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Production of haploid plants from ten hybrids of bread wheat (*Triticum aestivum* L.) through wide hybridization with maize (*Zea mays* L.)

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SUMMARY

The aim of this work was to obtain haploid plants of bread wheat through wide hybridization with maize. The experimental material included ten bread wheat hybrids (female parent) and one population of maize (pollen donor). Two assays were carried out in two different seasons (summer and winter). Wheat spikes were manually emasculated, each spike was pollinated twice with fresh pollen of maize and a solution of 2,4-D (100 mg l^{-1}) was sprayed on pollinated florets and injected in the upper internode. Fifteen and 21 days after pollination caryopses were removed and surface sterilized. Embryos were cultured in tubes containing B_5 medium. The ten hybrid combinations produced caryopses, but only eight of these hybrids produced embryos and, in six of them, the recovered embryos developed into haploid plantlets. The results showed that there is genotypic influence of the wheat parents on the percentage of haploid embryo formation, in accordance with the results obtained by other authors. Regardless of the genotype, the sowing season and the harvest date, 69.4% of the pollinated flowers gave place to the formation of caryopses, 5.5% of these caryopses developed into presumably haploid embryos (for their morphological phenotypes) and 26.1 % of the recovered embryos developed into haploid plantlets.

Key words:bread wheat, haploid plants, wide hybridization, maize pollen

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RESUMEN

El objetivo del presente trabajo fue obtener plantas haploides de trigo para pan mediante hibridación interespecífica con maíz. Se utilizaron 10 híbridos de trigo para pan (madre) y una población de maíz (donante de polen); se llevaron a cabo dos ensayos en distintas estaciones de cultivo. Cada espiga de trigo fue emasculada manualmente y polinizada dos veces con polen fresco de maíz; las flores polinizadas se pulverizaron con una solución de 2,4-D (100 mg l^{-1}),

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la que también se inyectó en la base de la espiga. Las semillas se cosecharon a los 15 y 21 días posteriores a la polinización. Los embriones recuperados se colocaron en tubos conteniendo medio de cultivo B_5. Las 10 combinaciones híbridas produjeron semillas, de ocho de los híbridos se recuperaron embriones y en seis de ellos los embriones desarrollaron plantas haploides. Estos resultados muestran que existe influencia del genotipo del trigo sobre el porcentaje de formación de embriones haploides. Independientemente del genotipo materno, la estación de cultivo y la edad de los embriones recuperados, 69,4% de las flores polinizadas formaron caryopses, 5,5% de los caryopses formados desarrollaron embriones presuntamente haploides (por sus fenotipos morfológicos) y 26,1% de los embriones recuperados desarrollaron plantas haploides.

Palabras clave: trigo para pan, plantas haploides, hibridación interespecífica, polen de maíz

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ABBREVIATIONS

DH: Doubled haploid
2,4-D: diclorofenoxiacetic acid
Hyb: hybrid combination
Cl: Chlorine
CC: Cooperación Calquín variety
PI-E: ProINTA Elite variety
DE-I: Don Ernesto INTA variety
PI-F: ProINTA Federal variety
PI-IV: ProINTA Isla Verde variety

INTRODUCTION

The use of doubled haploids (DH) improves the efficiency of cultivar development because it allows to reduce the time required to achieve homozygosis in breeding lines (Viscarra Torrico, 2001; Polci *et al*., 2005); besides, DH are helpful tools in genetic and molecular studies (Picca & Cardone, 2004). An essential step towards developing DH lines is the production of haploid plants. In cereals, haploid plants can be mainly obtained by *in vitro* anther culture or wide hybridization; both techniques have the advantage of allowing the development of completely homozygous lines from heterozygous parental lines in a single generation (Riera-Lizarazu *et al*., 1992; Lefebvre & Devaux, 1996; Bistch *et al*., 1998; Verma *et al*., 1999; Mehtá & Angra, 2000; Viscarra Torrico, 2001; Jobet *et al*., 2003; Chaudhary *et al*., 2005)

