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IMPROVING THE EFFICIENCY OF HAPLOID WHEAT PRODUCTION MEDIATED BY WIDE CROSSING

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SUMMARY

This study examined several possible means of improving the wheat (*Triticum aestivum* L.) x maize (*Zea mays* L.) method for haploid production in Australian hard white spring wheat genotypes. Two wheat varieties were pollinated by each of five maize lines, giving an average of 22 embryos per 100 florets. One maize inbred gave a significantly lower haploid embryo yield. Embryos rescued 12 or 15 days post pollination had a significantly higher germination percentage (51 to 80%) than embryos rescued 9, 17, 19 or 21 days after pollination (6.4 to 31%). Embryos 12 to 15 days old were found to be large and easily rescued. Haploid wheat embryos germinated at equal frequencies on four commercial tissue culture media and germination frequency was not affected by differing levels of 6-benzylaminopurine (BAP). However, the rate at which haploid embryos germinated was found to be significantly affected by both the type of medium and the cytokinin level. Haploid embryos germinated fastest on an MS based medium with 0.05 mg/L BAP.

Key words: wheat, maize, haploid, embryo culture, doubled haploid.

Doubled haploid technology has the potential to significantly accelerate wheat breeding programs because homozygous lines are available for selection in the generation following F_1 hybrids. Several haploid production systems have been reported for wheat, including anther culture (Schaeffer et al., 1979), microspore culture (Luckett and Darvey, 1992) and intergeneric crosses to other grasses such as *Hordeum bulbosum* L. (Barclay, 1975), maize (Laurie and Bennett, 1986), sorghum (*Sorghum bicolor* (L.) Moench; Laurie and Bennett, 1988*a*) and pearl millet (*Pennisetum glaucum* (L.) R.Br.; Laurie, 1989). Of the intergeneric crosses, maize appears to be the most efficient pollen donor when utilising a diverse range of wheat germplasm (Inagaki and Tahir, 1990; Kisana et al., 1993).

The production of embryos through wheat × maize crosses was first reported by Zenkteler and Nitzsche (1984). Laurie and Bennett (1986) cytologically examined embryos produced via this system and found maize chromosomes to be preferentially eliminated during the first three cell divisions, leaving a haploid complement of wheat chromosomes. Reports of haploid wheat plants soon followed (Laurie and Bennett, 1988*b*; Comeau et al., 1988). Several other investigations of haploid wheat production through wide crossing have since been reported (Laurie and Bennett, 1989; Riera-Lizarazu and Mujeeb-Kazi, 1990; Laurie and Reymondie, 1991; Matzk and Mahn, 1994; Suenaga, 1994; Morshedi and Darvey, 1995).

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It appears that a wide range of wheat and maize genotypes can be used to produce haploid wheats, although there is evidence to suggest that the efficiency of production is variable (Suenaga, 1994). Haploid production efficiency is affected by the proportion of pollinated florets which develop haploid embryos (yield). Yields of haploid embryos have been reported to be as high as 53% (Morshedi and Darvey, 1995) and as low as 1% (Suenaga and Nakajima, 1989) depending upon a wide range of variables. Factors that affect the yield of haploid embryos include genotypic differences between individual wheat and maize lines (Inagaki and Tahir, 1990; Suenaga, 1994), the timing and use of exogenous growth substances to stimulate ovule development (Suenaga and Nakajima, 1989) and environmental factors (especially temperature) during and following pollination.

Haploid production efficiency will also be influenced by the proportion of haploid embryos which germinate and develop into plantlets (germination percentage). One factor which is likely to influence the germination success of haploid embryos is the timing of embryo rescue. This has been demonstrated to affect the germination percentage of hexaploid embryos (Robertson and Curtis, 1967; Mukade et al., 1973; De Pauw and Clarke, 1976). In most recent reports, haploid embryos have been rescued from 2 to 3 weeks after pollination (Laurie and Reymondie, 1991; Kisana et al., 1993; Morshedi and Darvey, 1995).

Successful germination will further depend on nutritional and hormonal factors in the germination medium. Several types of solid media have been successfully used for germination of haploid embryos, including Norstog II (Comeau et al., 1988) orchid agar (Laurie and Bennett, 1988*b*), half strength MS (Murashige and Skoog, 1962; Suenaga and Nakajima, 1989) and B5 (Gamborg et al.,1968; Inagaki and Tahir, 1990). However, detailed comparisons between different media types have not been reported.

