Triticeae III

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Efficient techniques for polyhaploid production in hexaploid wheat using pearl millet crosses

M. N. Inagaki and A. Mujeeb-Kazi

A methodology for producing wheat polyhaploids using wide crosses followed by embryo rescue has been developed over the last two decades. Significant technical advances have been achieved by using pollen selected from different subfamilial species and applying plant growth regulators. Efficient crossing techniques were developed in pearl millet crosses using stored pollen and detached-tiller culture, and resulted in considerable savings in terms of labor and space required for growing parent plants. These techniques will also accelerate the production of doubled haploids from hybrid progenies that are used as recombinant inbred lines with favorable uniformity in breeding selection and genetic analysis.

Introduction

A methodology for producing polyhaploids in hexaploid wheat (*Triticum aestivum* L.) using wide crosses followed by embryo rescue was improved by successfully selecting pollen donors from different subfamilial species and applying a plant growth regulator (see Inagaki and Mujeeb-Kazi,1994a, for a review). At present, doubled haploid populations derived from hybrid progenies can be used as recombinant inbred lines with favorable uniformity in breeding selection and genetic analysis. However, this type of methodology always requires having viable pollen available at crossing sites where the wheat plants are growing. Storing pollen at ultra-low temperatures and/or detaching and artificially culturing wheat tillers could solve this problem. This paper presents the technical development of wheat polyhaploid production using stored pollen and detached-tiller culture in wide crosses.

Pollen source

Wide crosses of hexaploid wheat with members of the Panicoides subfamily have initially been attempted in alien genetic transfer (Zenkteler and Nitzsche, 1984). Maize (*Zea mays* L.) pollen can successfully hybridize with wheat egg cells and produce zygotes (Laurie and Bennett, 1986), irrespective of the presence of cross-incompatibility *Kr* gene(s) (Laurie and Bennett, 1987). The maize chromosomes are rapidly eliminated from hybrid zygotes, requiring the artificial rescue of proembryos at an early developmental stage (Laurie and Bennett, 1 988a). Treatment with 2,4-dichloro-phenoxyacetic acid (2,4-D) after maize pollination is critical to enhance embryo development in wheat x maize crosses

(Suenaga and Nakajima, 1989). Maize pollination and subsequent 2,4-D treatment of wheat florets result in production of immature wheat embryos capable of regenerating polyhaploid plants, even for wheat genotypes that are cross-incompatible with *Hordeum bulbosum* L. (Inagaki and Tahir, 1990). Wheat polyhaploid production through maize crosses has been confirmed using diverse wheat varieties (Inagaki and Tahir, I 990; Laurie and Reymondie, 1991; Ricra-Lizarazu et al., 1992). Some species related to maize, such as teosinte *(Zea mays* L. spp. *mexicana)* and Eastern gamagrass *(Tripsacum dactyloides* (L.) L.), are alternative pollen donors for wheat polyhaploid production (Ushiyama et al., 1991; Riera-Lizarazu and Mujeeb-Kazi, 1993).

Cytological evidence indicates successful fertilization and elimination of paternal chromosomes form hybrid zygotes in sorghum *(Sorghum bicolor* (L.) Moench) and pearl millet *(Pennisetum glaucum* (L.) R. Br.) crosses, which suggests that sorghum and pearl millet are potential pollen sources for wheat polyhaploid production (Laurie and Bennett, 1988; Laurie, 1989; Ahmad and Comeau, 1990). Wheat polyhaploids were obtained at high frequencies from sorghum and pearl millet crosses followed by 2,4-D treatment after pollination (Ohkawa et al., 1992; Inagaki and Bohorova, 1995). However, sorghum crosses expressed a strong genotypic barrier of wheat to embryo formation (Table 1) (Inagaki and Mujeeb-Kazi, 1995). Therefore, polyhaploid production through crosses with maize and pearl millet appears more stable than other methods because of its lesser genotypic effect on embryo formation.

Pollen storage and detached-tiller culture

Maize and pearl millet pollen have been successfully preserved at ultra-low temperatures for long periods (Barnabās and Rajki, 1981; Hanna, 1990). The process of storing pollen involves both drying and freezing. Understanding the effects of drying and freezing on pollen viability is essential for achieving successful crosses with wheat. Maize pollen dried to optimum water content range and stored at -80°C produced wheat polyhaploid embryos, but frequencies decreased to half compared to the case of fresh pollen (Inagaki and Mujeeb-Kazi, 1994b).

