Theor Appl Genet (1994) 89:559–566 © Springer-Verlag 1994

L. S. O'Donoughue · M. D. Bennett

Durum wheat haploid production using maize wide-crossing

Received: 10 March 1994 / Accepted: 18 April 1994

Abstract While anther culture or pollinations with *Hordeum bulbosum* have provided suitable methods for haploid production in bread wheat, they have been largely unsuccessful in durum wheat. Pollinations with maize were used in an attempt to produce haploid seedlings and, from these, fertile doubled haploids of durum wheats. Moreover, the effect of various concentrations and combinations of a synthetic auxin, 2, 4-dichlorophenoxyacetic acid (2,4-D), kinetin, and an ethylene inhibitor, silver nitrate ($AgNO_3$), on embryo recovery were also investigated. Haploid seedlings were recovered from *Triticum turgidum* ssp. *turgidum* cv 'Rampton Rivet' pollinated with maize following in-vivo treatment of ovaries with 2,4-D for 2 weeks and subsequent embryo culture. The recovery of haploid seedlings from *T. turgidum* ssp. *durum* cv. 'Wakona' pollinated with maize necessitated the addition of $AgNO_3$, to the 2,4-D treatment. Overall, haploid seedlings were produced in 1.7% and 3.3% of pollinated florets for 'Rampton Rivet' and 'Wakona' respectively. The success of the present work represents a significant breakthrough for haploid production in durum wheats. Wide hybridization with maize followed by in-vivo treatment of ovaries with 2,4-D alone, or in combination with $AgNO_3$, may provide a widely-applicable method of haploid production in tetraploid wheats.

Key words Haploids · Wide hybridization · Durum wheat · Maize · Embryo culture

Communicated by K. Tsunewaki

L. S. O'Donoughue (✉)[1]
Cambridge Laboratory, IPSR, Colney Lane, Norwich, NR4 7UJ, UK

M. D. Bennett
Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3DS, UK

Present address:
[1] Agriculture Canada, Research Branch, Central Experimental Farm, Building 50, Ottawa, Ontario K1A 0C6, Canada

Introduction

Tetraploid durum wheat is a major crop in several Mediterranean countries (Bennici 1986). Producing haploids and completely homozygous doubled haploids in this crop species would enhance its improvement by accelerating breeding programs, improving selection efficiency and facilitating genetic analyses (Snape 1989; Snape and Simpson 1981). The established methods of anther culture and pollinations with *Hordeum bulbosum*, used to produce haploids in bread wheats and barley, are largely unsuccessful for producing haploids from durum wheats (Zhu et al. 1980; Sharma et al. 1982; Hadwiger and Heberle Bors 1986; O'Donoughue and Bennett 1994). More recently, Hsam and Zeller (1993) have succeeded in obtaining haploids from the durum wheat cultivar 'Cando' using nucleo-cytoplasmic interactions. In plants with the cytoplasm of *Aegilops kotschyi* and the nucleus of the durum wheat 'Cando', carrying a 1BL/1RS interchange, they obtained up to 14% haploid plant frequency. However, the haploid production was dependent upon the presence of the foreign cytoplasm, which tends to induce male sterility, the presence of 1RS and the absence of 1BL. All these conditions tend, at this point in time, to restrict the widespread use of this technique. Therefore other methods are needed.

A more-recently-developed method of haploid production in bread wheat involves pollination with maize and subsequent elimination of the maize genome (Comeau et al. 1988; Laurie and Bennett 1988a; Suenaga and Nakajima 1989; Riera-Lizarazu and Mujeeb-Kazi 1990). Although similar to the bulbosum method, the new method has the added advantage of being genotype independent as it is largely unaffected by the presence of dominant alleles at the *Kr* loci which restrict crossability with *H. bulbosum* (Laurie and Bennett 1987). O'Donoughue and Bennett (1994) showed that tetraploid wheats could also be successfully fertilized by maize. In ten different genotypes pollinated with maize,

30.0–62.4% of the pollinated florets contained an embryo, and all these crosses were characterized by the rapid elimination of the maize genome resulting in haploid embryos. These results indicated that wide-crossing with maize could also be used to produce doubled haploid plants from durum wheats.

