al.*, 2005). Other inducer lines like, Stock6-BC4 inducer line showed the average induction rate of 7.53% and Ig-gene × Stock-6 × CMST) × (Stock-6- BC4) showed average performance of 2.38%. Stock-6 showed an average

induction rate of 1.01%, whereas Coe (1959) reported 2–3% induction rate for this line. So, lateral three inducer lines have induction rate less than 8.00%, which showed that commercial exploitation of these lines was not appropriate.

Colchicine and different levels of pendimethalin herbicide were used for inducing chromosome doubling, because rate of spontaneous chromosome doubling was very low and not appropriate to exploit for doubled haploid breeding (Rober *et al.*, 2005; Chang and Coe, 2009; Geiger, 2009). Spontaneous chromosome doubling was also not preferred because it may occur via somatic cell fusion, endoreduplication, endomitosis and possibly many other mechanisms (Jensen, 1974). Traditionally, seedlings have been treated with colchicine 0.06 to 0.50% solution (Eder and Chalyk, 2002; Han *et al.*, 2006) for chromosome aberrations but in current study solution with 0.04% colchicine and 0.5% DMSO was used due to hazardous nature of the chemical.

Pendimethalin is cost effective and less toxic than colchicine and easier to handle with safe disposal. Colchicine was used in current study because this is successfully adapted to large scale applications and 0.04% colchicine solution with 0.5% DMSO was used, in this case only 0.006% plants were partially fertile out of total treated seedlings. It was also visualized that besides chromosome doubling colchicine was also caused sterility and lethality in the plants subjected to treatment. Observations from this study showed that concentrations even lower than 0.04% colchicine should be practiced to reduce the problem of lethality and sterility. Total four different concentrations of pendimethalin i.e., 0.66, 0.33, 0.165 and 0.0825 ppm were used as chromosome doubling agent. In 0.66 ppm treatment, 0.011% plants were partially fertile out of total treated seedlings. While the other pendimethalin concentrations showed survival rate 26.66, 33.33 and 11.11%, respectively but plants showed 100% sterility with sectoral diploidization. These results showed that 0.66 ppm treatment of pendimethalin was relatively more effective because proportion of fertile plants was higher with doubled chromosomes relative to other treatment levels and colchicine. Pendimethalin at 0.66 ppm has proportionally lowest values for lethality and sterility based on the total number of seedlings treated and fertile matured plants.

Conclusion

Inducer line, Stock-6 × CMST × Ig-gene showed highest induction rate among the four studied inducer lines and this inducer line could be used comparably with TAILs for further homozygous and homogeneous inbred line development. The application of pendimethalin herbicide in DH line development as an anti-microtubular agent was proved effective method due to reduced risks of colchicine application, cost effectiveness and ease of application. Among different levels of pendimethalin, 0.66 ppm proved to be most effective for producing more mature fertile plants. So, it is recommended that the potential of pendimethalin herbicide may further be explored for doubled haploid maize inbred lines development.

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