E-ISSN: 2618-0618
P-ISSN: 2618-060X

NAAS Rating (2025): 5.20
www.agronomyjournals.com
2025; 8(8): 403-409
Received: 09-05-2025
Accepted: 12-06-2025

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In vivo and *in vitro* colchicine manipulations for enhancing doubled haploid production efficiency in bread wheat

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DOI: https://www.doi.org/10.33545/2618060X.2025.v8.i8f.3567

Abstract
The doubled haploidy breeding approach using chromosome elimination technique following wheat x maize system has provided the most suitable tool for doubled haploid production in bread wheat, but this has low success rate. Colchicines manipulations were done at *in vivo* and *in vitro* levels for enhancing the efficiency of doubled haploid production in bread wheat. For *in vivo* experiment colchicine concentrations ranging from 100 to 10000 ppm were injected with and without 2,4-D at first internode at different time intervals *viz.* 24, 48,72 and 96 hours after pollination. After 16-18 days embryos were excised and cultured on MS medium. For *in vitro* experiment the haploid embryos were rescued on MS medium containing colchicines at different concentrations ranging from 100 to 500 ppm and moved to colchicine free MS medium after different time intervals i.e. 24, 48, 72 and 96 hours. Control experiment was also conducted for both the methods in which colchicine treatment was not given neither in field nor in medium but at tillering stage by dipping the crowns of regenerated haploid plantlets in 0.1% colchicine solution supplemented with 1.5 ml/l dimethyl sulfoxide at 20 °C for 5 hours. Data on various parameters was analysed using three factorial RBD. The *in vivo* colchicine application was found to be most superior over standard method and *in vitro* method for enhancing doubled haploid production efficiency in bread wheat.

Keywords: Colchicine, doubled haploid production, bread wheat, *in vivo* colchicine treatment, *in vitro* colchicine treatment, wheat x maize system, chromosome elimination, haploid embryo culture, 2,4-D, embryo rescue, MS medium, chromosome doubling

Introduction
Wheat (*Triticum aestivum)* is a worldwide cultivated cereal from the Levant area of the Middle East and is the staple food for millions of people. But the low productivity which is the result of various biotic and abiotic factors has posed the threat to ever increasing population worldwide. Biotic factors include the incidence of diseases like rusts, powdery mildews, leaf blight. Abiotic stress comprises of drought, heat, cold, salinity etc. One such example is the attack of Ug99 the Ug99 fungus, called stem rust, could wipe out more than 80% of the world's wheat crops as it spreads from Africa, scientists fear. The International Maize and Wheat Improvement Center in Mexico estimates that 19% of the world's wheat which provides food for 1 billion people in Asia and Africa is in imminent danger. So, as to keep pace of the food production with population growth and for overcoming such havocks there is the need to develop biotic and abiotic stress resistant cultivars in the shortest possible time. The goal of any breeding programme is to produce pure line cultivars that are true breeding and homogenous in nature, so there is a need to develop homozygous lines. Producing haploids and doubled haploids in the crop species would enhance its improvement by accelerating the breeding programs, improving selection efficiency and facilitating genetic analysis. The doubled haploidy breeding approach using chromosome elimination technique following wheat x maize system (Laurie and benett 1988 and) [11] evolved as the most preferred route for doubled haploid production in wheat. For rapid isolation of homozygous lines of wheat doubled haploidy breeding approach is of immense importance which offers several advantages such as saving time and labour, development of 100 per cent homozygous lines at all loci. This technique as a potential haploid breeding approach for rapid isolation of homozygous lines (Inagaki and Tahir 1991; Sun and Khasa *et al*. 1995) [9].

The low efficiency of doubled haploids production has limited the exploitation of this technique in crop improvement. The usual approach of chromosome doubling in plants involves colchicine application to uprooted haploid plantlets at the tillering stage. As a result, there is high mortality rate after colchicine application. This limitation necessitates to look for other alternatives of colchicine application such as *in vivo* (in field) and *in vitro* (in laboratory) level. The objectives of the present investigation were therefore to study the effect of different colchicine concentration at *in vivo* and *in vitro* level for obtaining doubled haploid in bread wheat and standardize the protocol for enhancing doubled haploid production efficiency through colchicine manipulation in maize mediated system of chromosome elimination approach.

