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The Efficiency of Chromosome Doubling in Haploids Derived from Wheat X Maize Crossing

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Abstract: A significant effect of the plant development stage on fertility was observed at the time of colchicine treatment. Plants at the 5-7 tillers stage showed improved plant fertility and reduced plant mortality than those treated at the 2-3 tillers stage. The addition of colchicine to media resulted not only in higher plant fertility than did conventional methods, but furthermore led to a huge increase in the number of doubled-haploid seeds.

Key words: Poly haploids, wheat x maize crossing, chromosome doubling

INTRODUCTION

Wheat haploids derived from the wheat x maize crossing system require chromosome doubling to produce fertile homozygous plants. Doubled haploid production of wheat is now used routinely in many breeding programmes to achieve homozygosity from early generation hybrid material. Spontaneous chromosome duplication has been observed at a low frequency[1,2] thus artificial chromosome doubling is essential to enhance efficiency.

Colchicine may be applied at the seedling stage to double the chromosome complement. Kasha[3] treated barley seedlings at the 2-3 leaf stage with 0.1% colchicine for 5 h at 22°C in the culture vial. The treated seedlings were then thoroughly washed and left in water at 31°C for 3 h. The results improved from 56% chromosome doubling to 90%. Jensen[4] treated later stage seedlings (about 5 leaves) with 0.05% colchicine solution. The addition of colchicine to anther culture media[5,6] induced chromosome doubling in the plantlets regenerating from wheat anther-derived calli[7] and rice anther-derived calli[8].

Recently, another method of colchicine treatment, which involves the incorporation of colchicine in low concentrations in the induction medium for the first three days of culture was reported by Barnabas *et al.*[6], Navarro-Alvarez *et al.*[5] and Sood *et al.*[9].

The agent most commonly used to induce chromosome doubling is colchicine. One alternative to colchicine is the use of podophyllin[10]. Nitrous oxide (N_2O) has been applied at the time of fertilization and during the first zygote division to produce chromosome doubling[11]. The application of nitrous oxide soon after pollination also induced chromosome doubling, but this technique in wheat produced high levels of aneuploidy[12].

The present studies were conducted to replace the routine chromosome doubling method with a labour efficient alternative that gives a high frequency of chromosome doubling and overcomes the problem of high mortality of haploid plants.

MATERIALS AND METHODS

Developmental stage of haploid plants for colchicine application: Fifty haploids derived from each of six wheat F_1 combinations namely, Cross-1 (Punjab-96/Karwan-2), Cross-2 (Punjab-96/Crow), Cross-3 (Inqilab-91/Kentana), Cross-4 (MH-97/Tanager), Cross-5 (Auqab-2000/Fasan) and Cross-6 (Kohistan-97/Oasis), after pollination with maize F_1 hybrid 'FSH-399', were potted and grown in the greenhouse and the field during spring 2002-03. The haploid plantlets were treated for five h with 1.0 g L^{-1} colchicine solution at two stages, the 2-3 and 5-7 tiller stages.

The plants were grown in controlled microclimates, maintaining temperatures of 12-15°C (day/night) and having a 10 h photoperiod.

***In vitro* application of colchicine:** Four concentrations of colchicine (0.25, 0.50, 0.75 and 1.0 g L^{-1}) were added to the rescue medium of immature embryos of wheat x maize crosses. Twenty five haploid plants, derived from the cross "Cross-3 × FSH-399" were used in each treatment. The same number of haploids were grown without using colchicine as a control. At the last stage of growth in the rescue medium, the haploid plants were removed from the petri plates/test tubes and the roots and leaves were trimmed to 1 and 4 cm in length, respectively. The roots and crowns of the plants were then immersed into a ½ MS medium containing the colchicine for 24, 48, 72 and 96 h.

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The plants were subsequently rinsed in running tap water for 2-3 min. The plants were then re-potted and allowed to grow in the greenhouse. The spikes appeared on the doubled haploid plants were covered with butter paper bags to avoid any chance of out-crossing.

