EMASCULATION TECHNIQUES AND DETACHED TILLER CULTURE IN WHEAT X MAIZE CROSSES

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ABSTRACT

Intergeneric hybridization of wheat with maize has been exploited to achieve instant homozygosity in wheat with the production of doubled haploids. A study was conducted at Agricultural Biotechnology Research Institute, AARI, Faisalabad during 2007-08 and 2008-09 to enhance the efficacy of haploid production, in terms of frequency of seed and embryo development. In wheat crop manual emasculation of spikes is laborious and time consuming. Different emasculation techniques like hand emasculation with cut and intact glumes, hot water emasculation and no emasculation were compared. The results reflected that laborious exercise of hand emasculation can be substituted with no or hot water emasculation methods with above 10 percent embryo frequency. Alternative technology to reduce the space required for pollinated wheat heads was also studied by tiller culture method. Detached tillers were kept in liquid nutrient medium after emasculation to increase turnover for greenhouse materials. The results indicated that wheat spikes can be successfully cultured in a liquid medium before anthesis till embryo rescue. There is a close similarity for grain formation and embryo development in the detached wheat spikes in liquid medium and intact plant method. Detached tiller culture was found highly successful in wheat x maize crossing (17.15% embryo formation in emasculated florets). This technique had great edge over intact plant method with respect to saving of time and space, delaying of pollination due to storage of tillers under controlled conditions, simple hot water emasculation of detached tillers and application of plant growth regulators. Solution containing 40 g/L sucrose, 100 mg/L 2, 4-D and 8 ml/L sulphurous acid was identified as the best medium for culture of wheat x maize crossed tillers for getting maximum frequency of haploid embryos per pollinated floret.

KEYWORDS: ***Triticum aestivum; Zea mays;*** **plant emasculation; cross-breeding; embryo culture; Pakistan.**

INTRODUCTION

To cope with ever- increasing food demand due to rapid population growth in Pakistan, it is essential to increase the production of cereal grains. The wheat

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improvement endeavours in the past had not only made the country self-sufficient in wheat but some surplus has also been exported besides maintaining the annual reserves. Due to gradual increase in population of Pakistan, the demand of wheat is also increasing. Tremendous efforts are thus needed to improve wheat crop for meeting the demand of 22 to 28 million tons during the next 10-12 years.

Practical breeding programmes to evolve high yielding wheat varieties, should include a system of instant homozygosity of important characters after gene pyramiding to gain more variation (8). Many generation cycles are needed to achieve uniformity in different agronomic traits which is time consuming. Production of haploids has significant value as genetic and breeding tool for crop yield enhancement. The prime goal of haploid production is to achieve instant homozygosity by reducing time. True breeding pure lines can be developed rapidly using this technique. Further, the lines developed through doubled haploid method are pure and useful in maintaining pure genetic stocks or their utility in molecular work where 100 percent purity is essential for reconfirmation of experimental findings (2).

Haploid breeding has several advantages over the conventional method and equally important to molecular mapping and genetic studies. It also provides a way of combining and fixing the desirable features of diverse wheat genotypes into a common genetic background. At present haploid breeding technique is quite advanced and is being routinely employed to generate valuable homozygous breeding materials and creation of new cultivars in different crop plants throughout the world. For example China which ranks the first to use this technology, has alone released hundreds of rice and wheat varieties within a period of 15 years (7).

To be efficient as a breeding tool, doubled haploids need to be produced in a high frequency across all genotypes. In addition, production needs to be cost efficient, and the products need to be competitive with those produced by conventional breeding methods.

Several techniques are available for the production of haploids which include: wheat $\times$ maize crossing system, anther culture, isolated microspore (pollen) and megaspore (egg cell) culture and the *Hordeum bulbosum* method. Now-a-day's three haploid production methods are present i.e., *Hordeum bulbosum* method (21), anther culture (26) and wheat x maize crossing (16, 17, 23, 24). It was pointed out by Zenkteler and Nitzsche (27) for the first time that embryos can be frequently made by pollinating bread wheat with maize. This raised the considerable interest among wheat breeders. Laurie

and Bennett (13, 14) systematically studied to confirm the previous reports. They were cytologically able to demonstrate that maize pollen normally germinates and forms wheat embryo sac after the fertilization of wheat with maize resulting in zygote having 10 maize chromosomes and 21 wheat chromosomes.