Pearl millet pollen is relatively tolerant to drying and freezing, unlike maize pollen, which has a narrow water content range for maintaining viability during drying and freezing (Fig. 1). As a result, pollen storage at ultra-low temperatures does not affect embryo formation frequency in wheat x pearl millet crosses, but greatly reduces frequency in wheat x maize crosses (Inagaki and Mujeeb-Kazi, 1997; Inagaki et al., 1997). Stored pearl millet pollen can be used as an alternative medium for producing wheat polyhaploids when fresh pollen is not available.

A technique for artificially culturing detached wheat tillers has been developed through physiological research on immature 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 culture solution with 2,4-D is essential for developing embryos from wheat x maize crosses (Ushiyama et al., 1991; Riera-Lizarazu et al., 1992). Detached-tiller culture has been extensively applied in wheat polyhaploid production using maize crosses at the National Agriculture Research Center (NARC), Japan (Nagamine et al., 1995). The effectiveness of detached-tiller culture has also been confirmed in combination with pollen storage in wheat polyhaploid production (Table 2) (Inagaki et al., 1997). Detached-tiller culture makes it possible to collect spikes from wheat plants growing in distant sites and to handle them 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 growing parent plants They also provide

greater flexibility as to when and where wheat polyhaploid production through wide crosses can be performed. Two procedures were used for crossing, they are discussed below.

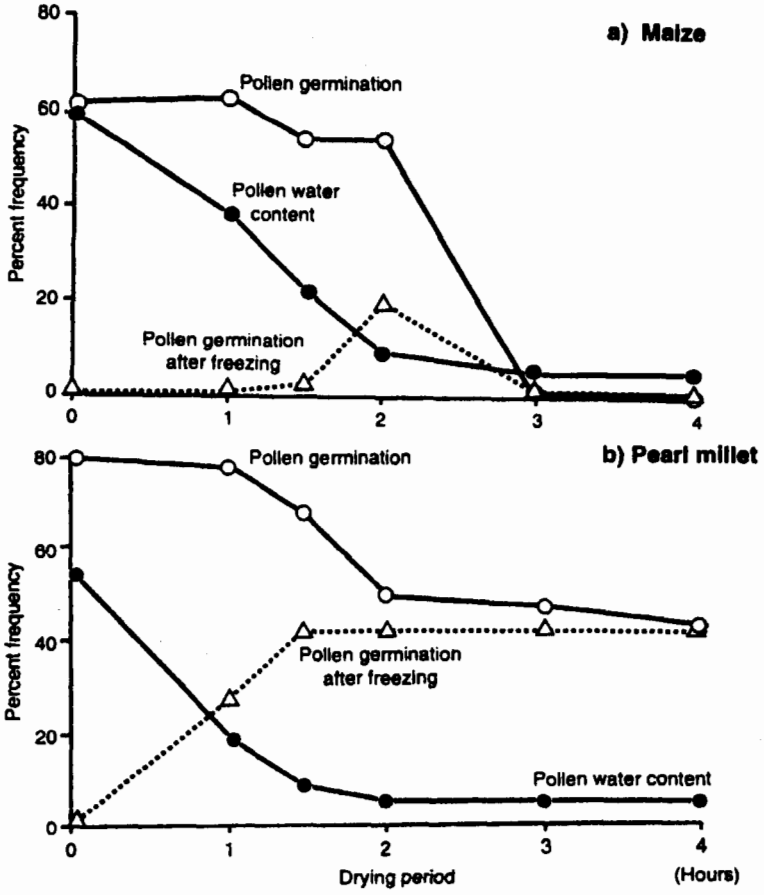

Fig. 1. Water content and germination frequency of (a) maize and (b) pearl millet pollen after drying and freezing. Maize; F1 hybrid line (CML-246 x CML-242), Pearl millet; inbred line NEC-7006 (Source: Inagaki and Mujeeb-Kazi, 1994b; 1997).