RESULTS

Developmental stage of haploid plants: The effects of growth stages and environments on the success of chromosome doubling are presented in Table 1.

The analysis of variance for plant fertility and plant mortality reflected significant differences ($p<0.01$) between the stages of growth. Significant differences ($p<0.05$) were obtained only for plant fertility in the "environments×stages of growth" interaction. Significant differences ($p<0.01$) for the rates of chromosome doubling between the greenhouse and field grown plants were also obtained (Table 2). The treatments of field grown plants produced fewer chromosome doublings than the treatments of the greenhouse grown plants (Table 3).

The overall means of fertile plants were 31.5% and 61.0% for the colchicine treatments at the 2-3 and 5-7 tiller stages, respectively (Table 3). Application of the colchicine solution at the 5-7 tiller stage produced about twice as many fertile plants as treatment at the 2-3 tiller stage. The frequency of dead plants as a result of treatment at the 2-3 tiller stage was much higher than for treatment at the 5-7 tiller stage (Table 3). No abnormalities were observed in both treatments regarding chromosome doubling (Fig. 1). The percentage fertility in the greenhouse was nearly twice that of the field grown plants (Fig. 2 and 3).

***In vitro* application:** The effects of different concentrations of colchicine used in culture media for 24, 48, 72 and 96 h are shown in Table 4. Both the duration and colchicine concentration influenced plant fertility. There were highly significant differences ($p<0.01$) in plant fertility, the number of tillers and the number of seeds per fertile plant (Table 5 and 6).

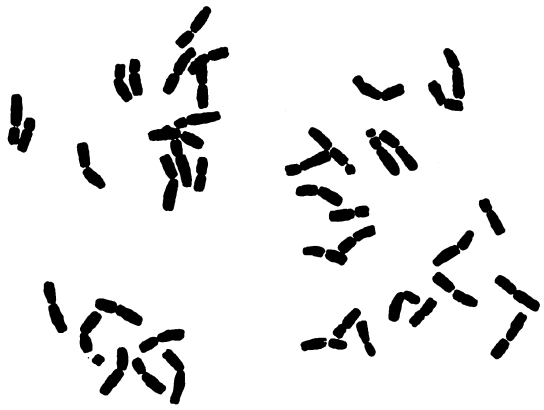

Fig. 1: 42 Chromosomes of a doubled haploid plant

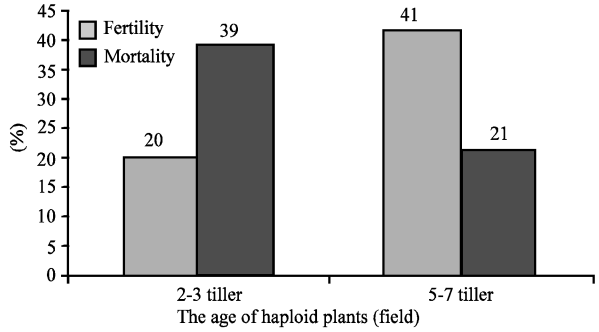

Fig. 2: Effect of the stage (2-3 and 5-7 tillers) of the haploid plants at the time of colchicine treatment on plant mortality and mature plant fertility in field

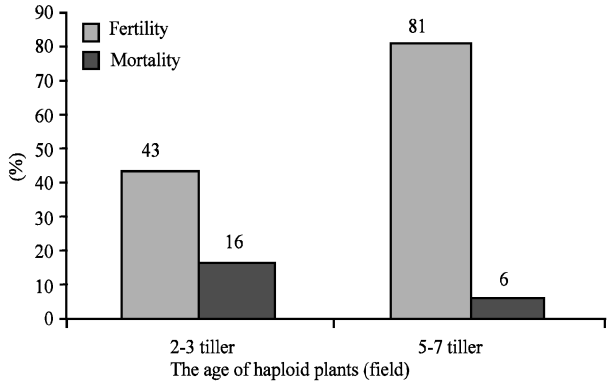

Fig. 3: Effect of stage (2-3 and 5-7 tillers) of haploid plants at time of colchicine treatment on plant mortality and mature plant fertility in glass house

There was wide variation in plant fertility among the different concentrations of colchicine (0.0 - 88.0%). As the concentration of colchicine decreased to 0.25 g L^{-1} the percentage of fertile plants increased to 62.0% (Fig. 4). Application of 0.25 g L^{-1} colchicine gave the highest plant fertility percentage in all durations (Table 4). The control did not produce any seed.