Materials and Methods

An elite doubled haploid DH 114 of bread wheat (*Triticum aestivum* 2n=42) parents VWFW 452 X WW 24 was used as female parent and a single variety of maize 'Early Composite' was chosen as a pollen source (*Zea mays* 2n=20). Maize plants were maintained in the polyhouse and wheat genotype (DH 114) was sown in two adjacent plots. Experiments were conducted during *rabi* 2007-2008. Three wheat spikes per treatment per replication for both *in vivo* and *in vitro* experiments were emasculated and pollinated.

In vivo colchicine manipulations

Two methods were employed for colchicine manipulations at *in vivo* level. First, manipulated method (Method I) in which different colchicine concentrations *viz*. 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 3000,5000, 7000 & 10000 ppm were injected with or without 2,4-D at different time intervals that is 24, 48, 72 and 96 hours after pollination and second, standard method (Method II) which was used as control where no colchicine was applied at field conditions but was applied at tillering stage by dipping the crowns of haploids in 0.1% colchicine solution supplemented with 1.5 ml per litre dimethyl sulfoxide at 20 °C for 5 hours. Spikes were harvested 15 to 18 days after pollination and seeds were examined for embryos, by using technique of Bains *et al*. (1998). The embryos were excised under aseptic conditions and cultured on nutrient medium comprised of MS medium supplemented with essential amino acids.

In vitro colchicine manipulations

Two methods were employed for colchicines manipulations at *in vitro* level. First, manipulated method (Method II) in which colchicine was applied in different concentrations *viz.* 100, 200, 300, 400 & 500 ppm in MS medium supplemented with various essential amino acids on which excised embryos were cultured. These cultured embryos were kept on this colchicine rich medium for 24, 48, 72 & 96 hours and then sub cultured to colchicine free medium. Second, standard method (Method II) was used as control experiment in which no colchicine was added to medium. Haploid embryos were kept on this colchicine rich medium as a control for each treatment for each time intervals. Colchicine was applied to regenerated haploids in the same way as above in control experiment of *in vivo* colchicine application.

Cultured embryos were given chilling treatment at 4 °C for 24hours immediately after embryo rescue and then shifted to complete darkness in the growth room for regeneration into green plantlets under controlled environmental conditions (2 °C$\pm 2^0$ C & 80% RH) for first 6-8 days. After that test tubes were transferred to 10hrs /14hrs light dark regime for 15-18 days until they developed to 3-4 leaflet stage, followed by their transfer to liquid rooting medium comprising half strength of MS salts (Murashige and Skoog, 1962), 1 mg per litre naphthalene acetic acid (NAA) and 1 mg/litre indole butyric acid (IBA), devoid of sucrose and agar. These plantlets were then transferred to pots containing potting mixture then to bigger pots (after 30-40 days) and finally to cage house. Data was recorded for percentage pseudo seed formation, embryo formation, plantlet regeneration, plant survival and doubled haploid (DH) seed production for *in vivo* experiment. For *in vitro* data on plantlet regeneration, plant survival and DH seed production was taken because no colchicine application was done before these parameters.

Results

In vivo colchicine manipulations

Method-II (standard method) was found to be s significantly superior over the method-I (manipulated method) for pseudo seed formation, embryo formation and plant regeneration. Method-I was significantly superior over method-II for plant survival and DH seed formation (Table). The effect of colchicine concentrations ranging from 100 to 10,000 ppm were statistically at par for pseudo seed formation whereas 600 ppm of colchicine concentration resulted in 9.17% embryo formation which is significantly higher than the other doses. Plantlet regeneration of (89.3%) was obtained by 100 ppm colchicine application whereas 1500 ppm which has resulted in significantly poor plant regeneration, for plant survival any of the treatment for plant survival all the colchicine concentrations are at par with each other whereas colchicine concentrations of 700, 200, 100 and 1000 ppm gave significantly higher doubled haploid seed production percentage i.e. 3.35, 2.69, 1.85, and 1.25% respectively.

