Use of Pollen Storage and Detached-tiller Culture in Wheat Polyhaploid Production through Wide Crosses

M. N. Inagaki, T. Nagamine[1] and A. Mujeeb-Kazi
International Maize and Wheat Improvement Center (CIMMYT),
Lisboa 27, Col. Juárez, Apartado Postal 6-641, 06600, Mexico, D.F., Mexico

Summary

The effects of pollen storage and detached-tiller culture on polyhaploid production of hexaploid wheat were examined using maize and pearl millet crosses. Pollen storage at ultra-low temperature did not affect polyhaploid production frequency in pearl millet crosses, but greatly reduced frequency in maize crosses. Stored pearl millet pollen can be used as an alternative medium for wheat polyhaploid production when fresh pollen is not available. Detaching wheat tillers with spikes at crossing time and culturing them in a solution containing sucrose, sulfurous acid and 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in no distinct reduction of polyhaploid production frequency in crosses with both maize and pearl millet. Hot-water emasculation after detaching wheat tillers was successful for these wide crosses. Detached-tiller culture makes it possible to collect the spikes from wheat plants growing in distant sites and handle them for wide crosses in a laboratory. These techniques avoid having to synchronize flowering times of both parents and result in considerable savings in terms of labor and space required for handling parent plants.

Index words: Hexaploid wheat, maize, pearl millet, haploid, wide cross, pollen storage, detached-tiller culture.

Introduction

A technique for producing polyhaploids from hexaploid wheat (*Triticum aestivum* L., $2n=6x=42$) through wide crosses followed by embryo rescue has been recently established (for a review, see Inagaki and Mujeeb-Kazi 1994a). Significant technical advances have been attributed to the selection of pollen donors from Panicoides subfamilial species and the application of plant growth regulators for developing immature embryos. Pollination of maize (*Zea mays* L.) or pearl millet (*Pennisetum glaucum* (L.) B. Rr.) followed by 2,4-dichlorophenoxyacetic acid (2,4-D) treatment onto wheat florets can be extensively used for wheat polyhaploid production (Inagaki and Mujeeb-Kazi 1995). However, this type of methodology always requires having viable pollen available at crossing site where the wheat plants are growing. Storing pollen at ultra-low temperatures and/or detaching and artificially culturing wheat tillers could solve this problem.

Maize and pearl millet pollen has been successfully preserved at ultra-low temperatures (Barnabás and Rajki 1981; Hanna 1990). Effects of drying and freezing on pollen viability may be different between maize and pearl millet (Inagaki and Mujeeb-Kazi 1994b; 1996). On the other hand, detached-tiller culture of wheat has been developed through physiological studies on immature

---

[1] *National Agriculture Research Center (NARC)*, Tsukuba, Ibaraki, 305 Japan

seed vernalization (Kato *et al.* 1990). Major components of the culture solution are sucrose as a nutrient and sulfurous acid for preventing fungal contamination. Supplementing the solution with 2,4-D is essential for developing haploid embryos from wheat x maize crosses (Ushiyama *et al.* 1991). This paper presents the effects of pollen storage and detached-tiller culture on wheat polyhaploid production frequencies in maize and pearl millet crosses.

Materials and Methods

Plant materials

Hexaploid wheat variety Norin 61 was used as the female parent. Pollen parents were a maize F1 hybrid line (CML-246 x CML-242) and pearl millet inbred line NEC-7006. Wheat plants were grown in potted soil in a greenhouse controlled at 29/14°C (max/min). Maize and pearl millet plants were grown in ground soil in a greenhouse without temperature control. Temperature conditions were approximately 35/10°C (max/min) at anthesis of maize and pearl millet.

Pollen storage

Fresh pollen was collected between 9:30 and 10:00 a.m., and screened through a 0.5-mm aperture sieve to remove anthers.Ten grams of pollen were spread on a paper tray and dried with gentle ventilation at 35°C and 40 - 35% relative humidity. Pollen water content was reduced to 11.8% in maize and 5.3% in pearl millet after approximately two hours of drying (Fig. 1). Pollen water content was determined from a 0.5-g pollen sample dried at 95°C for five hours. The dried pollen was distributed among cryopreservation tubes (1.5 ml volume). The sealed tubes were immediately immersed in liquid nitrogen (-196°C). Maize and pearl millet pollen were stored for 8 months and 10 months, respectively. After thawing the tubes in a water-bath at 38°C for five minutes, pollen was used for crossing.