Fertilization of bread and durum wheats by maize is possible but plant recovery can be difficult, as young seeds left on the plant rapidly abort (Laurie and Bennett 1988a). In durum wheat × maize crosses double fertilization, and consequently endosperm formation, were more than twice that in hexaploid wheats pollinated with maize (O'Donoughue and Bennett 1994). The authors postulated that the presence of an endosperm may aid embryo development and plant recovery. However, preliminary results indicated that early seed abortion is also a problem in durum wheat × maize crosses (O'Donoughue 1990). Three methods have been used to recover haploid seedlings from bread wheat × maize crosses. Laurie and Bennett (1988a) plated whole spikelets 2 days after pollination on an MS medium (Murashige and Skoog 1962) supplemented with sucrose and the synthetic auxin 2, 4-D (Mathias and Boyd 1988) followed by embryo culture 3 weeks later. Comeau et al. (1988) recovered plants using ovule culture on a medium high in sugars with subsequent transfer to a modified Norstog-II medium (Norstog 1973). The third method involves treating ovaries in-vivo with a solution of 2, 4-D until the embryos become big enough to be cultured and germinated on standard embryo-culture medium. These 2, 4-D applications have been successful through direct placement inside the floret (Laurie et al. 1990), spraying (Riera-Lizarazu and Mujeeb-Kazi 1990), injection into the uppermost internode of the wheat stem (Suenaga and Nakajima 1989; Inagaki and Tahir 1990; Riera-Lizarazu and Mujeeb-Kazi 1990) and by placement of detached spikes into an aqueous solution (Riera-Lizarazu and Mujeeb-Kazi 1990). The key ingredient for the recovery of bread-wheat haploid embryos following pollination with maize is apprarently 2, 4-D. Unfortunately, preliminary experiments (O' Donoughue 1990) indicate that 2, 4-D alone, though inducing swelling of the ovaries, may not be sufficient for the recovery of haploid embryos from durum wheat.

Treatment of ovaries in vivo with other growth regulators, such as kinetin and gibberellic acid, could possibly also enhance embryo survival and development. However, post-pollination treatment with gibberellic acid of bread-wheat ovaries pollinated with maize tended to reduce embryo recovery (Laurie 1989). Compounds which interfere with abscission and senescence may also prevent early embryo abortion. Abscission in plants is often associated with increased ethylene production (Salisbury and Ross 1978). Thus, treatment of ovaries with ethylene inhibitors such as CO_2 (Abeles 1973), salicylic acid (Romani et al. 1989), or silver ions (Beyer 1976; Davies et al. 1988), may improve embryo survival.

The aim of the present work was therefore to determine if kinetin or silver nitrate, an ethylene inhibitor, alone or in combination with 2, 4-D, could help the recovery of haploid seedlings from durum wheats pollinated with maize.

Materials and methods

Plant material and pollination methods

The tetraploid (2n = 4x = 28) wheats *Triticum turgidum* (L.) Thell. ssp. *durum* Desf. cv 'Wakona' and *T. turgidum* (L.) Thell. ssp. *turgidum* cv 'Rampton Rivet' (2n = 4x = 28), or the hexaploid (2n = 6x = 42) *T. aestivum* (L.) Thell. cv 'Chinese Spring' were used as female parents in crosses with *Zea mays* L. cv 'Seneca 60'. 'Rampton Rivet' seedlings were vernalized for 8 weeks at 4 °C with an 8-h day, 16-h night regime. Wheat plants were either grown in an unheated glasshouse or transferred to a growth cabinet at 20 °C with a 16-h day, 8-h night for about 1 week before anthesis (referred to hereafter as the two environments). Maize plants were maintained in an unheated glasshouse and experiments performed in summer. Emasculations and pollinations were as in Laurie and Bennett (1986). The glumes were left intact when emasculations were performed and pollinations were made when all the stigmas in the spike appeared feathery.

Treatment of spikes with growth regulators

Table 1 lists treatments carried out on plants growing in both environments. For each cross-treatment-environment combination, two to five spikes were used. The treatments were applied in vivo by filling each floret with the solution, daily, starting 2 days after pollination, for about 2 weeks. A 5-ml syringe with a blunted needle was inserted between the glumes. A distilled water (dH_2O) control was used in each of the three crosses. If plants were available, treated unpollinated florets were also used as controls (Table 1). After about 2 weeks of treatment, all ovaries were dissected and embryos cultured as in Snape et al. (1979), while a subsample of the ovaries (from two to four spikes) from each cross-treatment-environment combination were measured to give an indication of seed swelling.

Chromosome doubling of haploid seedlings

The ploidy level of seedlings was determined in metaphase spreads from root tips which were pretreated in ice water for 24 h, fixed in 3:1 absolute ethanol/glacial acetic acid, and either acetocarmine or Feulgen stained. Roots and crown of recovered seedlings were then immersed in 0.05% colchicine (Sitch and Snape 1986) at the three-tiller stage. 'Wakona' tillered poorly, so the treatment was applied at an equivalent age and height. The treated seedlings were grown to maturity (those from 'Rampton Rivet' × 'Seneca 60' were initially vernalized for 8 weeks). Spikes were then bagged before anthesis and the seeds harvested.