Considerable variation was observed in plant fertility over durations. The percentage plant fertility decreased from 62.0% following the 24 h treatment to 13.0% following the 96 h treatment (Fig. 4). Although the 24 h treatment gave the highest percentage of plant fertility among the durations, a concentration of 0.25 g L^{-1} for 48 h gave 88.0% fertility which was the best across all treatments (Table 4).

Table 1: Effects of environments and growth stages of haploid plantlets on success rates of chromosome doubling

	Greenhouse				Field			
	2-3 tillers		5-7 tillers		2-3 tillers		5-7 tillers	
Genotypes	Fertile plants (%)	Dead plants (%)	Fertile plants (%)	Dead plants (%)	Fertile plants (%)	Dead plants (%)	Fertile plants (%)	Dead plants (%)
Cross-1	24 (48)	6 (12)	44 (88)	3 (6)	11 (22)	18 (36)	22 (44)	9 (18)
Cross-2	25 (50)	8 (16)	39 (78)	1 (2)	9 (18)	16 (32)	24 (48)	10 (20)
Cross-3	18 (36)	10 (20)	38 (76)	2 (4)	6 (12)	24 (48)	19 (38)	10 (20)
Cross-4	20 (40)	8 (16)	44 (88)	4 (8)	13 (26)	17 (34)	24 (48)	9 (18)
Cross-5	26 (52)	6 (12)	43 (86)	3 (6)	12 (24)	19 (38)	15 (30)	10 (20)
Cross-6	16 (32)	11 (22)	35 (70)	4 (8)	9 (18)	24 (48)	19 (38)	14 (28)
Total/Average	129 (43)	49 (16.3)	243 (81)	17 (5.7)	60 (20)	118 (39.3)	123 (41)	62 (20.7)

Values in parenthesis are percentages

Table 2: Analysis of variance (MS) for plant fertility and plant mortality of colchicine treated haploid plants derived from wheat × maize crosses, as influenced by different environments (field and greenhouse) and two growth stages (2-3 tiller and 5-7 tiller stages)

		Mean squares	
Source	df	Plant fertility	Plant mortality
Environment (E)	1	38733.9**	13298.9**
Stages of growth (S)	1	35396.8**	8585.6**
Wheat (W)	5	643.5 Ns	296.0 NS
E × S	1	3129.7*	587.3 NS
E × W	5	246.3 Ns	79.0 NS
S × W	5	123.1 Ns	119.1 NS
E × S × W	5	169.1 Ns	87.5 NS
Error	216	2733.2	1807.3
Total	239		

NS = Non significant, * = p<0.05, ** = p<0.01

Table 3: Means and SE for plant fertility and plant mortality of colchicine treated haploid plants derived from wheat × maize crosses, as influenced by different environments (field and greenhouse) and two growth stages (2-3 tiller and 5-7 tiller stages)

	Plant fertility (%)		Plant mortality (%)	
	Mean	SE	Mean	SE
Environments				
Field	30.5	2.59	30.0	1.36
Greenhouse	62.0	2.50	11.0	2.17
Stage of growth				
2-3 tillers	31.5	2.41	27.8	2.16
5-7 tillers	61.0	2.72	13.2	1.49
Wheat genotypes				
Cross-1	50.5	4.89	18.0	3.18
Cross-2	48.5	4.75	17.5	2.83
Cross-3	40.5	4.66	23.0	3.69
Cross-4	50.5	5.5	19.0	3.89
Cross-5	48.0	5.06	19.0	2.85
Cross-6	39.5	4.16	26.5	3.57

Treatment with 0.25 g L^{-1} colchicine gave 22.0% plant mortality compared to 73.0% with 1.0 g L^{-1} colchicine (Fig. 5). Hence, 0.25 g L^{-1} concentration was considered an efficient concentration of colchicine to treat haploid plants. The duration of treatment, as well as colchicine concentration, influenced the frequency of dead plants. As expected the highest concentration of colchicine was the most lethal, especially over longer durations.