A lot of technical refinements have been made in the technique, to use this system in practical breeding programmes, mainly for embryo formation, germination and green plant regenerations components, culture media and chromosome doubling.There are two tedious steps involved in the technique viz. hand emasculation of wheat spikes and pollination and post pollination hormonal treatment of individual spikes. Emasculation of wheat spikes is the first step in haploid production from the wide cross system, which is a laborious and time consuming procedure. The most common method is clip emasculation which involves the removal of anthers from the florets with forceps following cutting of the glumes. However, the glumes may be left intact in wheat × maize crosses. Laurie (12) obtained higher embryo frequencies with the intact glume method (55.8%) than by traditional clip-emasculation (37.2%), where the exposed stigmas significantly reduced the frequency of embryo formation.

Although emasculation is one of the main steps in hybridization, it is not a prerequisite for crossing. In certain self-pollinated species such as soybean (25) and common bean (4), hybrid seeds can be obtained by pollinating the stigmas without emasculation. Matzk and Mahn (15) also demonstrated this in the wheat × maize system, where crosses without emasculation were successful if pollination was performed 1-2 day(s) before anthesis. Inagaki (8) also promoted the method of pre-anthesis pollination of wheat spikes with maize pollen for haploid production.

The present study was conducted to replace the routine emasculation procedure (cut glume technique) with a labour efficient alternative that gives a high frequency of embryos, and to overcome the need for keeping spikes on the plants and thus reduce both space and labour requirements.

MATERIALS AND METHODS

Emasculation methods

These studies were conducted at the experimental areas, laboratories and glass houses of Agricultural Biotechnology Research Institute, Wheat Research Institute, AARI and Department of Botany, GC University Faisalabad, Pakistan during the years 2007-08 to 2008-09. Two spring wheat

F_1-crosses, Cross-1 and 'Cross-2' (Table.1) were used as female parents to evaluate the effects of different emasculation methods on seed set, embryo formation and regeneration. Three maize parents i.e. Neelum, Golden and Sultan were used as pollinator.

Table 1. Wheat F_1s used as female parents, in crosses with maize.

Cross No.	Female parent	Male parent
Cross-1	Inqalab91	Chakwal-86
Cross-2	MH-97	Auqab-2000
Cross-3	Punjab-76	Parula

In Experiment-1, non-emasculated spikes (T_1) were compared with three methods of emasculation viz. cut glume emasculation (T_2) intact glume emasculation (T_3), and hot water emasculation (T_4). Emasculation of the wheat spikes was performed two days before anthesis (Fig. 1).

Two basal and upper spikelets were removed and only the primary and secondary florets of remaining spikelets were used. In T_2 and T_3, three anthers in each floret were removed with a fine forceps. In T_1, florets were pollinated two days before anthesis without removing the anthers. In other treatments, anthers were removed from each floret by carefully inserting the forceps between the lemma and palea and separating them. The three anthers were removed with the forceps, carefully to avoid their crushing or unduly injury the feathery stigmas. In T_4, spikes were immersed in water at 43°C for four minutes (hot water method-I) (Fig. 2). The glumes in T_3 were left intact. With the exception of T_1, pollination was performed on the day of anthesis.

Fig. 1. Wheat tillers at emasculation stage

Fig. 2. Hot water emasculation of wheat tillers

Maize pollens were collected by gently shaking the pollen producing tassel over a craft paper bag or petri dish. The collected pollen was transferred to the wheat stigmas within 10-15 minutes of pollen release, with the aid of a small camel hair brush.

Application of growth regulator

The 2,4-D stock solution was prepared by dissolving 1.0 g of powder in ten ml of absolute ethanol and making volume 100 ml by adding distilled water. The solution was diluted with distilled water when it was needed for the treatment of spikes. One day after pollination the spikes were treated as follows:

1. A hole was made in each end of the upper internode with a fine hypodermic needle (1.0 ml syringe).

2. 2, 4-D solution (0.01%) was injected gently through the lower hole until the solution started to drip out of hole at the top.

3. The holes were covered with wax to prevent leakage.

4. The same exercise was repeated for 2-3 consecutive days.

5. The treated spikes were covered with a butter paper crossing bag and left for 14-16 days.

Embryo rescue

After 12-14 days of pollination each floret was removed from the spike, palea and lemma opened, and intact caryopsis placed in a petri dish. These seeds were smaller in size and lack endosperm.

The caryopsis were sterilized with a solution of sodium hypochlorite (1-2%) and few drops/L of tween-20 for 15 minutes followed by 3-4 rinsing with autoclaved water.