Results

The range of treatments is given in Table 1 and the numbers and percentages of recovered embryos and seedlings in Table 2. No treatment lacking 2, 4-D caused swelling of the ovaries and these resulted in almost complete desiccation of the ovaries 2 weeks after pollination. Nevertheless, three embryos were recovered from cross-pollinated 'Chinese Spring' treated with 50 mg/l of $AgNO_3$ (two of which germinated), and three

Table 1 Treatments performed on tetraploid and hexaploid wheat ovaries pollinated with maize cv 'Seneca 60'[a]

Treatment	Female parent					
	'Wakona'		'Rampton Rivet'		'Chinese Spring'	
	CP	UP	CP	UP	CP	UP
dH_2O control	Y	–	Y	–	Y	–
$AgNO_3$ 25 mg/l	Y	–	–	–	Y	–
$AgNO_3$ 50 mg/l	Y	–	–	–	Y	–
$AgNO_3$ 100 mg/l	Y	–	–	–	Y	–
Kinetin 1 mg/l	Y	–	–	–	Y	–
2,4-D 0.5 mg/l	Y	–	Y	–	Y	–
2,4-D 5 mg/l	Y	Y	Y	Y	Y	Y
2,4-D 5 mg/l + $AgNO_3$ 25 mg/l	Y	Y	Y	–	Y	Y
2,4-D 5 mg/l + $AgNO_3$ 100 mg/l	Y	Y	Y	Y	Y	Y
2,4-D 5 mg/l + $AgNO_3$ 200 mg/l	Y	Y	–	–	–	–
2,4-D 5 mg/l + Kinetin 1 mg/l	Y	Y	Y	Y	Y	Y

[a] CP, cross pollinated; UP, unpollinated; Y, treatment performed. Treatments were performed both in the glasshouse and in the growth room

Table 2 Numbers and percentages of embryos and seedlings obtained in wheats pollinated with maize cv 'Seneca 60'. Only treatments which yielded some embryos are listed

Female parent[a]	Treatment	Environment[b]	No. of spikes	No. of florets	Embryos		Seedlings	
					No.	%	No.	%
W	Kinetin 1 mg/l	GL	2	52	3	5.8	0	0.0
		GR	2	54	0	0.0	0	0.0
	2,4-D 5 mg/l + $AgNO_3$ 25 mg/l	GL	4	96	14	14.6	5	5.5
		GR	4	92	10	10.9	0	0.0
	2,4-D 5 mg/l + $AgNO_3$ 100 mg/l	GL	3	76	17	22.4	5	6.6
		GR	4	92	22	23.9	0	0.0
	2,4-D 5 mg/l + $AgNO_3$ 200 mg/l	GL	4	104	17	16.3	9	8.7
		GR	4	94	13	13.8	3	3.2
Total			27	660	96	14.5	22	3.3
RR	dH_2O	GL	4	107	0	0.0	0	0.0
		GR	4	110	19[c]	17.3	0	0.0
	2,4-d 0.5 mg/l	GL	4	124	3	2.4	1	0.8
		GR	4	128	27	21.1	4	3.1
	2,4-D 5 mg/l	GL	4	143	11	7.7	2	1.4
		GR	4	130	58	44.6	3	2.3
	2,4-D 5 mg/l + $AgNO_3$ 25 mg/l	GL	4	138	16	11.6	1	0.5
		GR	4	119	22	18.5	8	6.7
	2,4-D mg/l + $AgNO_3$ 100 mg/l	GL	4	133	23	17.3	0	0.0
		GR	4	116	14	12.1	3	2.6
	2,4-D 5 mg/l + kinetin 1 mg/l	GL	4	134	9	6.7	1	0.7
		GR	4	116	32	27.6	3	2.6
Total			48	1498	234	15.6	26	1.7
CS	$AgNO_3$ 50 mg/l	GL	3	70	2	2.9	2	2.9
		GR	3	74	1	1.4	0	0.0
	2,4-D 0.5 mg/l	GL	4	115	15	13.0	5	4.3
		GR	4	108	1	0.9	0	0.0
	2,4-D 5 mg/l + $AgNO_3$ 25 mg/l	GL	4	92	2	2.2	2	2.2
		GR	4	102	6	5.9	3	2.9
	2,4-D 5 mg/l + $AgNO_3$ 100 mg/l	GL	4	92	7	7.6	7	7.6
		GR	4	95	19	20.0	9	9.5
	2,4-D mg/l + kinetin1 mg/l	GL	4	95	7	7.4	5	5.3
		GR	4	90	7	7.8	4	4.4
Total			38	933	67	7.2	37	4.0

[a] W, Wakona; RR, Rampton Rivet; CS, Chinese Spring
[b] Gl, glasshouse; GR, growth room
[c] Only 13 of the 19 embryos cultured

very small embryos were recovered from the 1 mg/l kinetin treatment of cross-pollinated 'Wakona'. These were undifferentiated and did not germinate. Cross-pollinated 'Rampton Rivet' ovaries were not treated with kinetin or $AgNO_3$ alone. However, 19 embryos were recovered from the dH_2O control.