Effects of time periods showed significant differences for pseudo seed formation that is colchicine when applied after 96 and 72 hrs. gave 40.12 and 39.82 per cent, respectively For plantlet regeneration colchicine doses when given after 72 hours of pollination resulted in (85.33%) significantly higher plantlet regeneration whereas, time period of 24 hours resulted in significantly poor plantlet regeneration (57.34%). Colchicine application after 48 hrs resulted in significantly higher plant survival (21.25%). Application of colchicine significantly lower plant survival after 24 hrs of pollination resulted in high doubled haploid seed production (1.46%)

Interaction effect of colchicine concentration x time period were found significant for all parameters except pseudo seed formation. Colchicine concentrations of 600 ppm when applied after 24 hrs. of pollination resulted in maximum embryo formation 17.04% (Table 2) followed by 1500 ppm after 72 hrs and 200 ppm and 100 ppm after 24 hrs gave 13.95, 12.04 and11.93% next significantly higher embryo formation frequency respectively. Plantlet regeneration 100% (Table 3) was obtained when colchicine concentration of 600 and 5000 ppm applied at 96 hrs., 200 ppm at 72 hrs. and 100 ppm at 48 hrs. after pollination followed by 1000 at and 400 ppm at 24 hrs, and 3000 ppm at 72 hrs. after pollination resulted in next higher frequency of 93.75, 87.50 and 87.50 per cent, respectively. Highest interaction effects (Table 4) were obtained when 100 ppm colchicine was applied at 72 hrs after pollination (50%) for plant survival followed by 300 ppm concentration when applied at 72 hrs after pollination (47.50%). For Doubled Haploid seed production (Table 5) colchicine dose of 700 ppm when applied at 48 hrs after pollination gave significantly higher interaction effects of 13 41% followed by of 100 and 200 ppm given after 24 hrs that is 10 75% and 13.25% respectively but were statistically at par with former interaction.

***In vitro* colchicine manipulations**

Method-II (standard method) was superior over Method-I (*in vitro* colchicine manipulations) for all the parameters studied namely plantlet regeneration, plant survival and DH production (Table 6) Colchicine concentration of 100 ppm resulted significantly higher plantlet regeneration of 62.71%. Highest plant survival of 23.50% was obtained by Colchicine concentration of 400 ppm. Effects of time periods were significantly higher for 24 hrs of colchicine application which resulted in 72.00% plantlet regeneration frequency whereas, colchicine treatment given for48 hours resulted in highest plant survival (14.00%)

Significant interaction effects of colchicine concentration x time period were significant for plant survival only in whichColchicine dose of 400 ppm applied for 48 hrs gave significantly higher plant survival 48.93% frequency (Table 7)

Discussion

***In vivo* colchicine manipulations**

The higher pseudo seed formation, embryo formation and plantlet regeneration in Method-II (Standard Method) as compared to Method-I (*in vivo* experiment) was because no colchicine was applied under field conditions. In Method-I, different doses of colchicine were applied along with and 2,4-D was applied at different time intervals in the field conditions which implies that colchicine had toxic effects due to which there was poor pseudo seed formation, embryo formation and plantlet regeneration in manipulated method. The higher number of plant survival and DH seed formation in Method-I and low frequency of these traits in Method-II was mainly attributed that no colchicine was applied in Method-I after regeneration whereas, colchicine application to the uprooted plants at tillering stage in Method-II resulted in high mortality.

Doubled haploid seed production was found to be high in 700 ppm followed by 200 and 100 ppm of colchicine. As 700 ppm was statistically at par with 100 & 200 ppm so 100 ppm being economical and safe was considered most effective in DH seed production. 400 ppm. Sood *et al*. (2003) who reported that 100 ppm colchicine concentration was most effective treatment for doubled haploid production when injected into uppermost internode of crossed tiller. For pseudo seed formation and plant regeneration time period of 72 hrs was giving highest results because by this time pseudo seed development has already taken place so, colchicine does not have any effect after a long time. Whereas, for plant survival and doubled haploid seed production 48 and 24 hours gave good results. This implies that doubling of chromosome number is more effective when applied during early time periods. Similar results were reported by Sood *et al.* (2003) while injecting 1000 ppm colchicine after 48 and 72 hrs of pollination.

Interaction effects are more important than individual effects of method, colchicine applications and time periods because these effects can help us to determine the appropriate colchicine concentrations and the time of its application. Amongst various colchicine concentrations x time periods for various parameters except pseudo seed formation was found to be significant. High plantlet regeneration was achieved when colchicine concentration of 600 and 5000 ppm applied after 96 hrs, 200 ppm after 72 hrs and 100 ppm after 48 hrs of pollination. High plantlet regeneration was achieved when colchicine was applied after 96 hrs followed by 72 and 48 hrs because after a long time embryo development has already has taken place and colchicine was not absorbed by the embryo at that time resulting the normal development of haploid embryo.

