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IN VITRO WHEAT HAPLOID EMBRYO PRODUCTION BY WHEAT x MAIZE CROSS SYSTEM UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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Haploids are helpful in studies for intergenomic relationship, identifying molecular markers, reducing time period of varietal development and increasing efficiency of breeding program. In case of bread wheat (*Triticum aestivum* L.), wheat x maize cross system is the most successful system due to its higher efficiency, more haploid embryo production and low genetic specificity. The haploid embryo production is affected by many factors i.e. light, temperature, relative humidity and tiller culture media. A study was carried out comprising 25 genotypes of bread wheat for haploid embryo production using 100 mgL^{-1} 2,4-D, 40gL^{-1} Sucrose and 8mlL^{-1} Sulphurous acid. Haploid embryo production was observed at various levels of environmental factors i.e. maize pollen collection temperature, time of pollination after tiller emasculation, light intensity and relative humidity during haploid seed formation. Maximum haploid embryo formation recorded was 9.52%. Best temperature observed for pollination was 21-26^{o}C, optimum time duration for pollination was 24 hours after emasculation , light intensity was 10,000 Lux and relative humidity was 60-65% at 20-22^{o}C .
Keywords: wheat, doubled haploid, wheat x maize cross, *in vitro*

INTRODUCTION

Wheat is major staple food worldwide originated from the Fertile Crescent region and cultivated all over the world. It is one of the most produced cereals. Wheat is also a source of vegetable protein in human diet, having higher protein content than either maize or rice.

Varietal development of wheat requires homozygosity and homogeneous lines which is a laborious and time consuming process and it delays varietal developmental programmes for many years. Recent advances in doubled haploid (DH) production enabled the development of homozygous lines instantly from a crop plant. DH in bread wheat can be used in a number of ways to identify molecular markers for improving quality traits (Perretant *et al*., 2000). DH plants could be produced for achieving homozygosity in single step by a number of ways such as anther/microspore culture, ovary/ovule culture, and chromosome elimination following hybridization, haploid inducer genes and chemicals. So duration of varietal development is shortened and resources are conserved.

Bread wheat DH are produced by various intergeneric crosses viz., wheat x miaize (Sun *et al.,* 1995) wheat x pearl millet (Ahmad and Comeau, 1990), wheat x tripsacum (Riera-Lizarazu and Mujeeb-Kazi, 1993), wheat x teosinte (Suenaga *et al*., 1998.), wheat x barely (Barclay, 1975), wheat x job's tears (Mochida and Tsujimoto, 2001) and wheat x sorghum (Ohkawa *et al*., 1992). Some earlier studies showed that Wheat x maize system has more efficiency of embryo formation rate as compared to other techniques. For haploid embryo production a system of wheat x maize crossing is widely used due to higher production of haploid embryos as compared to other grass species pollination systems (Inagaki and Mujeeb-Kazi, 1995).

This system is fast, economical as compared to other techniques, ease of application and more efficient than others due to low level of genetic specificity (Cherkaoui and Lamsaouri, 2000). Zenkteler and Nitzsche (1984) reported for the first time that embryos were frequently formed when hexaploid wheat pollinated with maize. Later on their results were confirmed by Laurie and Bennett (Laurie and Bennett, 1986). They cytologically demonstrated that the maize pollen normally germinated and grew into the wheat embryo sac where the wheat egg was fertilized by the maize pollen. A hybrid zygote with 21 wheat chromosomes and 10 maize chromosomes was produced (Laurie and Bennett, 1989). The hybrid zygotes were karotypically unstable; therefore, maize chromosomes fail to move towards the spindle poles during cell divisions. Possibly, their centromeres fail to attach to the spindle