Dissection of embryos from sterilized caryopsis was done under 10 × magnification in a stream of sterile air on a laminar flow bench. The caryopsis were placed on a sterile filter paper and slit opened at the side with a forceps to expose the liquid contents of caryopsis (Fig. 3). The embryo was lifted out by a needle or forceps and placed on the embryo rescue medium. The embryo culture medium was half strength MS with full vitamins, 30 g/L sucrose, 8 g/L agarose, and pH 5.5 as described by Ahloowalia (1) and Suenaga and Nakajima (23).

Fig.3. Haploid caryopsis with embryo

Fig.4. Germination and growth of haploid plantlets

Culture conditions

The immature embryos were incubated in a culture cabinet. The first phase of culturing for one to two weeks was done in the dark at 22±2°C. When properly differentiated, the embryos were transferred to a growth room at 25±2 °C with a 16-8 hour light-dark periods (Fig. 4).

Plantlets cultured *in vitro* require hardening and good development to cope with transplantation to the soil. About three to five days prior to transplanting, the test tubes were placed in the controlled greenhouse under the same growing conditions as are routinely used for young plants. The plantlets were carefully lifted from the tubes, and remaining media was washed off. The plantlets were then transferred to small pots filled with a light soil mixture (sand: peat: clay in 1:1:1 by volume). The potted plants were put in a tray and covered with a polyethylene sheet to prevent desiccation.

Once growth was evident, the sheet was removed and plants were allowed to grow normally (Fig. 5). Haploid status of the plants were tested by making squash preparation from root tips (Fig. 6).

Fig. 5. Haploids under controlled conditions

Fig. 6. Haploid chromosomes

Chromosome doubling

When the plantlets reached 2-3 tiller stage, following treatments were initiated:

1. Soil was washed off the roots
2. Roots were cut back to 2-3 cm below the crown.
3. The plants were placed in a glass beaker.

The colchicine (500 to 1000 mg) was dissolved in 20 ml of dimethyl sulfoxide (DMSO), and made upto 1 litre with water and few drops of Tween-20. The solution was placed in the beakers to cover the plant crowns and part of the leaf area (Fig. 7).

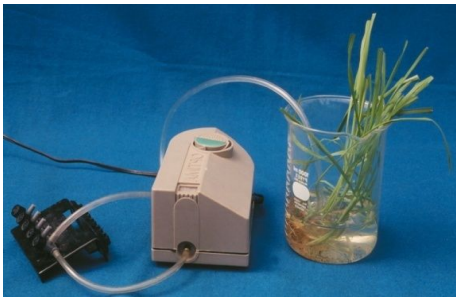

Fig. 7. Chromosome doubling with colchicine

Fig. 8: Doubled haploid plants

The plants under treatment were placed in a controlled microclimate area at 20±1 °C in the light for five hours. Roots were aerated with small air pump. After washing under running water for 5-10 minutes these were potted in a light soil. Given optimum conditions for growth, the colchicine treated plants flowered from one to three months later (Fig. 8). The root sections of the colchicine treated plants were examined under microscope for the confirmation of doubling of chromosomes.

Detached tiller culture

In tiller culture studies, two experiments were conducted. In Experiment-1, 'Cross-3' was used as the female parent (Table 1) to evaluate embryo formation and haploid production in this study. The male parents consisted of five maize synthetic cultivars and three F_1 hybrids (Table 7). The female parents were grown in the field while the pollen sources in a controlled-greenhouse environment. The same pollen sources were used to evaluate

seed set, embryo formation and haploid production using detached tiller culture.

In Experiment-2, 'Cross-2' was used as the female parent and FSH-399 as male to evaluate embryo formation and haploid production. Detached tiller culture method was used with different modifications of the culture media. In detached tiller culture technique, wheat tillers were cut off from plants on the day of emasculation having length of 40-50 cm. The detached wheat stems were cultured in tap water after trimming of the leaves on the stems. Emasculation was done as described in intact plant method. Pollination was performed one day before or on the initial day of anthesis. After pollination wheat stems were transferred into a solution, containing, 8 ml/L sulfurous acid (6% SO_2), 40 g/L sucrose, and 100 mg/L 2,4-D (for first 3 days only) following the methods of Inagaki (8). Tillers were kept at 21 °C under lights. Fresh culture solution was added to the containers every two to three days (Fig. 9). Rest of the procedure was same as done in intact plant case.