High plant survival at 72 hrs of 100 & 200 ppm colchicine application is due to the fact that there were toxic effect at initial time periods but these toxic effect decreased with increase in time period though at 96 hrs of colchicine application the plantlet regeneration was maximum but minimum plant survival. For doubled haploid seed production lower doses (100 & 200 ppm) of colchicine were more effective at 24 hrs after pollination, whereas higher dose of colchicine application (700, 1000 ppm) were more effective at 48 hrs after pollination. This attributed to the fact that the lower concentration can be easily absorbed at early stage of embryo development and effective in doubling the chromosome numbers but were not effective when applied at 48 hrs after pollination. On the other hand, the higher concentration might be toxic at early stages of cell division but able to double the chromosome number when applied after 48 hrs.

***In vitro* colchicine manipulations**

The main effects, Method-II was showing superiority over modified method (Method-I) indicating that the standard method was giving good result as compared to modified method of colchicine application for all parameters namely plantlet regeneration, plant survival and DH seed production. This was because embryos were directly exposed to colchicine on medium which has caused more mortality. The effects of different colchicine concentration were not encouraging as at none of the concentration DH seed was formed however, the colchicine concentrations of 100 and 400 ppm were giving significant effects for plantlet regeneration and plant survival respectively. The effects of time periods were also not influencing the doubled haploid seed production as none of the period effects were significant for doubled haploid seed production. Although 24 hrs and 48 hrs were showing significant differences for plantlet regeneration and plantlet survival.

The interaction effects of colchicine concentrations x time periods were found significant for plant survival only for 400 ppm colchicine when applied for48 hours. Hansen and Andersen (1998) have also reported that colchicine application at the rate of 300-1000 μm for 48 hrs in culture medium resulted higher number of doubled haploid plants during microspore culture. The appearance of non-significant results for interaction of treatment with different time period is due to large number of mortalities of embryo when cultured on the medium containing different concentrations of colchicine.

Table 1: Effects of methods, colchicine concentrations and time periods on different parameters in *in vivo* experiment

	Mean sum of squares				
	Pseudo seed formation (%)	**Embryo formation (%)**	**Plantlet regeneration (%)**	**Plant survival (%)**	**DH seed formation (%)**
Effects of methods					
M_I	27.94 (31.08)	3.89 (8.48)	56.80 (69.84)	19.13* (17.78)	1.08* (1.70)
M_{II}	46.82* (43.15)	10.56* (18.31)	80.17* (50.19)	6.12 (6.26)	0.30 (0.59)
Mean	37.38 (37.11)	6.99 (13.39)	68.58 (60.02)	12.63 (12.02)	0.69 (1.13)
CD (5%)	1.81	0.98	4.56	4.88	0.57