The number of seeds per fertile plant was high for all treatments and there were significant differences ($p<0.01$) among the treatments of both colchicine concentrations and the time of treatments (Table 5). The number of seeds ranged from 0 for 1 g L^{-1} colchicine concentration after 96 h treatment to 93.6 at the same concentration after 24 h treatment (Table 4). No abnormalities were observed in fertile spikes.

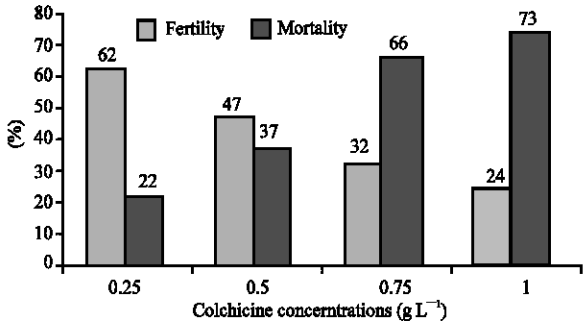

Fig. 4: Effect of different *in vitro* colchicine concentrations on plant fertility and plant mortality

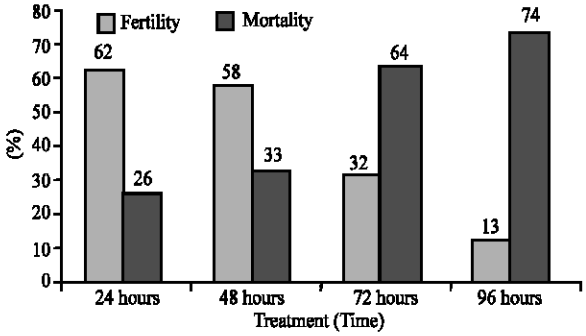

Fig. 5: Effect of duration of *in vitro* colchicine treatment on plant fertility

Table 4: The effect of the treatment period and concentration of colchicine on plant fertility, plant mortality, fertile spikes (%) and the number of seeds per fertile plants

Treatment period	Colchicine concentrations (g L^{-1})	No. of treated plants	Plant fertility (%)	Plant mortality (%)	No. of fertile spikes/plant	No. of seed/fertile plants
Control	0.00	25	0 (0.0)	0 (0.0)	0.0	0.0
24 h	0.25	25	20 (80.0)	0 (0.0)	2.3	49.3
	0.50	25	17 (68.0)	4 (16.0)	2.9	65.5
	0.75	25	13 (52.0)	10 (40.0)	3.7	74.4
	1.00	25	12 (48.0)	12 (48.0)	4.3	93.6
	Total/Average	100	62 (62)	26 (26)	3.3	70.7
48 h	0.25	25	22 (88.0)	1(4.0)	3.7	84.0
	0.50	25	15 (60.0)	5 (20.0)	3.7	87.5
	0.75	25	12 (48.0)	13 (52.0)	3.4	65.5
	1.00	25	9 (36.0)	14 (56.0)	2.8	56.2
	Total/Average	100	58 (58)	33 (33)	3.4	73.3
72 h	0.25	25	14 (56.0)	10 (40.0)	3.6	68.3
	0.50	25	10 (40.0)	13 (52.0)	3.5	71.9
	0.75	25	5 (20.0)	19 (76.0)	3.9	72.4
	1.00	25	3 (12.0)	22 (88.0)	4.2	80.5
	Total/Average	100	32 (32)	64 (64)	3.8	73.3
96 h	0.25	25	6 (24.0)	11 (44.0)	2.7	73.9
	0.50	25	5 (20.0)	15 (60.0)	3.9	91.1
	0.75	25	2 (8.0)	23 (92.0)	2.7	52.2
	1.00	25	0 (0.0)	25 (100.0)	0.0	0.0
	Total/Average	100	13 (13)	74 (74)	2.3	54.3