The 5 mg/l 2,4-D treatments alone, or in combination with $AgNO_3$ or kinetin, generally gave better ovary

development than the 0.5 mg/l 2,4-D treatment of both unpollinated and cross-pollinated ovaries (data not shown). ANOVA (Table 3) performed on the percentage of embryos obtained and the frequency of embryo germination for the treatments containing 2,4-D showed that, for embryo formation, the three female parents behaved differently ($P < 0.001$). Also significant were the interaction terms, environment-female parent ($P < 0.001$), female parent-treatment ($P < 0.001$) and environment-female parent-treatment ($P < 0.001$). Only the female parent effect ($0.001 < P < 0.01$) and the treatment effect ($0.01 < P < 0.05$) were significant for the embryo germination. Because of the significant effect of female parents and female parent-treatment interaction the results for each cross combination are described separately below.

No embryo was found in any of the unpollinated 'Wakona' controls. However, four seeds (from 5 mg/l 2,4-D + 100 mg/l $AgNO_3$ and 5 mg/l 2,4-D + 1 mg/l kinetin treatments) and one seed (from 5 mg/l 2,4-D + 100 mg/l $AgNO_3$ treatment) respectively were recovered from 'Rampton Rivet' and 'Chinese Spring' controls. These were markedly different from all those recovered from cross pollinations by containing a well-developed endosperm indicative of accidental self-pollination.

'Wakona' × 'Seneca 60'

The 2,4-D treatment alone was effective in swelling the ovaries but did not maintain live embryos on the plants for 16–18 days after pollination. The 5 mg/l 2,4-D treatment gave more swelling than 0.5 mg/l (data not shown) but all ovules inside the ovaries were dry and empty. Adding $AgNO_3$ to the 5 mg/l 2,4-D treatment overcame this problem, and embryos were recovered from all three $AgNO_3$ concentrations (Table 2). The frequencies of embryo formation and germination between the three different 2,4-D + $AgNO_3$ treatments were not significantly different. O'Donoughue and Bennett (1994) determined the egg-cell fertilization frequency for this cross as 41.5% of pollinated florets. Thus, in the highest yielding treatment (5 mg/l 2,4-D + 100 mg/l $AgNO_3$, in the growth room, Table 2) 57.6% of the expected embryos were maintained alive on the plants. There was no significant difference between environments for embryo yield.

Embryos recovered from this cross were small and poorly differentiated, often resulting in absent, precocious, or abnormal germination. However, normal germination was significantly higher ($0.001 < P < 0.01$) in embryos from glasshouse-treated spikes than from growth room-treated spikes (Table 2).

In all, 22 green seedlings were recovered (Table 2) and 17 were treated with colchicine. Root-tip chromosome counts were obtained from 14 of them. Thirteen had the expected haploid complement ($n = 2x = 14$) and one was a 14/28 chromosome chimera presumably resulting from spontaneous doubling in some cells early in embryo development. Nine seedlings survived the colchicine treatment and seven produced some seeds. However, the mean number of seeds recovered per fertile plant was only 3.9 (Table 4).

'Rampton Rivet' × 'Seneca 60'

Here treatment with 2,4-D alone sustained embryo growth on the mother plants for 16–18 days. The 5 mg/l 2,4-D treatment performed in the growth room gave the highest embryo recovery (44.6% of pollinated florets containing an embryo, Table 2). The embryo frequency observed by O'Donoughue and Bennett (1994) 3–4 days after pollination for this cross was 41.3% of pollinated florets. Thus, this treatment probably maintained all embryos alive on the plants. Natural seed survival in this cross was better than in the 'Wakona' and 'Chinese Spring' crosses as 19 embryos (17.3%) were recovered from the dH_2O control performed in the growth room. However, without the 2,4-D treatment, enlargement of the ovaries was poor and the embryos recovered were small, undifferentiated, and did not germinate. Treatment with 2,4-D at the low concentration of 0.5 mg/l improved ovary swelling, but many (33.3–36%) of the embryos recovered were small and did not germinate. The ovaries treated with 5 mg/l 2,4-D alone, or in combination with kinetin or $AgNO_3$, were consistently enlarged, and their embryos were large and well differentiated. There was no significant difference between 2,4-

Table 3 The partitioned ANOVA on the frequency of embryos formed and the frequency of embryo germination after pollination with maize cv 'Seneca 60' and in-vivo treatment of ovaries (data transformed to angles)

Source of variation	Degrees of freedom	Mean square	
		Frequency of embryos formed	Frequency of embryo germination
Environment (E)	1	396.12*	1 735.7
Female parent (Fp)	2	2 050.77***	4 121.2**
Treatment (T)	4	965.01***	2 166.5*
E × Fp interaction	2	597.45***	468.9
E × T interaction	4	28.33	240.1
Fp × T interaction	8	410.31***	1 323.5
E × Fp × T interaction	8	285.55***	739.7
Residual	89	77.12	656.3