Crossing

At the time of ear emergence, wheat spikes on plants were emasculated following removal of the central florets of each spikelets and cutting off the top halves of the outer florets. Emasculated spikes were pollinated with maize and pearl millet one day before predicted wheat anthesis. On the two consecutive days after pollination, the uppermost internodes of the wheat culms with pollinated spikes were needle-injected with a 100 mg/l 2,4-D solution (crossing method A), according to the method of Inagaki and Tahir (1990).

Detached-tiller culture

Wheat tillers with spikes were cut off at the base of the tiller and cultured in a flask containing tap water. The wheat spikes were emasculated and pollinated as described above (crossing method B). The other group of spikes was immersed in hot water set at 43°C for three minutes one day before pollination, and pollinated after cutting off the upper parts of the florets but remaining the anthers (crossing method C).

After pollination, wheat tillers were cultured for two days in a solution containing 40 g/l sucrose, 8 ml/l sulfurous acid (6% SO2) and 100 mg/l 2,4-D. They were then transferred to a solution containing only sucrose and sulfurous acid, and cultured until embryo rescue (Fig. 2). Culture conditions were 22.5°C, 12-h daylength and 70 - 60% relative humidity in a growth chamber. Procedures of hot-water emasculation and detached-tiller culture are minor modifications of the method of Nagamine *et al.* (1995). Five wheat spikes (approximately 30 florets/spike) were used for each crossing treatment in combination with pollen storage with two replications.

Plant regeneration

At 14 days after pollination, immature embryos were aseptically excised from wheat seeds, and transferred onto half strength Murashige and Skoog (1962) culture medium supplemented with 20

g/l sucrose and 6 g/l agarose. Embryo length was measured using a micrometer. The embryos were incubated at 25°C, 12-h daylength and ca. 5000 lux light intensity. Plants regenerated from embryos were randomly selected and cytologically examined in squashed preparations of root-tips stained with aceto-orcein, according to the method of Mujeeb-Kazi and Miranda (1985).

Results

Embryo formation in wide crosses

Most wheat florets pollinated with maize and pearl millet produced plump seeds that were somewhat smaller than selfed seeds, but lacking the solid endosperm found in selfed seeds. Some of the crossed seeds contained immature embryos (Fig. 3). Embryo formation frequencies (percent frequencies of embryos obtained from pollinated wheat florets) in crosses with maize and pearl millet are shown in Table 1. In crosses using fresh pollen of maize and pearl millet, embryos were obtained at frequencies of 20.4 - 18.9% and 35.6 - 19.7%, respectively, across three crossing methods. Statistical data analyses of embryo formation frequencies after angular transformation indicated that crosses with stored maize pollen produced embryos at lower frequencies (8.5 - 2.8%) than other crosses (35.6 - 18.9%), but embryo formation frequencies in crosses with stored pearl millet pollen were comparable to those with fresh pollen of maize and pearl millet.

Fungal contamination was not found in detached-tiller cultures. After pollination with maize and pearl millet, hand-emasculated wheat spikes developed plump seeds. Wheat spikes that were treated by hot water produced seeds but contained few selfed seeds, which were easily distinguished from crossed seeds. No significant differences in embryo formation frequency were found among crossing methods.

Table 1. Embryo formation frequencies (%) in crosses of hexaploid wheat with maize and pearl millet[1)]

Pollen donor	Pollen storage	Crossing method (A) Hand/ on plant	(B) Hand/ detached	(C) Hot water/ detached
Maize	Fresh	20.4[b]	19.4[b]	18.9[b]
	Stored	2.8[d]	7.0[c]	8.5[c]
Pearl millet	Fresh	19.7[b]	21.2[b]	35.6[a]
	Stored	20.4[b]	27.7[b]	23.3[b]

[1)] Numbers followed by the same letter are not significantly different at the 5% probability level.