Effects of Treatment					
Colchicine concentration (ppm)					
100	35.40 (36.23)	8.44 (15.22)	89.30* (78.85)	19.12 (19.29)	1.85* (2.35)
200	39.18 (39.27)	6.90 (13.41)	79.10 (70.00)	12.50 (12.75)	2.69* (4.23)
300	35.99 (36.41)	6.78 (12.85)	57.92 (52.01)	16.04 (15.68)	0 (0.04)
400	40.11 39.10)	6.54 (13.12)	65.10 (57.25)	11.45 (10.05)	0.20 (1.41)
500	37.73 (37.53)	7.75 (14.57)	81.04 (71.75)	13.75 (13.95)	0 (0.04)
600	35.29 (35.95)	9.17* (16.03)	67.71 (60.33)	9.27 (10.38)	0.75 (1.80)
700	35.16 (35.92)	7.22 (13.92)	70.83 (61.51)	14.58 (14.28)	3.35* (4.40)
800	37.57 (37.46)	6.09 (11.89)	69.79 (61.59)	10.94 (11.19)	1.67 (1.32)
900	41.08 (39.51)	6.82 (13.22)	63.54 (55.39)	15.20 (15.10)	0 (0.04)
1000	38.41 (38.06)	8.20 (15.19)	72.19 (69.39)	14.69 (9.84)	1.25* (2.19)
1500	33.55 (34.82)	6.95 (12.05)	42.41* (44.56)	8.33 (8.24)	0 (0.04)
3000	39.81 (38.76)	6.94 (13.58)	57.60 (48.82)	11.45 (11.28)	0 (0.04)
5000	40.16 (39.01)	6.06 (12.34)	74.58 (67.03)	14.06 (14.60)	0 (0.04)
7000	34.42 (35.83)	6.93 (12.06)	63.54 (54.90)	8.33 (8.78)	0 (0.89)
10000	31.98 (33.86)	5.90 (11.58)	67.71 (60.69)	9.38 (13.87)	0 (0.04)
Mean	27.29 (37.12)	7.05 (13.6)	68.48 (60.02)	12.62 (12.61)	0 (0.60)
CD (5%)	NS	2.78	12.49	NS	1.56
Effects of Time Period (Hrs)					
24	31.12 (34.70)	7.78 (13.68)	57.34* (51.07)	11.46 (11.78)	1.46* (2.53)
48	35.12 (35.88)	7.27 (14.13)	70.36 (64.85)	21.25*(18.91)	1.23* (1.58)
72	39.82* (39.92)	6.62 (12.89)	85.33* (63.15)	16.94 (15.66)	0.02* (0.14)
96	40.12* (39.97)	6.41 (12.70)	71.92 (62.90)	1.67* (1.73)	0.44* (0.26)
Mean	36.54 (37.36)	7.04 (13.35)	68.40 (60.02)	12.63 (12.01)	0.69 (1.56)
CD (5%)	2.56	NS	6.45	6.9	0.81

*P≤0.5 Figures in parentheses are transformed values

Table 2: Effects of colchicine concentration x time period interactions on embryo formation (%) in *in vivo* experiment

Colchicine concentration (ppm)	**Time periods (hrs)**			
	24	**48**	**72**	**96**
100	11.93* (19.52)	8.95 (15.30)	5.56 (12.12)	7.31 (13.95)
200	12.04* (19.84)	6.50 (14.43)	4.96 (11.12)	4.09 (9.16)
300	7.04 (11.01)	7.25 (15.47)	9.32 (17.35)	3.52 (8.48)
400	9.29 (17.62)	6.32* (12.33)	6.32 (14.09)	4.25 (9.35)
500	5.77 (12.36)	9.35 (17.64)	8.66 (14.20)	7.23 (13.93)
600	17.04* (24.27)	8.16 (16.14)	5.21 (9.43)	6.27 (14.26)
700	7.62 (15.92)	10.24 (18.11)	4.44 (10.21)	6.68 (13.11)
800	5.35 (9.61)	7.65 (14.35)	4.40 (8.54)	7.03 (15.10)
900	5.53 (9.81)	7.41 (15.39)	5.09 (11.31)	9.26 (16.46)
1000	9.61 (18.05)	3.81* (7.96)	10.00 (17.35)	9.35 (17.35)
1500	4.61 (8.84)	3.81* (7.96)	13.95* (21.79)	5.42 (10.50)
3000	5.53 (9.73)	10.44 (18.69)	5.86 (12.85)	5.89 (12.57)
5000	7.04 (11.90)	4.45 (10.78)	5.89 (12.19)	6.83 (14.53)
7000	5.29 (9.60)	6.19 (12.90)	4.36 (8.54)	7.34 (15.44)
10000	3.85* (8.05)	8.40 (14.58)	5.51 (12.02)	5.78 (10.79)
Mean	7.83 (13.68)	7.26 (14.13)	6.63 (12.89)	6.42 (13.00)
CD (5%)	5.36	5.36	5.36	5.36

*P≤0.5 Figures in parentheses are transformed values

Table 3: Effects of colchicine concentration x time period interactions on plantlet regeneration (%) in *in vivo* experiment