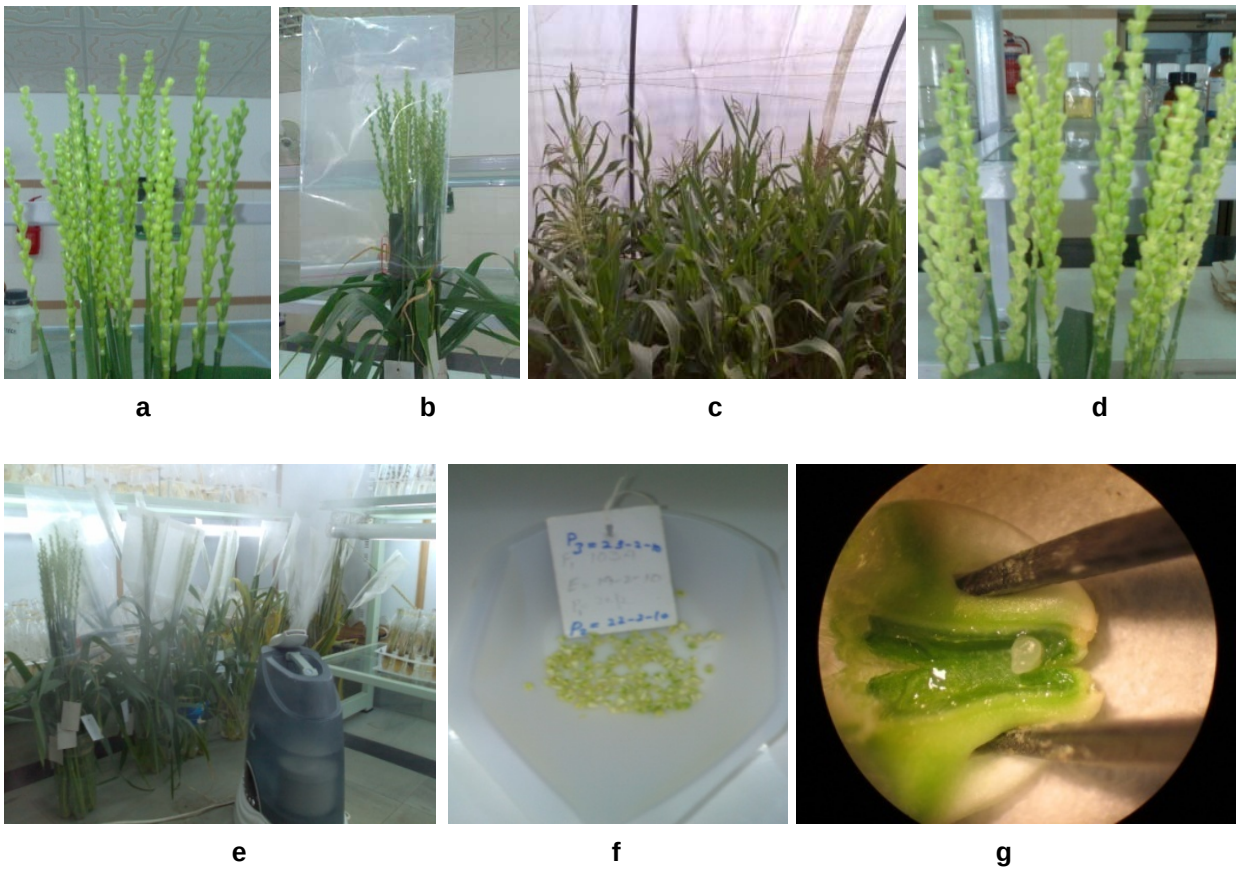
microtubules due to progressive loss of centromere activity, resultantly in maize chromosomes were eliminated after 3-4 mitotic cell divisions from genetic mechanism of haploid embryo forming wheat haploid embryo with n=21 chromosomes (Laurie and Bennett, 1989). The embryo was rescued and grew on nutrient medium for development because endosperm was absent which may result in nutritional shortage (Zhang *et al*., 1996). The objective of this study was to ascertain the effects of different environmental factors at various levels of haploid embryo development.