Fig. 9. Detached tiller culture of wheat x maize crosses

Data analysis

Data were recorded from both experiments and manipulated for statistical analysis using method of Steel *et al.* (22) for analysis of variance using the MINITAB computer package. DMR test was applied to mean values of the treatments.

RESULTS AND DISCUSSION

Emasculation methods

The data on the efficiency of emasculation methods based on the seed set, embryo formation and embryo regeneration across wheat and maize parents in Experiment-1 are presented in Table 2 and summarized in Table 3.

Table 2. Effect of emasculation on seed set, embryo formation, and haploid plant production in wheat × maize crosses.

Wheat genotypes/ pollen source	Emasculation methods	No. of pollinated florets	Seed set as % of pollinated florets	Embroys as % of pollinated florets	Haploid plants as % of embryos
Cross-1 /Neelum	T_1	100	88.0	11.3	52.7
	T_2	100	92.0	16.0	46.9
	T_3	100	95.3	17.2	58.1
	T_4	100	79.7	12.2	45.9
Cross-2 / Neelum	T_1	100	86.0	9.2	54.5
	T_2	100	84.7	15.0	51.6
	T_3	100	87.3	15.2	54.7
	T_4	100	93.3	8.8	47.4
Cross-1 / Golden	T_1	100	92.3	12.0	45.8
	T_2	100	95.7	15.0	47.7
	T_3	100	93.0	16.3	56.0
	T_4	100	85.0	13.0	44.9
Cross-2 / Golden	T_1	100	86.0	10.7	49.1
	T_2	100	84.7	14.0	57.0
	T_3	100	93.7	16.0	58.4
	T_4	100	83.7	9.8	45.3
Cross-1 / Sultan	T_1	100	83.7	10.3	53.3
	T_2	100	94.0	16.0	52.1
	T_3	100	93.3	15.0	52.0
	T_4	100	88.0	11.7	47.1
Cross-2 / Sultan	T_1	100	82.0	9.2	45.4
	T_2	100	97.0	15.3	54.2
	T_3	100	92.7	16.0	57.2
	T_4	100	84.7	7.7	45.3

Emasculation methods: T_1 = No-emasculation, T_2 = Cut glumes, T_3 = Intact glumes and T_4 = Hot water (43^{o}C, 3 minutes).

Table 3. Mean seed, embryo formation and haploid production in two wheat genotypes after pollination with three maize genotypes, following non-emasculation and three methods of emasculation.

Emasculation methods	Seeds (%)	Embryos/ florets (%)	Haploids/ embryos (%)
No emasculation (T_1)	86.33	10.44	50.15
Cut glume method (T_2)	91.33	15.22	51.60
Intact glume method (T_3)	92.56	15.94	56.09
Hot water method (T_4)	85.72	10.53	46.00

The analysis of variance for seed set and haploid production was non-significant among maize parents and differences among emasculation methods were significant for wheat crosses. There were highly significant differences among emasculation methods and all interactions except for embryo formation with respect to maize parents x wheat crosses x emasculation methods (Table 4).

Table 4. Analysis of variance (mean squares) of seed set, embryo formation and haploid production in crosses between two wheat and three maize genotypes as influenced by different methods of emasculation.

Sources	DF	Mean squares		
		Seeds	Embryos/seeds	Haploids/embryos
Maize (M)	2	8.85 N.S	3.1 *	5.56 ns
Wheat (W)	1	74.01 **	45.92**	39.01*
M x W	2	27.60 **	1.35ns	29.78*
Emasculation (E)	3	215.64 **	157.48**	312.18**
M x E	6	50.7 **	2.9 **	31.12**
W x E	3	55.35 **	8.18 **	34.61*
M x W x E	6	74.60 **	0.94 ns	26.05*
Error	48	4.53 **	0.94	9.17
Total	71			

NS = Non-significant, *$p < 0.05$, **$p < 0.01$, CV = 2.39%

Two wheat crosses, three maize cultivars and four emasculation methods differed significantly for the means of embryo formation ($p < 0.01$). Maize parent Golden resulted in the highest frequency of embryos formation (13.35%). 'Cross-1' gave a higher mean embryo response than 'Cross-2' (Table 5.). The intact glume method (T_3) (15.94%), cut glume technique (T_2) (15.22%) and hot water technique (T_4) (10.53%) showed significant differences for embryo formation (Fig. 10 and Table 3).