* $0.01 < P < 0.05$,
** $0.001 < P < 0.01$,
*** $P < 0.001$

Table 4 Number of fertile doubled haploids obtained from colchicine-treated seedlings derived from hybridization with maize

Female parent, treatment and environment[a]	No. of seedlings treated	No. of surviving seedlings	No. of fertile doubled haploids	Total no. of seeds recovered	Mean no. of seeds per plant
W 2,4-D 5 mg/l + $AgNO_3$ 25 mg/l Gl	4	2	1	14	14.0
W 2,4-D 5 mg/l + $AgNO_3$ 100 mg/l Gl	4	2	1	2	2.0
W 2,4-D 5 mg/l + $AgNO_3$ 200 mg/l Gl	6	5	4	10	2.5
W 2,4-D 5 mg/l + $AgNO_3$ 200 mg/l GR	3	1	1	1	1.0
Total[b]	17	10 (58.8)	7 (41.2)	27	3.9
RR 2,4-D 0.5 mg/l Gl	1	1	1	12	12.0
RR 2,4-D 0.5 mg/l GR	4	2	2	4	2.0
RR 2,4-D 5 mg/l GR	3	1	1	42	42.0
RR 2,4-D 5 mg/l + $AgNO_3$ 25 mg/l GR	5	3	3	89	29.7
RR 2,4-D 5 mg/l $AgNO_3$ 100 mg/l GR	3	3	1	24	24.0
RR 2,4-D 5 mg/l + kinetin 1 mg/l Gl	1	1	1	59	59.0
RR 2,4-D 5 mg/l + kinetin 1 mg/l GR	3	3	2	70	35.0
Total	20	14 (70.0)	11 (55.0)	300	27.3
CS 2,4-D 5 mg/l Gl	4	2	0	0	0.0
CS 2,4-D 5 mg/l + $AgNO_3$ 25 mg/l Gl	2	2	2	19	9.5
CS 2,4-D 5 mg/l + $AgNO_3$ 25 mg/l GR	3	1	1	31	31.0
CS 2,4-D 5 mg/l + $AgNO_3$ 100 mg/l Gl	5	3	3	34	11.3
CS 2,4-D 5 mg/l + $AgNO_3$ 100 mg/l GR	8	6	2	25	12.5
CS 2,4-D 5 mg/l + kinetin 1 mg/l Gl	4	3	1	8	8.0
CS 2,4-D 5 mg/l + kinetin 1 mg/l GR	3	2	2	12	6.0
Total	29	19 (65.5)	11 (37.9)	129	11.7

[a] CS, 'Chinese Spring'; W, 'Wakona'; RR, 'Rampton Rivet'; Gl, glasshouse; GR, growth room
[b] Percentages in parentheses

D-containing treatments in the frequency of embryos obtained.

The growth-room environment was significantly more favourable ($P < 0.001$) than the glasshouse for embryo recovery (Table 2). Embryo germination was also better, though not significantly so, from growth room-treated plants. Despite the good embryo yield, germination was poor. At best 36.4% of embryos gave green seedlings (5 mg/l 2,4-D + 25 mg/l $AgNO_3$, growth room). Poor germination from the dH_2O control and the low 2,4-D concentration may reflect the small undifferentiated state of the embryos recovered. Embryos recovered from the treatments containing 5 mg/l 2,4-D were large and well differentiated, but aberrant types of germination were common, especially abnormal development of the shoot meristem.

Twenty-six green seedlings (Table 2) were recovered, 20 of which were colchicine-treated. Chromosome counts were obtained in all but two. All had only 14 chromosomes, confirming their haploid (n = 2x) nature. Fourteen seedlings (70%) survived the colchicine treatment and 11 fertile doubled haploids (55.0%) were recovered which produced an average of 27.3 seeds per plant (Table 4).

'Chinese Spring' × 'Seneca 60'

In this hexaploid wheat × maize cross, the highest frequency of embryos recovered 2 weeks after pollination was 20.0% (Table 2, 5 mg/l 2,4-D + 100 mg/l $AgNO_3$, growth room). Based on the observations of Laurie and Bennett (1988b) 2 days after pollination, 26.8% of the pollinated florets are expected to contain an embryo in this cross. Therefore, 74.6% of the expected embryos were recovered 2 weeks after pollination following this treatment. Except for three small embryos obtained from the 50 mg/l $AgNO_3$ treatment, no embryo was recovered from treatments containing less than 5 mg/l 2,4-D. All treatments containing 5 mg/l 2,4-D were equally effective as no significant difference in embryo frequency and germination was detected between these treatments. Also no significant environmental effect was detected for embryo frequency or germination.

In general, the germination of embryos from this cross was high compared to those crosses involving tetraploid wheats as female parents (overall mean; 59.7%, 27.5%, 15.3% of embryos germinating normally from 'Chinese Spring', 'Wakona' and 'Rampton Rivet', respectively). Overall, germination was normal, though occasional precocious germination was noted.