Plant regeneration from wide crosses

Embryo length and plant regeneration frequencies (percent frequencies of plants regenerated from cultured embryos) obtained from crosses with maize and pearl millet are given in Table 2. Embryo length varied from 1.42 to 0.79 mm. Embryos obtained from maize crosses were larger than those from pearl millet crosses. Except for crosses with stored maize pollen in which embryos were few, embryos grown on plants were smaller than those of detached tillers. Plant regeneration frequencies varied from 67.0 to 42.5%, but were not statistically different among crossing treatments.

A total of 108 plants regenerated from maize and pearl millet crosses were randomly selected for cytological examination. Of these, 104 plants were wheat polyhaploids carrying a complement of 21 chromosomes (Fig. 4). Three plants were hexaploids with 42 chromosomes. The other plant was aneuploid with 43 chromosomes. These four plants were obtained from crosses using hot-water emasculation and detached-tiller culture techniques, that is, crossing method C.

Table 2. Length (mm) of obtained embryos and plant regeneration frequencies (%, in parentheses) in crosses of hexaploid wheat with maize and pearl millet[1)]

Pollen donor	Pollen storage	Crossing method (A) Hand/ on plant		(B) Hand/ detached		(C) Hot water/ detached	
Maize	Fresh	1.21^{b}	(67.0^{f})	1.40^{a}	(42.5^{f})	1.42^{a}	(47.3^{f})
	Stored	1.16^{bc}	(65.0^{f})	1.22^{b}	(46.5^{f})	1.21^{b}	(55.6^{f})
Pearl millet	Fresh	0.79^{e}	(45.8^{f})	0.95^{d}	(56.7^{f})	1.02^{cd}	(50.7^{f})
	Stored	0.79^{e}	(44.3^{f})	1.01^{cd}	(54.5^{f})	1.07^{bcd}	(48.3^{f})

1) Numbers followed by the same letter are not significantly different at the 5% probability level.

Discussion

Pollen storage

Pollen of the Gramineae is less amenable to long-term storage than that of other familial species (Towill 1985). However, maize and pearl millet are species whose pollen is successfully preserved at ultra-low temperatures for long periods, and thus can be used as pollen donors for polyhaploid production of hexaploid wheat through wide crosses. Barnabás and Rajki (1981) reported that 50% of maize pollen grains could be kept viable for a year with seed setting ability of 30% when pollen water content was reduced to 14.4%. Hanna (1990) reported that pearl millet pollen stored for several years showed 100% seed setting when pollen water content was reduced to below 7.2%. These data suggest that

optimum range of pollen water content for stored pollen, as well as pollen viability after drying and freezing, is different between maize and pearl millet. Previous studies (Inagaki and Mujeeb-Kazi 1994b; 1996) indicated that optimum water content range was 12 - 10% in maize and 7 - 5% in pearl millet, and that pearl millet pollen was more tolerant to drying and freezing than maize pollen. The present study confirmed that pollen storage at ultra-low temperature did not reduce polyhaploid production frequency in pearl millet crosses. Therefore, stored pearl millet pollen can be used as an alternative for wheat polyhaploid production when fresh pollen is not available.