Table 5. Means for seed set, embryo formation and haploid production in crosses between two wheat genotypes and three maize F_1 hybrids as influenced by different methods of emasculation (Experiment No. 1).

	Seed set (%)		Embryo formation (%)		Haploid production (%)	
Maize	Mean	SE	Mean	SE	Mean	SE
Neelum	88.29	1.03	13.10	0.64	51.49a	0.98
Golden	89.25	1.02	13.35	0.51	50.55a	1.23
Sultan	89.42	1.16	12.65	0.67	50.84a	1.0
Wheat						
Cross-1	90.0 a	0.87	13.83 a	0.40	50.22 a	0.80
Cross-2	87.97 b	0.85	12.24 b	0.54	51.70 a	0.93

Emasculation						
No-emasculation	86.33 b	0.89	10.44 c	0.32	50.15 b	1.02
Cut glume	91.33a	1.25	15.22 b	0.25	51.60 b	1.03
Intact glume	92.56a	0.80	15.94 a	0.28	56.09 a	0.93
Hot water	85.72b	1.09	10.53 c	0.49	46.00 c	0.54

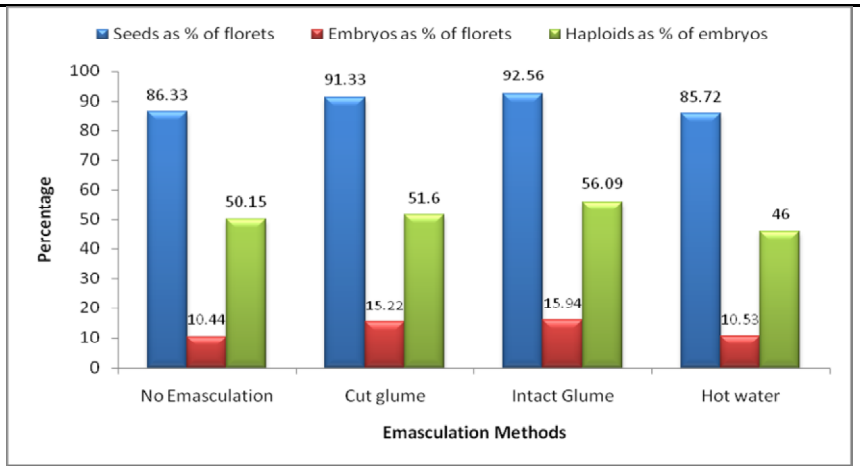

Fig. 10. Effect of emasculation techniques on seed set, embryo formation and haploid production in wheat x maize crosses

There were highly significant differences between the wheat cultivars regarding regeneration of their haploid embryos ($p < 0.01$), whereas the effect of maize parents did not show significant differences. Statistical analysis showed no significant difference in regeneration of embryos produced by the non-emasculated method (50.15%) and cut glume method (51.60%) (Table 3). However, there were significant differences ($p < 0.05$) in the regeneration of embryos between intact glume method (56.09%) and other emasculation methods. The most effective treatment was the intact glume method (T_3), which gave the highest seed set (92.56%), embryo formation (15.94%) and embryo regeneration (56.09%). The hot water technique (T_4) showed the poorest response in these traits (85.72%, 10.5 and 46.00%) (Table 3 and Fig. 10).

The results of Experiment-1 showed that emasculation methods affected the seed set, embryo formation and regeneration. The intact glume method gave the highest response, both in embryo formation and embryo regeneration. This is in accordance with the results of Laurie (12) who obtained a higher percentage of embryos with intact glumes (55.8%) than with the cut glume method (37.2%). It is suggested that this is the result of not exposing the stigmas to adverse environmental conditions such as high temperature (11).

However, if number of haploid plants per unit time is considered, the benefits of intact glume method might be offset by the time saved in preparing the wheat florets by cut glume method (12). Statistical analysis of the present results, revealed that cut glume, non-emasculated and hot water methods gave similar responses. It is, therefore, suggested that non-emasculation and early pollination is the most efficient method in terms of time and labour. The hot water technique is feasible to inactivate the pollen if right temperature and time of treatment effectively reduce the frequency of selfed seeds. This technique is also quite simple to apply under detached tiller method.