We are currently engaged in the development of wheat × maize-mediated doubled haploid lines both for use in a major wheat breeding program and in related research projects. One of our objectives is to rapidly generate large, random, inbred populations from particular F_1 crosses and to use these populations to search for molecular markers linked to highly desired quality traits and disease resistances. The study reported here has sought to increase the efficiency of production of haploids from spring wheat parents relevant to many Australian breeding programs. The specific objectives of this study were:

i) To test a range of maize genotypes for their effects on the yield of haploid wheat embryos;

ii) To investigate the effect of timing of embryo rescue post-pollination on subsequent embryo germination *in vitro,* and;

iii) To compare embryo development and germination under a variety of nutrient and phytohormone conditions in culture.

MATERIALS AND METHODS

Wheat lines

Four wheats (Hartog, Janz, QT2200-20 and Cunningham) were selected from lines produced by the Queensland Wheat Research Institute. Janz (3Ag3/4*Condor//Cook) has a maximum classification of Australian prime hard and is widely grown in New South Wales and South Australia. Hartog (Vicam71//Ciano'S'/

Siete Cerros/3/ Kalyansona/Bluebird) is currently the principal early maturing cultivar in the northeastern region of the Australian wheat belt with a maximum classification of Australian prime hard. The unrelated line QT2200-20 (Frocor/Kenya Farmer//Gabo /3/Frocor/McMurchy//Kentana/Yaqui/4/ Frocor/5/Timgalen) is also a quick maturing, high baking quality line. Cunningham (3Ag3/4*Condor//Cook) is an intermediate maturing, prime hard sister line of Janz widely grown in Queensland and northern New South Wales.

Maize lines

The maize lines used as pollen donors were the hybrids Terrific, Seneca 60 and Honeysweet and two inbred selections, Hi27 and BL46. Honeysweet and Terrific are commercially available maize hybrids. Seneca 60 seed was kindly provided by the Tropical Field Crops Genetic Resource Centre, Biloela, Australia, and seed of Hi27 and BL46 was supplied by Dr Ian Martin, Kairi Research Station, Kairi, Australia.

Effect of maize parent on embryo yield

Wheat plants were raised in a glasshouse, and maize plants for pollen production were field-grown. Spikes were emasculated approximately one day before anthesis, leaving the primary and secondary florets of ten spikelets, and covered with a glassine bag. Emasculation was conducted without cutting back of the glumes, lemma and palea. Any awns were cut back to the top of the glumes.

Early on the morning of the fourth day after emasculation of wheat spikes, maize tassels undergoing anthesis were shaken to remove old pollen and then bagged to collect fresh pollen for crossing. Pollination of wheat spikes was conducted at noon on the same day. Two wheat parents, Hartog and Janz, were each pollinated by the five maize parents. Maize pollen was transferred to wheat stigmas with a camel hair brush, spikes were rebagged and the pollinated plants were placed in a glasshouse where temperatures ranged between 9°C and 34°C.

One day after pollination, the internode below the spike was filled with 10 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) as described by Suenaga and Nakajima (1989) and a drop of the 2,4-D solution was placed in each pollinated floret (Laurie and Reymondie, 1991). Fifteen days after pollination the numbers of caryopses developing in all spikes were recorded.

Treatments were applied to five emasculated spikes of each wheat parent. The yield of haploid embryos was measured as the number of embryos formed per pollinated wheat spike (20 florets). The data were then analysed as a completely randomised experiment with up to ten observations within each replication, using a residual maximum likelihood analysis (REML) performed by Genstat5 software (Payne et al., 1993).

The timing of embryo rescue

Embryos derived from the crosses QT 2200-20 x Terrific and Cunningham x Terrific were used in this study. All crossing procedures were as previously described except that from emasculation onwards plants were placed in an air conditioned perspex growth cabinet set at 25°C with natural lighting. Embryos were rescued at 9, 12, 15, 17, 19 and 21 days after pollination and cultured on MS media (Murashige and Skoog, 1962) containing 30g/L sucrose, 8 g/L agar and 0.05 mg/L 6-benzylaminopurine (BAP). All

treatments were replicated six times with one to seven embryos cultured in each replication. Embryos were placed in the dark at 20°C and observed daily. Embryo germination was scored as successful once the tip of the first leaf had emerged from the coleoptile. REML analysis by Genstat 5 was again used to generate best linear unbiased estimates of the timing of embryo germination.