Pollen Storage

Fresh pollen is collected from donors at anthesis and screened through a sieve to remove anthers. Ten grams of pollen are spread on a paper tray and dried with gentle ventilation at 35°C and 35 - 40% relative humidity. Pollen water content is determined from a 0.5 g pollen sample dried at 95°C for five hours. Optimum water content for storing pollen is 10-12% for maize and 5-7% for pearl millet. The dried pollen is distributed among cryopreservation tubes. The sealed tubes are stored in liquid nitrogen (-196°C) until they are used. After thawing sample tubes containing pollen in a water-bath at 38°C for five minutes, pollen is checked for its germination viability and used for crossing. The drying and freezing process reduces pollen viability and may affect embryo formation frequency in wide crosses.

Detached-tiller culture

At ear emergence wheat tillers are cut off at the base and cultured in a flask containing tap water. Wheat spikes are emasculated conventionally by hand or incompletely using hot water (Nagamine et al., 1995), and covered with polyethylene bags for maintaining high humidity.

After pollination, polyethylene bags are replaced with paper bags, and the tillers are cultured in a solution containing 40 g/l sucrose, 8 ml/l sulfurous acid (6% SO_2) and 100 mg/l 2,4-D until embryo rescue. This process takes approximately 12 days (in maize crosses) and 14 days (in pearl millet crosses) after pollination. The culture conditions are 22.5°C, 12-h daylength and 60 - 70% relative humidity in a growth chamber.

Table 1. Effect of pollen donors on embryo formation frequencies (%) in crosses of hexaploid wheat with maize, pearl millet and sorghum.

Pollen donor		Wheat variety		
		Chinese Spring	Norin 61	Siete Cerros
Maize	Population-93A	26.0	22.4	12.6
	Hybrid-33	18.4	26.8	13.1
Pearl millet	NEC-7006	39.4	37.6	13.6
	NEC-7268	23.3	15.6	0.8
Sorghum	ICSR-LM-90166	22.1	42.1	0.0
	Toluca-1	20.4	41.1	0.0

Source: Inagaki and Mujeeb-Kazi (1995).

Table 2. Effect of pollen storage and detached-tiller culture on polyhaploid production frequencies (%) in crosses of hexaploid wheat with maize and pearl millet.

Pollen donor	Pollen storage	Tiller culture	Embryo formation (a)	Plant regeneration (b)	Polyhaploid production (a x b)
Maize	Fresh	On plant	20.4	67.0	13.7
		Detached	19.4	42.5	8.3
	Stored	On plant	2.8	65.0	1.8
		Detached	7.0	46.5	3.3
Pearl millet	Fresh	On plant	19.7	45.8	9.0
		Detached	21.2	56.7	12.0
	Stored	On plant	20.4	44.3	9.0
		Detached	27.7	54.5	15.0

Wheat; variety Norin 61; F1 hybrid line (CML-246 x CML-242), Pearl millet; inbred line NEC-7006. Source: Inagaki, Nagamine and Mujeeb-Kazi (1997).

Polyhaploid production

At CIMMYT, fresh maize pollen is available during the summer crop cycle. Table 3 shows efficiency of wheat polyhaploid production using maize crosses over a four-year period.

It is suggested that polyhaploid production using detached-tiller culture is as efficient as the conventional crossing method. Production of wheat polyhaploids is efficient enough to obtain 1.5 polyhaploid plants per wheat spike (one doubled haploid per wheat spike after chromosome doubling). The process takes approximately nine months from sowing of plant material (with spring growth habit) to harvesting of doubled haploid grains. However, it must be noted, in particular, that production efficiency in the process of plant regeneration from rescued embryos and chromosome doubling after colchicine treatment, varies depending on the use of environment-controlled facilities.

Table 3. Efficiency of polyhaploid production in hexaploid wheat using maize crosses.

Culture	Year	Wheat material	Spikes pollinated	Embryos obtained	Plants regenerated
On plant	1993	F1 plants	284	1449	810
	1994	F3 plants	456	1219	898
	1995	F1 plants	501	1894	1267
	Total		**1241**	**4562**	**2975**
Detached-tiller	1996	F1 plants	255	1046	668
	1996	F3 plants	1611	4634	2884
	Total		**1866**	**5680**	**3552**

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