The results of Experiment-2 confirmed the observations made in Experiment-1 with an additional information that hot water emasculation method-II (43^{o}C for 4 minutes) is more effective than the method-I (45^{o}C for 3 minutes). In wheat x maize crossing system selfed seeds can easily be distinguished and separated from crossed seeds on the basis of their size and endosperm development. There is no or very poor / abnormal endosperm development in crossed seeds. The selfed seeds can be identified by visual observation, hence there is no need of chromosomal study at this stage. To summarize, hand emasculation of the female parent is less economically feasible than hot-water-treated or non-emasculated spikes for the large scale production of haploid embryos in wheat $\times$ maize system. As there was only a small difference in the overall efficiency of embryo formation between non-emasculated and intact glume methods, it seems that hand emasculation can be replaced by non-emasculated early pollination method. This technique not only saves time and labour, but in addition, the stigmas are not exposed to environmental stresses and are better protected against desiccation. Methods that eliminate emasculation increase the number of crosses that can be made without having a major impact on efficiency of haploid plant production.

Intact plants vs detached tiller

Intact plants: The data (Table 6) on number and percentage of seed set, embryos and haploid plants produced in 'Cross-3' showed significant differences in seed set ($p < 0.01$) among the pollen sources (Table 7). The florets setting seeds on 'Cross-3' ranged from 77.7 percent for 73Q3 hybrid to 95.3 percent for Neelum while Golden (93.3%), FSH-399 (93.0%) and Sultan (92.3%) showed similar seed setting (Table 6).

There were marked differences in embryo formation among pollen sources in intact plants ranging from 11.0 percent for Golden to 37.0 percent for Neelum. The percentages of embryos resulting from pollination with Sadaf

(31.0%) and Agaiti 2002 (21.0%) were better than those resulting from other pollen sources. It was noted that synthetic cultivar Sultan resulted in high percentage of haploid plants (77.9%) while Golden regenerated haploid plantlets (70.6%). Hybrid 3335 and Neelum regenerated 69.5 and 67.1 percent haploid plantlets, respectively (Table 6).

Table 6. Effect of different pollen sources on seed set, embryo formation and haploid production using intact wheat plants in cross-3 with maize parents (Experiment-2).

Genotypes/ pollen sources	No of pollinated florets	Seed set (as % of pollinated florets)	No. of embryos (as % of pollinated florets)	Embryos as % of seeds	Haploid plants (as % of pollinated florets)	Haploid plants (as % of embryos)
Synthetics or open pollinated maize cultivars						
Neelum	100	95.3a	37.0a	38.9a	24.7a	67.1ab
Sadaf	100	87.3b	31.0a	35.7a	11.3c	33.0d
Sultan	100	92.3a	14.7bc	15.9b	11.3c	77.9a
Agaiti2002	100	85.0bc	21.0b	24.6ab	11.7c	56.4bc
Golden	100	93.3a	11.0c	11.9b	7.7c	70.6ab
Average		90.64	22.94			61.0
Hybrids						
3335	100	82.0c	11.7c	23.9ab	8.0c	69.5ab
FSH-399	100	93.0a	37.0a	39.9a	17.3b	48.1cd
73Q3	100	77.7d	12.0c	15.5b	7.0c	59.7abc
Average		84.35	20.23			59.1
Overall average		88.00	21.8			60.1

Table 7. Analysis of variance (mean squares) of seed set, embryo formation, and haploid production, on intact plants in crosses between hexaploid wheat and maize.

Source	DF	Mean squares				
		Seed set (%)	Embryos/ florets (%)	Embryos/ seeds (%)	Haploids/ florets (%)	Haploids/ embryos (%)
Maize (M)	7	1105.048*	296.167*	796.839*	233.756*	609.766*
Error	16	22.625	7.542	43.367	5.583	61.595
Total	23					

* $p < 0.05$, ** $p < 0.01$

Detached tiller culture: For most pollen sources the percentage seed set was lower after tiller culture than on intact plants. The percentage seed set in wheat Cross-3 ranged from 40.0 percent in cross with 73Q3 to 94.0 percent in cross with FSH-399 (Table 8). Embryos recovered as a percentage of pollinated florets differed greatly in crosses with different pollen sources ($p<0.01$) ranging from 5.3 to 35.7 percent (Table 8). Sultan resulted in higher

embryo formation (24.7%) than others open pollinated varieties (Table 8). There were significant differences between two groups of pollen sources (synthetic lines and hybrids) in embryo formation. The statistical analysis revealed significant differences between pollen sources for embryo survival ($p < 0.01$).