The effects of nutrient conditions on embryo germination

In one experiment, Hartog × Seneca 60 embryos were germinated on four different media including full strength MS, half strength MS, B5 (Gamborg et al., 1968) and Knudson C orchid medium (Knudson, 1946). All media contained 30 g/L sucrose, 0.05 mg/L BAP and were solidified with 8 g/L agar. MS and B5 are common culture media containing macronutrients, micronutrients and vitamins. Knudson's C medium is much simpler and contains no vitamins. Crossing procedures were as described above and all embryos were rescued 15 days after pollination.

The numbers of embryos rescued on a particular day were recorded and the rescued embryos distributed over the four treatments. The trial was replicated on 41 occasions with one to seven embryos placed on each media treatment. Embryos were germinated in the dark at 20°C. The success and timing of embryo germination was recorded. A generalised linear model (GLIM) was used to test the hypotheses that the haploid embryos would germinate at equal times and at equal frequencies on the four different nutrient media.

In a similar, second experiment, embryos derived from Hartog x Seneca 60 crosses rescued on the same day were evenly distributed over six media treatments which included MS and half strength MS basal nutrients each containing BAP at either 0.05, 0.5 or 2.0 mg/L. The experiment was replicated 16 times with from one to seven embryos cultured per treatment. GLIM was once again used to test for effects of the medium on the success and timing of embryo germination.

RESULTS

Effect of maize parent on embryo yield

In experiments examining the effects of pollen parent genotype on embryo formation, all ten different crosses produced haploid wheat embryos, with embryo

Table 1. Best linear unbiased estimates of the percentages of pollinated florets that produced viable haploid wheat embryos for two wheat parents and five maize pollen donor lines.

Wheat parents	% Haploid embryos	Maize pollen donor lines	% Haploid embryos
Hartog	23.45a[1]	Terrific	32.00a
Janz	19.45a	Seneca 60	27.50a
		Honeysweet	21.00a
		BL46	19.25a,b
		Hi27	7.50b

[1] Values within a column accompanied by the same letter are not significantly different.

production averaging 22% over all treatments. No significant differences were found between the wheat parents in their response to maize pollen (Table 1). Embryo production levels ranged from 7.5 to 32% of pollinated florets, depending on the maize parent used. Of the five maize lines tested, crosses involving Hi27 produced significantly fewer haploid embryos than three of the other four lines when crossed to both wheat varieties. All other maize parents gave statistically similar embryo production (Table 1).

The timing of embryo rescue

The percentage of haploid embryos which subsequently germinated varied significantly according to the number of days between pollination and embryo rescue (Table 2). Similar responses were observed for both wheat parents. The majority of embryos rescued at 9 days after pollination (DAP) did not respond to culture and degenerated. Older embryos (19 to 21 DAP) became swollen and often produced callus, which appeared to impede germination. Embryos rescued 12 to 15 DAP rarely exhibited these responses and germinated at significantly higher frequencies than other treatments.

Table 2. Best linear unbiased estimates of the percentage germination of haploid wheat embryos that were excised at differing periods after pollination.

No. days from pollination to embryo excision	Wheat parents	
	QT 2200-20	Cunningham
9	22.43b,c[1]	28.87b
12	51.44a	80.03a
15	54.66a	77.67a
17	31.04b	29.05b
19	6.38d	13.51b,c
21	7.76c,d	10.87c

[1] Numbers accompanied by the same letter are not significantly different.

Effect of nutrient conditions on embryo germination

There were no significant differences between the four media in the percentages of haploid Hartog embryos that germinated. Of the 113 embryos cultured on each medium, 70 - 74% germinated, and 64% of the 452 embryos cultured grew to form haploid plants. Of these haploid plants, 44% formed doubled haploids following colchicine treatment (data not presented).

While the percent germination of cultured embryos was independent of the medium used, the timing of germination after rescue (Fig.1) was significantly influenced by the culture medium ($P<0.05$). The four media can be divided into two groups according to the timing of embryo germination. Haploid wheat embryos germinated significantly earlier on Gamborg's B5 and full strength MS than on the orchid medium or half-strength MS.