Colchicine concentration (ppm)	**Time periods (hrs)**			
	24	**48**	**72**	**96**
100	78.03 (65.44)	100.00* (90.00)	87.51 (78.75)	91.67 (81.19)
200	81.25 (71.25)	9.67 (81.19)	100.00* (90.00)	43.75* (37.52)
300	33.75 (27.71)	70.83 (65.06)	77.08 (64.88)	50.00 (45.02)
400	87.50* (78.75)	85.42 (73.69)	62.50 (54.05)	25.00* (25.52)
500	68.75 (60.00)	66.67 (61.13)	93.75 (82.50)	95.00 (83.36)
600	56.25 (48.76)	70.83 (65.06)	43.75* (37.52)	100.00* (90.00)
700	79.17 (63.57)	75.00 (67.50)	37.50* (33.77)	91.67 (81.19)
800	43.75 (37.52)	41.67 (81.19)	50.00 (45.00)	93.75 (82.50)
900	37.50 (33.77)	85.42 (73.69)	87.49 (54.10)	68.74 (60.00)
1000	93.75* (82.50)	37.50* (33.71)	68.75 (61.44)	88.75 (75.86)
1500	43.75 (37.52)	37.50* (33.71)	93.75 (60.00)	68.75 (33.38)
3000	37.50 (33.77)	55.42 (48.31)	87.50* (75.16)	50.00 (35.19)
5000	33.75 (27.72)	70.83 (65.06)	93.75 (82.51)	100.00* (90.00)
7000	43.75 (37.52)	66.67 (48.89)	50.00 (45.02)	93.75 (82.50)

10000	41.67 (37.53)	87.50 (78.75)	72.50 (82.50)	64.58 (38.27)
Mean	57.34 (49.55)	70.36 (64.47)	74.72 (63.14)	75.36 (62.90)
CD (5%)	24.97	24.97	24.97	24.97

*P≤0.5 Figures in parentheses are transformed values

Table 4: Effects of colchicine concentration x time period interactions on plant survival (%) in *in vivo* experiment

Colchicine concentration (ppm)	Time periods (hrs)			
	24	**48**	**72**	**96**
100	11.90 (16.99)	14.58 (16.33)	50.00* (45.02)	0 (0.04)
200	25.00 (28.28)	25.00 (22.52)	0 (0.04)	0 (0.04)
300	0 (0.04)	16.67 (15.15)	47.50* (44.99)	0 (0.04)
400	16.67 (17.64)	0.02 (0.04)	25.00 (22.52)	0 (0.04)
500	0 (0.04)	16.67 (22.52)	37.50 (38.08)	0 (0.04)
600	29.83 (34.52)	0 (0.04)	6.25 (8.90)	0 (0.04)
700	33.33 (34.52)	25.00 (22.52)	0 (0.04)	16.67 (17.64)
800	12.50 (12.04)	12.50 (15.02)	0 (0.04)	0 (0.04)
900	0.00 (0.04)	40.83 (39.23)	22.50 (21.07)	0 (0.04)
1000	20.00 (19.63)	16.65 (17.21)	20.00 (19.63)	0 (0.04)
1500	8.33 (10.39)	0 (0.04)	25.00 (22.52)	0 (0.04)
3000	0 (0.04)	50.00 (45.00)	0 (0.04)	0 (0.04)
5000	0 (0.04)	50.00 (45.02)	0 (0.04)	10.00 (13.31)
7000	0 (0.04)	25.00 (25.02)	8.33 (10.04)	0 (0.04)
10000	0 (0.04)	50.00 (45.02)	6.25 (10.37)	0 (0.04)
Mean	10.17 (11.48)	21.67 (21.19)	16.55 (16.23)	1.78 (2.10)
CD (5%)	26.71	26.71	26.71	26.71

*P≤0.5 Figures in parentheses are transformed values

Table 5: Effects of colchicine concentration x time period interactions on doubled haploid seed production (%) in *in vivo* experiment

Colchicine concentration (ppm)	Time periods (hrs)			
	24	**48**	**72**	**96**
100	10.75* (13.32)	0 (0.04)	0 (0.04)	0 (0.04)
200	3.25* (15.46)	0 (0.04)	0 (0.04)	0 (0.04)
300	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
400	0.79 (3.66)	0 (0.04)	0 (0.04)	0 (0.04)
500	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
600	3.00 (5.21)	0 (0.04)	0 (0.04)	0 (0.04)
700	0 (0.04)	13.41* (15.61)	0 (0.04)	0 (0.04)
800	0 (0.04)	0 (0.04)	0 (0.04)	3.59 (3.32)
900	0 (0.04)	0 (0.04)	0 (0.04)	0.00 (0.04)
1000	0 (0.04)	5.00* (6.80)	0 (0.04)	0 (0.04)
1500	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
3000	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
5000	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
7000	0 (0.04)	0 (0.04)	0.25 (1.60)	0 (0.04)
10000	0 (0.04)	0 (0.04)	0 (0.04)	0 (0.04)
Mean	0.97 (2.54)	1.23 (1.53)	0.02 (0.15)	0.44 (0.26)
CD (5%)	3.13	3.13	3.13	3.13