Values in parenthesis are percentages

Table 5: Analysis of variance (MS) for the number of tillers, fertile spikes/plant and the number of kernels/plant of colchicine treated haploid plants derived from wheat x maize crosses, as influenced by different durations of colchicine treatment and different concentrations of colchicine (0.25, 0.50, 0.75 and 1.0 g L^{-1})

Source	df	No. of tillers	No. of fertile spikes	No. of seeds
Duration (D)	3	172.2**	22.0**	7787.0**
Colchicine (C)	3	416.6**	33.6**	17998.6**
D x C	9	29.8Ns	4.7Ns	2038.7**
Error	384	117.8	1.9	5281.0
Total	399			

Ns = Nnon significant, ** = $p<0.01$

Table 6: Means and SE for the number of tillers, fertile spikes/plant and the number of seeds/plant of colchicine treated haploid plants derived from wheat x maize crosses, as influenced by different durations of colchicine treatment and different concentrations of colchicine (0.25, 0.50, 0.75 and 1.0 g L^{-1})

	Tillers		Fertile spikes		Seeds	
	Mean	SE	Mean	SE	Mean	SE
Durations (day)						
24	6.5	0.83	2.0	0.23	44.4	5.23
48	5.8	0.50	2.0	0.22	44.5	4.96
72	6.0	0.89	1.8	0.23	35.2	4.70
96	3.5	0.54	1.1	0.19	25.8	4.40
Colchicine concentration						
0.25	7.2	0.48	2.4	0.23	53.5	5.00
0.50	7.3	0.57	1.9	0.21	43.4	5.19
0.75	3.6	0.47	1.5	0.22	29.5	4.32
1.0	3.8	1.09	1.1	0.21	23.7	4.45

DISCUSSION

Developmental stage of haploid plants: Chromosome doubling depends on the stage of plant development, environment and genotypes. In this study, significant environmental differences were observed for the plant fertility and plant mortality percentage. The greenhouse grown plants showed significantly increased plant fertility and decreased plant mortality over the field grown plants.

Statistical analysis clearly indicated the effects of the stage of plant development on plant fertility. Plants at the 5-7 tiller stage not only had a marked influence on increasing plant fertility but also had significantly less detrimental effects on plant mortality than plants at the 2-3 tiller stage. This result is consistent with that of Arzani[13], but is in contrast to Thiebaut *et al.*[14] who indicated that the stage of plant growth has no significant effects on chromosome doubling or plant survival. This suggested

the optimisation of the growing environment was one of the reasons for better results.

***In vitro*:** The immediate genetic fixation which accompanies the doubling of the chromosome complement is an attractive feature of haploid breeding, especially in wheat where the conventional method takes several years. Recently the addition of low concentrations of colchicine to the wheat anther culture media for the first three days of culture was reported by Barnabas *et al.*[6], Hassawi and Liang[7] and Navarro-Alvarez *et al.*[5].

The application of colchicine as a pre-treatment in the induction medium in anther culture was carried out by Arzani[13]. He did not obtain a positive effect of the colchicine pre-treatment on the frequency of fertile regenerants, since the number of regenerated green plants derived from anther culture was inadequate.

Doubled haploid production by the *in vitro* method offers certain advantages over the conventional method. In the present study, haploid plants were treated *in vitro* for 48 h (treatment 2), which not only provided high plant fertility (88.0%) using a low concentration of colchicine (0.25 g L^{-1}) but also led to an increased number of seeds per fertile plant (73.3 seeds). This is potentially relevant to breeding programmes which require optimal seed set for the subsequent evaluation of doubled haploid lines. Furthermore, no abnormalities were observed in the fertile spikes.

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