The wide hybridization process leading to haploid recovery was first reported by Kasha & Kao (1970), who observed that crosses between barley (*Hordeum vulgare*) x *Hordeum bulbosum* leaded to egg fertilization and subsequent chromosome elimination of *H. bulbosum* during the initial stages of embryo development. Using *in vitro* culture techniques, they could recover those embryos and obtain barley haploid plants. In 1975, Barclay demonstrated that this method was also applicable to wheat, but the effect of the Kr1 and Kr2 crossability genes of wheat restricted the use of the bulbosum technique only to those wheat genotypes with recessive alleles at the Kr loci (Snape, 1989). In 1988, Laurie & Bennett found that it was possible to regenerate haploid plants from wheat x maize crosses; moreover, maize fertilization has shown to be relatively insensitive to the action of dominant Kr1 and Kr2 alleles, being the wide hybridization with maize also applicable in oat, triticale, barley and rye. Although the wide hybridization technique allows to obtain a maximum of one haploid embryo per floret, it results more efficient and less genotype-dependent than other haploid production methods (i. e. anther culture) in cereals.

Wheat haploid plant production by hybridization with maize pollen has been widely used in many countries; in fact, by applying this technique haploid plants were successfully obtained in bread wheat (*Triticum aestivum* L.) (Riera-Lizarazu *et al*., 1992; Matzk & Mahn, 1994; Lefebvre & Devaux,

1996; Suenaga *et al.*, 1997; Bistch *et al.*, 1998; Verma *et al.,* 1999; Mehtá & Angra, 2000; Martins-Lopes *et al.*, 2001; Jobet *et al.*, 2003; Biesaga-Koscielniak *et al.*, 2003; Kaushik *et al.,* 2004; Sirohi *et al.*, 2004; Chaudhary *et al.*, 2005) as well as durum wheat (*Triticum durum* L.) (O`Donoughue & Bennett, 1994; Cherkaoui *et al.*, 2000; Garcia-Llamas *et al.*, 2004). In Argentina there are only a few reports about wheat haploid production (Viscarra Torrico, 2001; Polci *et al.*, 2005).

Taking into account the importance of the cultivation of wheat for human consumption as well as the possibility of reducing the time required for obtaining new cultivars, the aim of this work was to obtain haploid plants of bread wheat through wide hybridization with maize from ten F_1 hybrids derived from crosses between commercial varieties with excellent yield potential and good industrial quality, developed in Argentina.

MATERIALS AND METHODS

This work was developed in the Laboratorio de Biotecnología Vegetal, Facultad de Ciencias Agropecuarias – Universidad Nacional de Córdoba (Argentina).

The experimental plant material included ten bread wheat hybrids, derived from crosses between commercial varieties chosen for their excellent yield potential and good industrial quality, developed in Argentina (Table 1), and one population of maize with a 90 days cycle (selected from a maize seed bulk provided by Ing. Carlos Biasutti, Facultad de Ciencias Agropecuarias – UNC)

Two assays were carried out in two different seasons. The summer assay was sown in December 2004 and both species were grown under field conditions. The winter assay was sown in May 2005; while maize was grown in a greenhouse, wheat was grown under field conditions. In both assays, the wheat spikes were manually emasculated 1-2 days before anthesis. Each spike was pollinated twice on successive days (two and three days after emasculation) with fresh pollen of maize, collected right before pollination. The day after the second pollination, a solution of 2.4-D (100 mg l^{-1}) was sprayed on the pollinated florets and an injection of the same solution was applied in the upper internode, sealing the holes with vaseline to avoid the leakage of the 2.4-D solution. In order to verify that the recovered embryos were not a product of selfing or the parthenogenetic division of egg cells, 2 spikes were pollinated and they not received 2.4-D solution treatment, while 2 spikes received the hormone treatment without being pollinated. Fifteen and 21 days after pollination, caryopses were removed from the maize-pollinated spikes, surface sterilized with a household bleach solution ([Cl] 16.5 g l^{-1}) during 15 minutes and rinsed three times with sterile distilled water. Embryos were dissected out from the sterilized caryopses and cultured in tubes containing Gamborg`s B_5 medium (Gamborg *et al.*, 1968) supplemented with sucrose (20 g l^{-1}), without growth regulators. Excised embryos were first incubated in the dark for 7 days at 4 °C and then at 20-25 °C until germination. When coleoptiles reached 1 cm long, they were transferred to 16/8 hours photoperiod. Since they were removed from the caryopses, all recovered embryos were placed on fresh medium every 2 weeks. The variables measured were: number of pollinated spikes, number of pollinated flowers, number of caryopses formed, number of embryos recovered and number of haploid seedlings (according to morphological phenotypes). From these data, the percentage of caryopses formed (N° caryopses/N° flowers pollinated*100), the percentage of embryos induced (N° embryo/N° seed formed *100) and the percentage of haploid plant produced (N° seedling haploid/N° embryo induced *100) were estimated.