MATERIALS AND METHODS

Twenty five F_1 crosses were made involving 21 wheat varieties/lines during the month of February and March, 2009. These crosses were sown in December, 2009 and used for wheat x maize cross.
A commercial hybrid of maize was sown in November, 2009 on three dates with 15 days interval to synchronize wheat anthesis and ensure maize pollen availability throughout the reproductive stage of the wheat crop.
For doubled haploid embryo production following procedure was adapted:

a) ***Emasculation*****:** Healthy tillers at booting stage were selected from field and cut at appropriate length (Riera-Lizarazu *et al.*, 1992). The central floret of each spikelet was removed by forceps to produce more space for lateral florets. Anthers were removed manually with the help of fine forceps (Fig. 1. a) and then emasculated tillers were placed in water and spikes were covered with plastic bag to conserve moisture (Fig.1.b).

b) ***Pollination:*** Fresh maize pollen was collected in petri-dish at various temperature levels ranging from 23-30^{o}C (at 11:00 am to 2:00 pm) (Fig.1.c). Emasculated spikelets were pollinated with freshly collected maize pollen after 24, 48 and 72 hours of emasculation under controlled conditions (Fig.1.d).



a **b** **c** **d**

e **f** **g**

Figure 1. Schematic presentation of *in vitro* haploid production of wheat x maize croses. a) emasculated tillers; b) tillers covered with plastic bag; c) pollen donor maize plants; d) pollinated tillers; e) tillers under high humidity; f) harvested haploid wheat seeds; g) haploid wheat embryo

c) ***Haploid seed formation*****:** After pollination, tillers were kept in tiller culture media containing 100mgL^{-1} 2, 4-D, 40gL^{-1} Sucrose and 8mlL^{-1} Sulphurous acid for 14-16 days (Mujeeb-Kazi *et al.,* 2006). Haploid seed formation was recorded under various levels of environmental factors i.e. light intensity below and at 10,000 Lux, relative humidity at 40-50, 60-65 and above 70% (Fig.1.e).

d) ***Embryo rescue:*** After 14-16 days of pollination, haploid seeds were collected from pollinated spikelets (Fig.1.f). Seeds were washed with solution of 1-2% sodium hypochlorite, tween-20 and autoclaved water for 10 minutes. Finally the seeds were rinsed three times with autoclaved water. Haploid seeds were dissected under stereoscope (Fig.1.g) and haploid embryo was rescued. Haploid embryos were transferred to artificial nutrition media (½ MS).

RESULTS

Twenty five F_1 crosses of bread wheat were used for haploid embryo production under 16 groups made according to their emasculation date. In each group, 3-25 spikes were emasculated and pollinated after 24, 48 and 72 hours of emasculation. The temperature at the time of maize pollen collection was ranged between 23-29.4^{o}C. Seeds were harvested after 14-16 days of pollination and dissected to rescue embryo. Percentage of embryo was calculated from seed produced (Table 1).

Under the three different time intervals between emasculation and pollination 24 hour periods was optimal. Haploid embryo formation was also observed under 48 and 72 hrs intervals but in some crosses these two intervals showed no haploid embryo production. The overall production was consistent in 24 hrs time period. The three different temperature regimes showed that embryo formation declined with increase in temperature from 25^{o}C and under this range haploid embryo production was remarkable.

DISCUSSION

There are two major methods to produce wheat haploids, microsporogenesis and megasporogenesis (Kisana *et al.,* 1993). Megasporogenesis using egg cells is superior over microsporogenesis using anthers due to absence or low genetic specificity, absence of albinism (as in case of anther culture) and efficiency of application (Lefebvre and Devaux, 1996) Wheat x Maize cross system is one of the most efficient and economical method for production of haploid wheat embryo (Morshedi and Darvey, 1995). Cut plants under *in-vitro* conditions showed better results than intact plants grown in the field (Inagaki and Mujeeb-Kazi, 1995; Ballesteros *et al*., 2003).