No clear difference ($p < 0.01$) in seed set between intact plants (95.3%) and tiller culture (94.0%) was found in best performing maize parents (Table 6, 8). Seed development in cultured wheat tillers paralleled to intact plants. Even between two groups of pollen sources, the synthetics gave a lower seed set (65.1%) in tiller culture than maize hybrids (73.8%) (Table 8). Among intact plants, there were significant differences between two groups of pollen sources, in which synthetics gave higher seed set (90.64%) and hybrid gave lower (84.35%) seed set (Table 6).

Table 8. Effect of different pollen sources on seed set, embryo formation and haploid production using detached tiller culture in Cross-3 with maize parents.

Pollen sources	No. of pollinated florets	No. of seeds (as % of pollinated florets)	No. of embryos (as % of pollinated florets)	Embryos (as % of seeds)	No. of haploid plants (as % of pollinated florets)	Haploid plants (as % of embryos)
Synthetic or open pollinated maize cultivars						
Neelum	100	71.0c	15.3cd	21.0c	12.7c	81.9a
Sadaf	100	50.7d	10.3e	20.6c	8.0d	77.3ab
Sultan	100	55.0d	24.7b	44.6ab	20.0b	81.2a
Agaiti2002	100	84.0b	9.0ef	10.8cd	6.3de	69.2ab
Golden	100	64.7c	11.3de	17.4cd	4.3de	40.1c
Average	100	65.1	14.1	22.9	10.3	70.0
Hybrids						
3335	100	87.3ab	5.3f	6.1d	3.3e	64.0b
FSH-399	100	94.0a	35.7a	38.0b	29.0a	81.2a
73Q3	100	40.0e	19.7c	50.8a	15.3c	78.2ab
Average	100	73.8	20.2	31.4	15.9	74.5
Overall average			17.15			72.6

Highly significant differences ($p < 0.01$) in wheat embryo formation per pollinated floret were obtained (Table 9). Pollen sources in crosses with 'Cross-3' as an intact plant produced higher frequencies of embryos (21.8%) per pollinated floret (Table 6) than tiller culture method (17.15%). Considerable differences in embryo formation occurred between two groups of pollen sources (Table 8). Synthetic maize lines produced more (14.10%) wheat embryo formation in intact plants (22.94%) than tiller culture. The hybrid maize group gave the same frequency of embryo formation in both intact and tiller culture method (20.23%) (Table 6 and 8). Under both

conditions, pollen sources FSH-399 (37.0 and 35.7%), Neelum (37.0 and 15.3%), Sultan (14.7 and 24.7%), Sadaf (31.0 and 10.3%) and 73Q3 (12.0 and 19.7%) resulted in more wheat embryos than 3335 hybrid (11.7 and 5.31%). In addition, on the intact plant, the pollen source Agaiti 2002 gave better results (21.0% wheat embryo formation) than 3335 hybrid (11.7%) (Table 6).

Table 9. Analysis of variance (mean squares) of seed set, embryo formation, and haploid production in crosses between the wheat 'Cross-2' with different maize pollen sources.

Source	DF	Mean squares			
		Seed set %	Embryos/ florets %	Embryos/seeds %	Haploids/embryos %
Maize (M)	3	75.25*	325.0**	210.05**	163.32**
Error	8	15.3	6.0	2.75	19.01
Total	11				

* $p < 0.05$, ** = $p < 0.01$

Pollination with synthetic maize cultivars regenerated near about similar wheat haploid plants per cultured embryos (61.0%) as the hybrids in intact plant (59.1%) (Table 6) indicating more positive effects on embryo regeneration. The efficiency of embryo regeneration was higher in embryos from tiller culture (72.2%) (Table 8) than those from intact plants (60.1%) (Table 6). The Cross-3 x Neelum with 67.1 and 81.9 percent embryo regeneration, and Cross-3 x Sultan with 77.9 and 81.2 percent embryo regeneration in intact plant and tiller culture, respectively, were the good haploid producers, while the Cross-3 x Sadaf with 33.0 percent in intact plants and Golden 40.1 percent in tiller culture regenerated embryos per cultured embryos was the poorest (Table 6, 8).

The growth of seeds on the spikes of detached wheat tillers in liquid culture closely resembled that observed in intact plants. As mentioned earlier, seed per floret was low for most pollen sources. Reduced seed in tiller culture may have been a consequence of the grains being at a developmental stage which was vulnerable to moisture stress or osmotic stress (3, 19). Another factor may be a limited sucrose supply, because reductions in the supply of sucrose through the culture medium likewise reduce kernel number (20). Kato and Hayashi (9) reported that optimum culture temperature was 15°C, as both the single seed mass and seed mass per spike decreased at higher temperatures. Kato *et al.* (10) further found two optimum temperatures; 15°C to obtain large seed, and 20 to 25°C to maintain high germination. They suggested that the easiest way to get a higher number of germinating seeds from detached wheat spikes is to culture the tillers at room temperature on

the liquid medium containing 100 g/L sucrose and 0.75% sulphurous acid without any sterilization procedure.