A total of 37 green seedlings was obtained (Table 2). Chromosome counts were performed on 30. All contained the haploid complement of 21 chromosomes (n = 3x). Of the 29 treated seedlings, 19 (65.5%) survived colchicine treatment but only 37.9% of these produced seeds. The mean number of seeds recovered per fertile doubled haploid plant was 11.7 (Table 4).

Discussion

Growth regulator effects

When *Triticum* species are pollinated with maize, the ovaries remain small and gradually dry out on the plants

whether they are fertilized or not (Laurie and Bennett 1988a; O'Donoughue 1990). In the present study two compounds were identified as beneficially retarding seed abortion, namely, the synthetic auxin, 2,4-D and the ethylene inhibitor, $AgNO_3$.

Auxin-induced parthenocarpy is well known. It has been shown to occur in unfertilized maize (Britten 1950; Rogers 1973) and bread wheat (Marshall et al. 1983) ovaries. Interestingly, Brewbaker and Emery (1962) and Pienaar and van Niekerk (1973) obtained parthenocarpic fruit development in members of the Triticeae following pollination with irradiated pollen in which pollen germination and growth occurred but fertilization was impaired. Perhaps the maize pollen tubes are unable to provide the hormonal stimulus required for fruit development and 2,4-D application compensates partly for this.

The effect of 2,4-D on ovary enlargement is directly correlated with its concentration (Marshall et al. 1983). In the present work the 5 mg/l 2,4-D concentration gave more consistent ovary enlargement than did the 0.5 mg/l concentration. However, genotypic differences were noted in 2,4-D response. While some embryos were recovered with a treatment of 0.5 mg/l 2,4-D from the the cross 'Rampton Rivet' × 'Seneca 60', a higher concentration of 5 mg/l was required for adequate ovary swelling in 'Wakona'.

For tetraploid 'Rampton Rivet' and the hexaploid 'Chinese Spring', 2,4-D alone was sufficient to cause both ovary enlargement and survival of the embryos. However in 'Wakona' × 'Seneca 60', embryo survival required the addition of $AgNO_3$ to the 2,4-D treatment.

Silver ions applied as $AgNO_3$ and other silver salt solutions inhibit the action of ethylene exogenously applied to whole plants and plant parts (Beyer 1976). Davies et al. (1988) postulated that silver ions inhibited ethylene-induced ripening of tomato fruits by interacting with the ethylene-perception mechanism. Ethylene is involved, alone or with other plant growth regulators, in flower senescence, and, leaf, flower and fruit abscission (Salisbury and Ross 1978). The phenomenon seen in untreated ovaries of *Triticum* species pollinated with maize seems to involve abscission of the ovary from the mother plant and senescence. This might be due to ethylene production in the mother plant and the applied $AgNO_3$ might counteract this effect. Auxins can promote ethylene production in plants (Salisbury and Ross 1978) and the rate of ethylene production may be regulated by the internal level of auxin (Abeles 1973). Perhaps $AgNO_3$ inhibits the effect of ethylene produced in response to the exogenously-applied 2,4-D.

Silver nitrate at the low concentration of 25 mg/l in conjunction with 5 mg/l 2,4-D was sufficient to maintain live embryos in the cross 'Wakona' × 'Seneca 60'. Though not required, addition of $AgNO_3$ to the 2,4-D treatment of 'Rampton Rivet' and 'Chinese Spring' cross-pollinated ovaries had no detrimental effect. Therefore, a combination of 2,4-D and $AgNO_3$ may be advisable when the requirements of a particular genotype are unknown.

Possible improvements

While the present work represents a considerable improvement over previous methods for obtaining durum haploids, the yield of haploid seedlings (1.7–3.3% of pollinated florets) is low compared to the best yields obtained in bread wheat using the bulbosum method, which are around 10% of pollinated florets (Sitch and Snape 1986). However, yields from the maize method may be expected to increase with further technical improvements. The present study showed clear genotypic differences between female parents in their response to the application of different growth regulators. Such genotypic differences involved three distinct steps of the doubled haploid plant recovery process, namely, (1) extension of embryo survival on the mother plant, (2) embryo germination and (3) production of fertile doubled haploids.

(1) Extension of embryo survival on the mother plant. In this study embryo survival seemed inherently better in 'Rampton Rivet' than in the other tetraploid cultivar 'Wakona'. Embryos were recovered from 'Rampton Rivet' × 'Seneca 60' from water-treated ovaries, though these were too small to germinate. Further work should investigate if the better ability of 'Rampton Rivet' to retain embryos resulting from maize pollination can be transferred to other tetraploid wheat genotypes.