*P≤0.5 Figures in parentheses are transformed values

Table 6: Effects of methods, colchicine concentrations and time periods on different parameters in *in vitro* experiment

	Plantlet regeneration (%)	**Plant survival (%)**	**DH seed formation (%)**
Effects of methods			
M_I	26.56 (25.57)	12.50 (2.27)	3.33 (11.59)
M_{II}	81.78* (69.99)	21.86* (24.85)	10.21* (16.89)
Mean	54.14 (47.78)	12.18 (13.56)	6.77 (8.78)
CD (5%)	6.38	4.75	3.02
Effects of treatment			
Colchicine concentration (ppm)			
100	62.71* (58.80)	8.67 (10.90)	4.93 (8.31)
200	57.29 (53.77)	12.50 (13.38)	4.91 (8.37)
300	47.07 (44.09)	5.34 (7.79)	2.81 (5.93)
400	52.09 (43.51)	23.50* (20.86)	14.99 (11.59)
500	45.00 (39.98)	13.23 (14.87)	6.22 (9.86)
Mean	52.83 (47.76)	12.50 (13.56)	6.77 (8.78)
CD (5%)	10.09	7.51	NS
Effects of time period (hrs)			

24	72.00* (57.68)	11.67 (15.73)	5.27 (8.56)
48	53.08 (48.44)	14.00* (20.01)	7.78 (10.63)
72	54.50 (49.14)	11.59 (13.59)	5.28 (8.87)
96	51.08 (47.00)	6.83 (8.21)	3.78 (7.12)
Mean	52.19 (48.01)	12.26 (13.17)	5.55 (8.80)
CD (5%)	9.03	6.72	NS

Table 7: Effects of colchicine x time period interaction on plant survival (%) in *in vitro* experiment

Colchicine Concentration (ppm)	Time period (hrs)			
	24	48	72	96
100	8.34 (10.25)	14.58 (16.32)	11.25 (14.52)	0 (0.03)
200	12.50 (12.70)	12.50 (12.69)	8.34 (10.26)	16.67 (17.88)
300	8.34 (10.38)	0 (0.03)	6.25 (8.95)	6.25 (8.95)
400	20.84 (20.07)	48.83* (42.56)	17.50 (17.90)	0 (0.03)
500	8.34 (10.26)	18.75 (18.76)	14.59 (16.32)	11.25 (14.16)
Mean	11.67 (12.73)	18.93 (18.13)	1.59 (13.59)	6.83 (8.21)
CD (5%)	15.02	15.02	15.02	15.02

*P≤0.5 Figures in parentheses are transformed values

Table 8: Effects of method x colchicine concentration x time period on plantlet regeneration (%) in *in vitro* experiment

Concentration (ppm)	Method I	Method II
100	40.00 (39.23)	85.42 (75.57)
200	25.00 (29.43)	100.00* (90.00)
300	20.00 (23.04)	74.17 (65.13)
400	30.00 (29.47)	67.70 (57.56)
500	5.00 (6.41)	85.00 (73.56)
Mean	14.24 (25.51)	80.37 (70.15)
CD (5%)	14.27	14.27

*P≤0.5 Figures in parentheses are transformed values

Conclusion

The study demonstrated that both *in vivo* and *in vitro* colchicine treatments significantly influence the efficiency of doubled haploid (DH) production in bread wheat. Among the strategies tested, optimized colchicine concentrations and treatment durations played a critical role in improving chromosome doubling rates and plant survival. *In vitro* colchicine application, when precisely timed and carefully administered, resulted in higher doubling efficiency compared to *in vivo* treatments, with reduced toxicity and better control over treatment conditions.

These findings underscore the importance of refining colchicine protocols to enhance DH line development, thereby accelerating breeding cycles and genetic gains in wheat improvement programs. The study offers valuable insights into practical colchicine manipulation techniques that can be adopted by wheat breeders for more efficient and reliable doubled haploid production.

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