In the second experiment which examined the effect of cytokinin level on Hartog haploid embryo germination, final germination percentages ranged from 61 to 85. While there were no significant effects of the six different treatments on germination percentage, the timing of embryo germination was significantly influenced by different BAP levels ($P<0.05$). Embryos germinated earlier on full strength MS medium containing 0.05 mg/L

BAP than on any other treatments. Germination rates in all other treatments were similar to each other (Fig. 2).

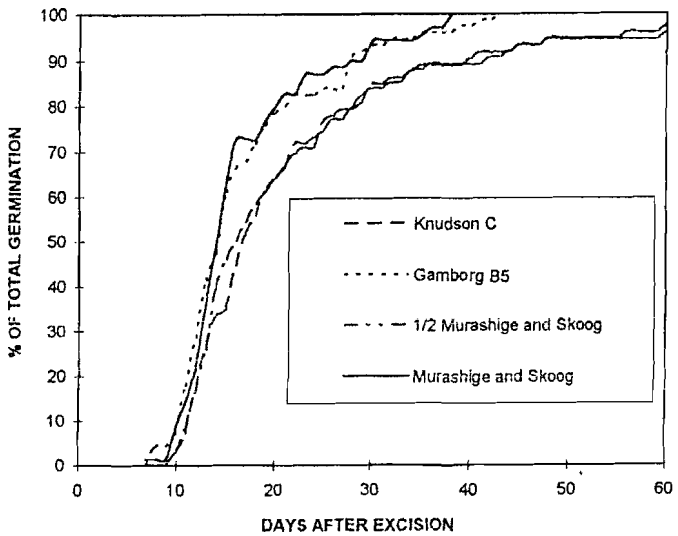

Figure 1. Germination rates of haploid Hartog wheat embryos on four culture media.

DISCUSSION

The results of this study have been useful for improving the method for the production, from wheat × maize crosses, of haploid plants in culture, with particular emphasis on choice of maize pollen, timing of embryo rescue and culture conditions for haploid embryo germination and growth *in vitro*.

Several previous studies have examined the effects of maize pollen genotype on haploid wheat embryo production, emphasising genotypes relevant to northern hemisphere environments (Suenaga and Nakajima, 1989; Inagaki and Tahir, 1990; Suenaga et al., 1991; Kisana et al., 1993). The most extensive work was that of Suenaga (1994), who investigated a series of crosses involving 47 wheat and 52 maize parents in total. While all crosses had the ability to produce haploid embryos, the yield of embryos varied from 0.9 to 36% depending upon the parents. This range of values is wider than that reported in this study (7.5-32%; Table 1), and wider than the range recently reported by Morshedi and Darvey (1995), who also worked on Australian genotypes. These authors observed that between 24 and 53% of pollinated florets produced embryos in the F_1 wheat cross M 2369/Sun 234A with three different sources of hybrid maize pollen.

In general, the hybrid maize parents appeared to marginally outperform the inbred lines for haploid embryo production (Table 1). However, the differences were not statistically significant with regard to both inbred maize parents perhaps due to the small

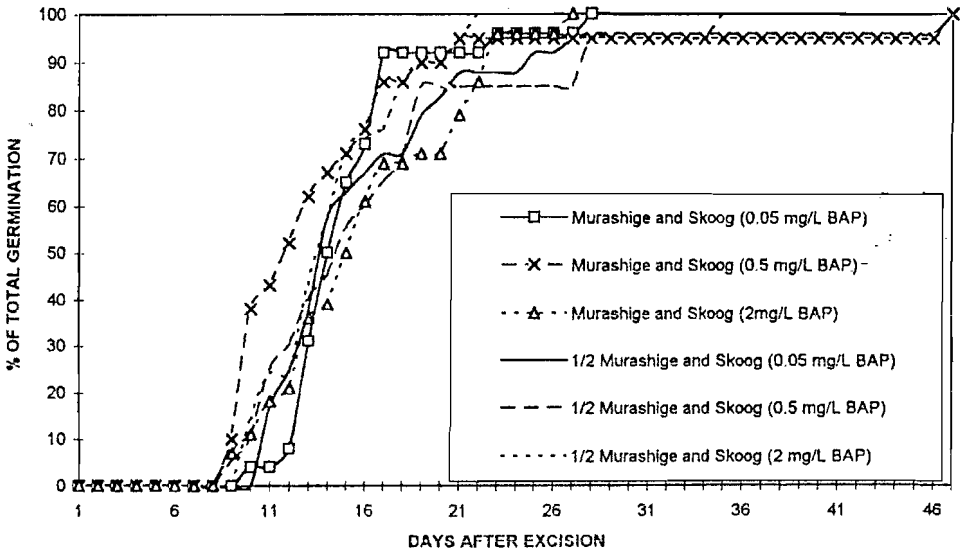

Figure 2. Germination rates of haploid Hartog wheat embryos on two levels of MS basal salts and three levels of 6-benzylaminopurine.