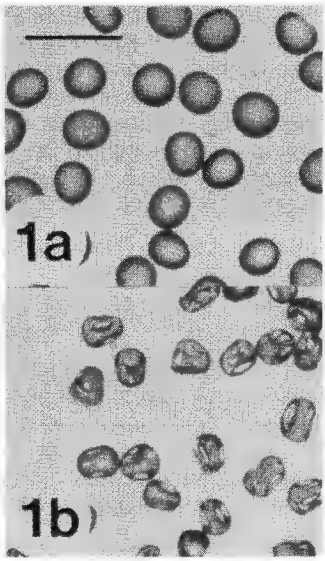
Detached-tiller culture

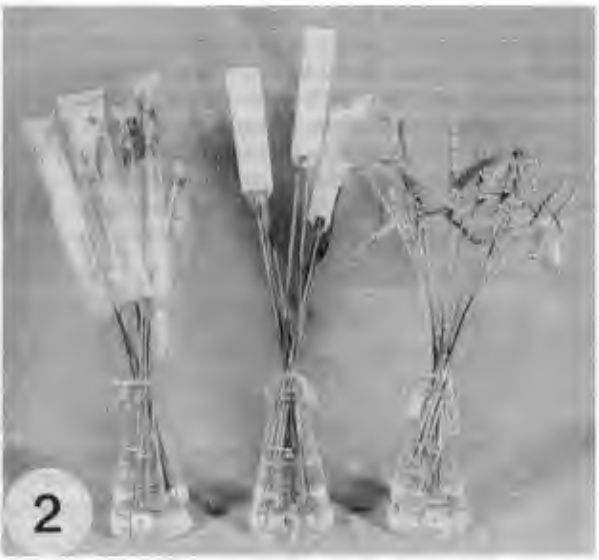
Detached wheat tillers cultured in a nutient solution without sulfurous acid were severely damaged by fungal contamination (Riera-Lizarazu *et al.* 1992). Supplementing the culture solution with sulfurous acid is essential for preventing fungal contamination. Successfully culturing wheat tillers with spikes resulted in no reduction of embryo formation frequency for crosses with both maize and pearl millet. Wheat spikes of detached tillers that were treated by hot water and pollinated with maize and pearl millet produced embryos at frequencies as high as those obtained from hand-emasculated spikes. In addition, culturing wheat tillers in a nutrient solution under controlled conditions resulted in production of larger embryos than growing tillers on plants. Embryos in maize crosses developed more rapidly than those in pearl millet crosses. Embryo size may affect plant regeneration frequency, as reported in pearl millet crosses (Inagaki and Bohorova 1995). Optimum timing of embryo rescue is determined depending on culture conditions and pollen donors used for wide crosses. Detached-tiller culture thus makes it possible to collect spikes from wheat plants growing in distant sites and handle them in a laboratory.

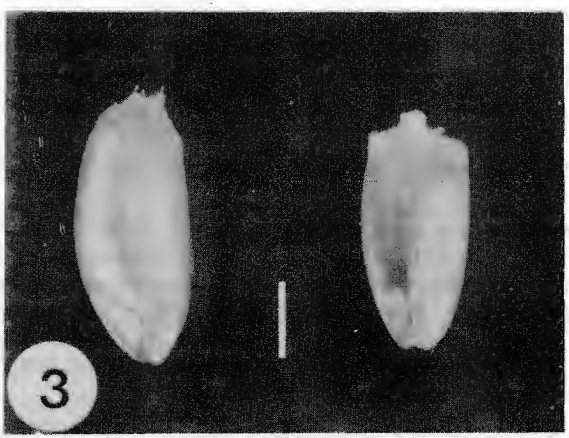
Efficient production of wheat polyhaploids using stored pollen and detached tillers

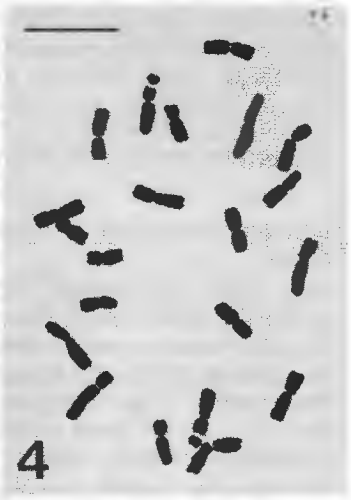
The wheat polyhaploid production process consists of two steps: embryo formation from wide crosses and plant regeneration from the embryos obtained. Present results across different crossing methods give polyhaploid production frequencies of 14 - 8% in maize crosses and 18 - 9% in pearl millet crosses using fresh pollen. When using stored pollen, frequencies are 5 - 2% in maize crosses and 15 - 9% in pearl millet crosses, indicating a frequency reduction only in crosses with stored maize pollen.

Use of pollen storage and detached-tiller culture did not give distinct increases in production frequency of wheat polyhaploids. However, they can greatly increase production efficiency. Stored pearl millet pollen can be used successfully for wheat polyhaploid production when fresh pollen is not available for crosses. Detached-tiller culture following crossing gives considerable savings in terms of labor and space required for handling wheat plants. Hot-water emasculation of a large number of wheat spikes requires only three minutes, whereas hand emasculation takes three minutes per spike. These techniques, used alone or in combination, provide greater flexibility in when and where polyhaploid production of hexaploid wheat through wide crosses is performed.