Various environmental factors were studied at different levels and their effect on haploid embryo production was recorded to reveal maximum haploid production conditions. These conditions were: time interval between emasculation and pollination, temperature at the time of maize pollen collection, light intensity and humidity levels (Table 1).

Table 1. Embryo formation %age under different environmental conditions

Group No.	No. of Spikes pollinated	No. of florets pollinated	Time of pollination after emasculation (Hrs)	Temperature (^{o}C)	No. of Seeds formed	No. of embryos rescued	Embryo %age
1	11	287	24	26.7	25	1	4.00
2	23	686	24	29.0	37	2	5.41
3	22	549	24	28.1	44	4	9.09
4	16	287	24	29.3	45	1	2.22
5	20	302	24	23.2	99	9	9.09
6	24	408	24	24.0	243	23	9.47
7	15	422	48	25.3	11	0	0.00
8	15	479	48	29.4	33	1	3.03
9	6	168	48	27.4	42	3	7.14
10	16	280	48	24.1	107	8	7.48
11	12	226	72	24.3	84	8	9.52
12	16	243	72	23.0	151	8	5.30
13	12	193	72	23.7	92	6	6.52
14	12	264	72	26.5	64	5	7.81
15	3	56	72	26.5	12	0	0.00
16	13	425	72	28.0	17	0	0.00

The time interval between emasculation and pollination was kept 24, 48 and 72 hours. Group numbers 3, 5 and 6 showed maximum values of embryo formation for all the crosses in case of 24 hours after emasculation. Although there were some high embryo production rate in some crosses with time interval of 48 and 72 hours but the overall performance of 24 hours time period was better, so increase or decrease in the time period will reduce the haploid embryo formation. This study establishes that wheat ovary matures and starts response for fertilization after 24 hrs of emasculation. Delay in this period may lead to over maturity of ovary and reduces its fertilization capacity.

Optimum temperature at the time of maize pollen collection was observed between 21-26^{o}C. It is depicted from the Table 1 that few or even no embryo was developed when temperature was above 26^{o}C. Increase in temperature reduced pollen water content and resulted in pollen death. So pollen should be collected before intense sun-light (before noon) because high temperature will reduce the pollen viability thus causing the failure of pollen to produce embryos.

Effect of high and low light intensity during seed formation revealed that haploid seed and haploid embryo formation was reduced drastically when light intensity was less than 10,000 Lux (data not shown). Therefore, 10,000 Lux light intensity was sufficient for haploid as well as embryo formation. Variation in light intensity can affect many physiological processes in plants (Campbell et al, 1998). Change in light intensity disturbs pollen tube growth. Under low light intensity it may reduce speed of pollen tube formation and pollen nuclei die before reaching the ovary due to energy shortage. High light intensity continues photosynthesis in detached tiller and produces photosynthates for developing embryo. This increases recovery of haploid embryo.

Three levels of humidity were also studied (data not shown) , which reveals that below 60 % relative humidity, tillers lost their moisture and produced shriveled seeds without embryo. Because in the absence of roots plant is unable to uptake water efficiently from tiller culture media and decrease in humidity of surrounding atmosphere reduces water contents in plants. In case of above 70% relative humidity, severe fungal attack was noted on tillers and especially on spikes under *in vitro* conditions. The best relative humidity for haploid seed formation and haploid embryo production ranged from 60-65%.

CONCLUSION

From the present results it was concluded that different environmental factors affected wheat haploid embryo production at large scale. A simple protocol was observed that tillers were cut, emasculated and placed in water for 24 hours while spikes were covered with plastic bag. Then these tillers were pollinated with fresh maize pollen collected at 21-26^{o}C. After pollination tillers were kept in tiller culture media at 20-22^{0}C for 14-16 days at 60-65% relative humidity. Finally, haploid seeds were harvested from tillers and dissected under stereoscope with aseptic conditions and haploid embryos were shifted in ½ MS media.

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