sample size. The genotypic specificity of the wheat × maize-mediated haploid production system is not of the magnitude reported for other haploid production systems including anther culture (Kisana et al., 1993) and wheat × *H. bulbosum* (Sitch and Snape, 1986). The genotypic specificity may reduce the efficiency of these systems to a level where they are not applicable to a breeding program (Inagaki and Tahir, 1990; Kisana et al., 1993).

Pollen from field-grown maize was used in the current investigation. Pollen taken from plants grown under controlled, glasshouse conditions may be more suitable for haploid embryo production. Physical conditions vary in the field and factors such as high temperatures are known to reduce maize pollen viability. Temperatures above 26°C have been shown to affect maize pollen viability as well as rate of pollen tube growth (Lyakh et al., 1991). At the time of these trials both field and glasshouse temperatures exceeded 26° and this may have adversely affected final embryo yields. Using methods similar to those in this investigation, Copas and Kammholz (unpublished) in March 1996 were able to produce 929 haploid embryos from 2151 pollinated florets (43%) in crosses using maize pollen produced in a temperature-controlled glasshouse.

It is evident that the most suitable timing of embryo rescue is from 12 to 15 days after pollination in our environment. Embryos younger than 10 days were quite small, difficult to manipulate and frequently failed to respond in culture. Rescuing of 15 day old embryos is recommended as this provides the largest embryos of the highest germination class. The only other reported study which has examined the timing of haploid embryo rescue is that of Suenaga (1994). Embryos were rescued at 7, 10 to 11 and 14 days after pollination. Embryos rescued 10 to 11 days after pollination gave a very high germination result (78%), but only 23 florets had been pollinated.

Investigations to compare optimal *in vitro* culture conditions have not been reported for the wheat × maize system. Several basal nutrient solutions have been used successfully including half strength MS (Suenaga, 1994; Suenaga and Nakajima, 1989;

Riera-Lazarazu and Mujeeb-Kazi, 1990), B5 (Inagaki and Tahir, 1990; Kisana et al., 1993) and orchid agar media (Laurie and Bennett, 1988b; Laurie and Reymondie, 1991). Comparisons between these investigations are made difficult by the use of different sucrose concentrations, the presence or absence of phytohormones, and variations in light and temperature regimes. It was observed in this study that the percentage of rescued embryos which germinated in culture was insensitive to variation of the culture medium between several major commercial formulations. The overall germination success of the embryo rescue procedure was similar to that reported elsewhere (Laurie and Bennett, 1988b; Riera-Lazarazu and Mujeeb-Kazi, 1990; Laurie and Reymondie, 1991; Suenaga, 1994). Full strength MS and B5 gave faster embryo development than Knudson's C or half strength MS (Fig. 1). This is an important result in terms of production efficiency for large scale development of doubled haploid families for breeding programs. One major difference between the two groups of media is their vitamin content. Knudson's C orchid medium (containing no vitamins) and half strength MS medium are significantly lower in vitamins than the other two media. Further studies are required to determine whether this difference is a significant factor.

In comparing the four media, the same low level of the cytokinin BAP (0:05mg/L) was added in each formulation. BAP has been shown to enhance the speed of shoot differentiation in wheat (Kato et al., 1991). Tanzarella and Greco (1985) used BAP effectively at 1 mg/L to germinate durum wheat embryos, while 5 mg/L induced proliferation of axillary shoots. However, in this study, when similar higher levels of BAP (0.5 or 2.0 mg/L) were substituted into a full strength MS medium (Figure 2), the time required for maximum embryo germination was lengthened, without changing the final yield of germinated embryos. This reinforces the observation that optimisation of the medium conditions will shorten the timing of plantlet production but may have only small effects on overall haploid plantlet numbers.

The current method for routine application of the wheat × maize system at the Queensland Wheat Research Institute uses pollen from a glasshouse-grown hybrid maize source (e.g. cv. Terrific), embryos rescued at 12 to 15 days after pollination, and culture of embryos on full strength MS medium containing 0.05mg/L BAP.

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