The environment also influenced embryo survival in a genotypically-dependent manner. Thus embryo recovery was significantly better from 'Rampton Rivet' grown in the growth room at 20 °C than in the glasshouse. However, there was no significant environmental effect on embryo recovery in the two other crosses. Nevertheless, manipulating the environment may also improve embryo recovery in some genotypes.

(2) Embryo germination. Embryo germination differed significantly between crosses. The highest levels of germination obtained for 'Rampton Rivet', 'Wakona' and 'Chinese Spring' were 42.1%, 60.0% and 100.0% respectively. Embryos from 'Wakona' × 'Seneca 60' were small and mostly undifferentiated. Perhaps harvesting the embryos later, and/or increasing the temperature at which the plants are grown, could improve germination. The fact that embryo germination was significantly better for this cross from the glasshouse environment indicates that a higher temperature may favour embryo development and therefore germination. The problem with germination of 'Rampton Rivet' haploid embryos is believed to be different, as embryos from this cross were mostly large and well differentiated. Perhaps, they had started undergoing physiological

changes leading to dormancy. If so, harvesting these earlier may improve germination. Similarly, embryos from *T. aestivum* cv 'Fokuho-komugi' × maize harvested 10–12 days after pollination had better germination than embryos harvested at 14 days (Suenaga and Nakajima 1989).

(3) Production of fertile doubled haploids. Though 'Rampton Rivet' and 'Chinese Spring' haploid seedlings were efficiently doubled with the colchicine treatment used here, a large proportion of the 'Wakona' seedlings did not survive the treatment and those which did, produced comparatively little seed. Moreover, 'Wakona' seedlings did not tiller much. The colchicine treatment usually killed the first tiller in all genotypes and the chances of recovery from secondary tillers in 'Wakona' were considerably reduced. If growth conditions which promote tillering could be found, the recovery of doubled haploids from this cross may be improved. Lowering the colchicine concentration used or using alternative compounds such as nitrous oxide (Kihara and Tsunewaki 1960) may also help.

General conclusions

The recovery of haploids and from these, fertile doubled haploids of durum wheats achieved in this study is a very significant development as previous attempts were all largely unsuccessful. Sharma et al. (1982) obtained up to 6.3% callus initiation from anther culture of the cultivar 'Cando', but were unable to regenerate plants. Zhu et al. (1980) succeeded in regenerating haploid durum wheats through anther culture, but the plants were all albino. Similarly, Hadwiger and Heberle-Bors (1986) obtained only two green plantlets from 7 325 anthers.

The bulbosum method has been of limited use for haploid production of durum wheats. O'Donoughue and Bennett (1994) obtained an average fertilization frequency of only 13.4% in crosses between tetraploid wheat genotypes and *H. bulbosum*, and the level of crossability was dependent on genotype. Observations of pollen tube growth revealed that an incompatibility reaction, similar to that produced by the *Kr* genes in hexaploid wheat, inhibited pollen tube growth in some durum wheat genotypes and was partly responsible for the low fertilization frequencies obtained.

Therefore, wide hybridization with maize represents a breakthrough for haploid production of durum wheats. The fertilization frequencies obtained by O'Donoughue and Bennett (1994) in ten tetraploid wheat genotypes crossed with maize ranged from 43 to 67% and were not significantly different. Together with the present work these results indicate that wide hybridization with maize followed by in-vivo treatment of ovaries with 2,4-D alone, or in combination with $AgNO_3$, may provide a widely-applicable method of haploid production for tetraploid wheats.

Acknowledgments L.S. O'Donoughue is grateful for funding by a Commonwealth Scholarship. The authors would also wish to thank Dr. D. A. Laurie for valuable discussions during the course of this study.

References

Abeles FB (1973) Ethylene in plant biology. Academic Press, New York

Bennici A (1986) Durum wheat (*Triticum durum* Desf.) In: Bajaj YPS (ed) Biotechnology in agriculture and forestry vol 2. Crops. Springer Verlag, Berlin, pp 89–104

Beyer EM (1976) A potent inhibitor of ethylene action in plants. Plant Physiol 58:268–271

Brewbaker JL, Emery GC (1962) Pollen radiobotany. Radiat Bot 1: 101–154

Britten EJ (1950) Natural and induced parthenocarpy in maize and its relation to hormone production by the developing seed. Am J Bot 37:345–352

Comeau A, Plourde A, St Pierre CA, Nadeau P (1988) Production of doubled haploid wheat lines by wheat × maize hybridization (abstract). Genome 30, Suppl 1:482

Davies KM, Hobson GE, Grierson D (1988) Silver ions inhibit the ethylene-stimulated production of ripening related mRNAs in tomato. Plant Cell Environ 11:729–738

Hadwiger MA, Heberle-Bors E (1986) Pollen plant production in *Triticum turgidum* ssp. *durum*. In: Horn W, Jensen CJ, Odenbach W, Schieder O (eds) Genetic manipulation in plant breeding. Proc Int Symp organized by EUCARPIA, 1985. Walter de Gruyter, Berlin, pp 303–305