Figs. 1 - 4. Polyhaploid production using stored pollen and detached-tiller culture in hexaploid wheat x pearl millet crosses. (**1a**) Fresh (53.1% water content) and (**1b**) stored (5.3% water content) pollen of pearl millet. Bar 0.1 mm. (**2**) Detached-tiller cultures at emasculation (*left*), pollination (*center*) and seed setting (*right*). (**3**) Wheat seeds obtained from self-pollination (*left*) and from crosses with pearl millet (*right*). At 14 days after pollination. Bar 2 mm. (**4**) Somatic chromosomes ($2n=3x=21$) of a plant regenerated from wheat x pearl millet crosses. Bar 10 μm.

Acknowledgments

This report is a contribution from the collaborative research project of CIMMYT with the *Japan International Research Center for Agricultural Sciences (JIRCAS)*, Tsukuba, Japan. The authors would like to thank Ms. Alma L. McNab, wheat program editor, CIMMYT, for her editorial review of the manuscript.

References

Barnabás, B. and E. Rajki (1981). Fertility of deep-frozen maize (*Zea mays* L.) pollen. Ann. Bot. 48: 861-864.

Hanna, W. W. (1990). Long-term storage of *Pennisetum glaucum* (L.) R. Br. pollen. Theor. Appl. Genet. 79: 605-608.

Inagaki, M. N. and N. Bohorova (1995). Factors affecting the frequencies of embryo formation and haploid plant regeneration in crosses of hexaploid wheat with pearl millet. Breed. Sci. 45: 21-24.

Inagaki, M. N. and A. Mujeeb-Kazi (1994a). Progress in polyhaploid production techniques of hexaploid wheat through wide crosses. Proc. 2nd Triticeae Symp., Utah, USA, 65-69.

Inagaki, M. N. and A. Mujeeb-Kazi (1994b). Storage of maize pollen for use in haploid production of hexaploid wheat. Breed. Sci. 44: 387-390.

Inagaki, M. N. and A. Mujeeb-Kazi (1995). Comparison of polyhaploid production frequencies in crosses of hexaploid wheat with maize, pearl millet and sorghum. Breed. Sci. 45: 157-161.

Inagaki, M. N. and A. Mujeeb-Kazi (1996). Production of polyhaploids of hexaploid wheat using stored pearl millet pollen. Proc. 5th Int. Wheat Conference, Ankara, Turkey. (in press).

Inagaki, M. N. and M. Tahir (1990). Comparison of haploid production frequencies in wheat varieties crossed with *Hordeum bulbosum* L. and maize. Japan. J. Breed. 40: 209-216.

Kato, K., S. Tomo, S. Yamazaki and K. Hayashi (1990). Simplified culture method of detached ears and its application to vernalization in wheat. Euphytica 49: 161-168.

Mujeeb-Kazi, A. and J. L. Miranda (1985). Enhanced resolution of somatic chromosome constrictions as an aid to identifying intergeneric hybrids among some Triticeae. Cytologia 50: 701-709.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nagamine, T., K. Kaneko and T. Yamada (1995). Improvement in "maize method" for efficient and labor-saving wheat haploid production. Proc. 2nd Asian Crop Science Conference, Fukui, Japan, 772-773.

Riera-Lizarazu, O., A. Mujeeb-Kazi and M. D. H. M. William (1992). Maize (*Zea mays* L.) mediated polyhaploid production in some Triticeae using a detached tiller method. J. Genet. Breed. 46: 335-346.

Towill, L.E. (1985). Low temperature and freeze-/vacuum-drying preservation of pollen. In: K. K. Kartha (Ed.), Cryopreservation of pollen cells and organs, CRC Press, Inc., Florida, USA, 171-198.

Ushiyama, T., T. Shimizu and T. Kuwabara (1991). High frequency of haploid production of wheat through intergeneric cross with teosinte. Japan. J. Breed. 41:353-357.

Received 3 September, 1996, accepted 7 January, 1997