The culture of detached plant (e.g. ear/tiller) is a beneficial method and has been applied in nutritional and environmental investigations (6), vernalization (10) and caryopsis development (6). Kato and Hayashi (9) attempted to simplify ear culture for chemical and physiological treatments of immature embryos. Germiable seeds were easily produced by the culture of detached tillers in sucrose solution without sterilization. The addition of sulfurous acid to the culture solution and cold treatment of the solution to overcome several problems such as small grain size and decay of the culture solution and stem base, were carried out by Donovan and Lee (5), Kato *et al.* (10) and Riera-Lizarazu *et al.* (18). Inagaki (8) also successfully used detached tiller culture in wheat x maize crossing system of haploid production. Detached tiller culture has special advantage in wheat x maize crossing as in this technique the essential hormones like 2, 4-D may be added into the culture solution and highly laborious work of injecting hormones to the upper most internode of crossed spikes may be avoided. In addition distantly grown wheat genotypes may easily be brought to the laboratory and emasculated in a pleasant environment.

In Experiment-2, three treatments significantly ($p<0.01$) differed from each other for all the traits studied (Table 10.). Maximum caryopsis development (74.3%) was observed in treatment-2 (40g sucrose, 100 mg 2,4-D and 6ml sulfurous acid/L) followed by treatment-1 (20g sucrose, 100 mg 2,4-D and 6ml sulfurous acid/L) with non-significant differences among them.

Table 10. Analysis of variance (mean squares) of seed set, embryo formation, and haploid production in crosses between the wheat 'Cross-2' with maize pollen source FSH-399.

Source	DF	Mean squares			
		Seed set (%)	Embryos/florets(%)	Embryos/seeds(%)	Haploids/ embryos (%)
Treatments	3	72.97*	706.0**	204.08**	152.34**
Error	8	10.5	10.0	2.58	18.62
Total	11				

*$p < 0.05$, **$p < 0.01$

Treatment-3 (60g sucrose, 100 mg 2,4-D and 6ml sulfurous acid/L) was significantly low seed producer. In treatment-2 maximum of its produced

seeds had embryos (31.7%) but on the basis of pollinated florets, treatment-3 produced maximum embryos (44.2%). Same trend was observed for regeneration of haploid plants from embryos. Maximum rate of regeneration of embryos was observed for treatment-2 and on the basis of florets pollinated, treatment-2 produced maximum percentage of embryos (Table 11).

Table 11. Effect of different media compositions on the seed set, embryo formation and haploid production using detached tiller culture in wheat × maize crosses.

Treatments	No. of pollinated florets	No. of seeds (as % of pollinated florets)	No. of embryos (as % of pollinated florets)	Embryos (as % of seeds)	No. of haploid plants (as % of pollinated florets)	Haploid plants (as % of embryos)
T_1	100	71.7a	17.3b	24.4b	11.0b	65.1
T_2	100	74.3a	31.7a	43.1a	23.0a	70.5
T_3	100	50.3b	22.3b	44.2a	15.3b	68.3

The data further indicated (Experiment-2) that low concentrations of 2,4-D, sucrose and sulphurous acid resulted in low caryopsis development but the rate of embryo recovery and regeneration was slightly better than other treatments. The high concentration of 2,4-D resulted in high percentage of caryopsis development but also caused malformation of regenerating embryos, hence resulted in low haploid recovery. The best percentage of haploid plants was recovered from treatment-2. So it is recommended that 40 g/L sucrose, 100 mg/L 2,4-D and 8 ml/L sulphurous acid should be used for making tiller culture solution. Almost the same media has already been recommended by Inagaki (8).

CONCLUSION

It is concluded that detached tillers of wheat can be used successfully in wheat × maize crosses. This technique has many advantages over intact plants (6). Main advantages of the present study are: (i) travel to and from the field can be reduced if tillers are detached from field grown plants, (ii) delaying pollination by storing tillers under cooler conditions, (iii) easy hot water emasculation of detached tillers and (iv) easy application of growth hormones like 2,4-D.

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