Hsam SLK, Zeller FJ (1993) Haploid production in durum wheat by the interaction of *Aegilops kotschyi* cytoplasm and the 1BL/1RS chromosomal interchange. Theor Appl Genet 86: 951–954

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

Kihara H, Tsunewaki K (1960) Production of polyploid wheat by nitrous oxide. Proc Jpn Acad 36:658–668

Laurie DA (1989) Factors affecting fertilization frequency in crosses of *Triticum aestivum* cv 'Highbury' × *Zea mays* cv 'Seneca 60'. Plant Breed 103:133–140

Laurie DA, Bennett MD (1986) Wheat × maize hybridization. Can J Genet Cytol 28:313–316

Laurie DA, Bennett MD (1987) The effect of the crossability loci *Kr1* and *Kr2* on fertilization frequency in hexaploid wheat × maize crosses. Theor Appl Genet 73:403–409

Laurie DA, Bennett MD (1988a) The production of haploid wheat plants from wheat × maize crosses. Theor Appl Genet 70: 100–105

Laurie DA, Bennett MD (1988b) Chromosome behaviour in wheat × maize, wheat × sorghum and barley × maize crosses. In: Brandham PE (ed) Kew Chromosome Conference III, proceedings of the third chromosome conference 1987. Her Majesty's Stationary Office, London, pp 167–177

Laurie DA, O'Donoughue LS, Bennett MD (1990) Wheat × maize and other sexual wide-hybrids: their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Gene manipulation in plant improvement II. Plenum Press, New York, pp 400–500

Marshall DR, Molnàr-Làng M, Ellison FW (1983) Effects of 2.4-D on parthenocarpy and cross-compatibility in wheat. Cereal Res Commun 11:213–218

Mathias RJ, Boyd LA (1988) The growth in culture of detached ovaries of wheat (*Triticum aestivum* L. em. Thell.) Plant Breed 100: 143–146

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

Norstog KJ (1973) New synthetic medium for the culture of premature barley embryos. In Vitro 8:307–308

O'Donoughue LS (1990) Chromosome behaviour and reproductive physiology in cereal wide-crosses, PhD thesis, Cambridge University, UK

O'Donoughue LS, Bennett MD (1994) Comparative responses of tetraploid wheats pollinated with *Zea mays* L. and *Hordeum bulbosum* L. Theor Appl Genet 87:673–680

Pienaar R de V, van Niekerk HA (1973) The effect of gamma-irradiation on wheat gametophytes. In: Sears ER, Sears LMS (eds) Proc 4th Int Wheat Genet Symp. University of Missouri, Columbia, pp 289–294

Riera-Lizarazu O, Mujeeb-Kazi A (1990) Maize (*Zea mays* L.)-mediated wheat (*Triticum aestivum* L.) polyhaploid production using various crossing methods. Cereal Res Commun 18:339–345

Rogers OM (1973) Growth regulator induction of parthenocarpy in maize. Maize Genet Coop Newslett 47:157–159

Romani RJ, Hess BM, Leslie CA (1989) Salicylic acid inhibition of ethylene production by apple discs and other plant tissues. J Plant Growth Regul 8:63–69

Salisbury FB, Ross CW (1978) Plant physiology, 2nd edn. Wadsworth Publishing Company, Belmont

Sharma GC, Wang WC, Sapra VT (1982) Effect of genotype, media and temperature pretreatment on callus initiation in triticale, wheat and rye anther culture. Cereal Res Commun 10:143–150

Sitch LA, Snape JW (1986) Doubled haploid production in winter wheat and triticale genotypes. Euphytica 35:1051–1054

Snape JW (1989) Doubled haploid breeding: theoretical basis and practical applications. In: Mujeeb-Kazi A, Sitch LA (eds) Review of advances in plant biotechnology 1985-88. 2nd Int Symp on genetic manipulation in crops. CIMMYT and IRRI, Mexico and Manila, pp 19–31

Snape JW, Simpson E (1981) Uses of doubled haploid lines for genetical analysis. In: Asher et al. (eds) Barley genetics IV. Proc 4th Int Barley Genet Symp. Edinburgh University Press, Edinburgh, pp 704–709

Snape JW, Chapman V, Moss J, Blanchard CE, Miller TE (1979) The crossabilities of wheat varieties with *Hordeum bulbosum*. Heredity 42:291–298

Suenaga K, Nakajima K (1989) Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). Plant Cell Rep 8:263–266

Zhu ZQ, Wang JJ, Sun JS (1980) The induction of albino pollen plants and preliminary observation of their ploidy in *Triticum durum* Desf. In: Davies DR, Hopwood DA (eds) The plant genome. 4th John Innes Symp and 2nd Int Haploid Conf. The John Innes Charity, Norwich, pp 254–255