Finally, in order to verify the ploidy level of seedlings, root tips of each seedling were cut and pretreated with an aqueous saturated solution of p-dichlorobenzene (PDB) for 3 hours at room temper-

Table 1 Bread wheat hybrids used as female parent in controlled wheat x maized crosses

Assay	Hybrid Material
Summer Assay (Dec. 2004)	Hyb-1 (PI-E x CC); Hyb-2 (DE-I x CC); Hyb-3 (DE-I x PI-E); Hyb-4 (PI-F x PI-E); Hyb-5 (PI-IV x CC); Hyb-6 (PI-IV x DE-I)
Winter Assay (May 2005)	Hyb-7: (PI-E x CC) x (DE-I x CC) Hyb-8 [(PI-E x CC) x (CC x PI-F)] x [(PI-E x CC) x (PI-IV x PI-E)] Hyb-9 [(DE-I x CC) x (CC x PI-F)] x [(DE-I x CC) x (PI-IV x PI-E)] Hyb-10 [(CC x PI-F) x (PI-IV x PI-E)] x [(PI-E x CC) x (PI-IV x PI-E)]

CC: Cooperación Calquín, PI-E: ProINTA Elite, DE-I: Don Ernesto INTA, PI-F: ProINTA Federal, PI-IV: ProINTA Isla Verde

ature, fixed in Farmer solution (3 ethylic alcohol:1 acetic acid) for 24 hours and preserved in 70% ethanol at 4° C until use. Squashes were made with material previously hydrolyzed in 1N HCl for 1 hour at room temperature, then washed with distilled water for 10 minutes and stained with Schiff's reagent for 2 hours in dark at room temperature (Matzke *et al*., 1994).

Statistical Analysis: the data were analyzed using the InfoStat software (InfoStat, 2009). In order to determine whether the observed differences among hybrids and harvest date (regarding to the percentage of caryopses formed, embryos induced and haploid plant produced) were significant or not, the data were statistically analyzed using logistic regression and contingency test, respectively.

RESULTS AND DISCUSSION

As a result of wheat x maize crosses, caryopses with a watery endosperm were formed in both assays. The haploid embryos, when present, were small compared with those of normal wheat caryopses.

Summer Assay – From 58 pollinated spikes, 58 embryos were obtained (1 embryo per spike). The six hybrid combinations produced caryopses with frequencies ranging from 65.5 to 79.3% (Table 2a), but only four of these F_1 hybrids produced embryos. The percentage of induced embryos ranged from 1 to 8.4% and the rate of those embryos that developed into haploid plantlets ranged from 15.4 to 28.6% depending on the genotype. The logistic regression test showed that the differences observed among the hybrids were significant regarding to the percentage of caryopses formed and embryos induced, hybrid 1 being the one with the highest percentage of caryopses and embryos formed. These results confirm that there is a genotypic influence of the wheat parents on the percentage of haploid embryo formation, in accordance with the results obtained by Suenaga *et al*. (1997) and Sirohi *et al.* (2004), who also observed differences between wheat genotypes in the percentage of embryo induction.

According to the contingency test, and considering only the rescue date (Table 2b), the percentage of caryopses recovered was significantly higher when spikes were harvested 21 days after pollination (76.7%) than they were harvested at day 15 (70.6%); however the percentage of induced embryos was significantly higher at day 15 (6.5%) than at day 21 (2.7%) because at the latter many times caryopses were empty. No significant differences were observed regarding to the percentage of induced embryos that developed into haploid plantlets, which was almost the same for both rescue dates. These results show that the time from pollination to embryo rescue seems to be a key stage in the recovery of haploid embryos. In this regard, several authors support that the right age for embryo rescue varies from one genotype to another and needs to be standardized in each particular case. Cherkaoui *et al*. (2000) found that the optimal

Table 2: Number of pollinated florets and percentages of caryopses, embryos and presumably haploid plantlets obtained in six hybrid combinations (a) and two embryo rescue dates (b); summer assay (2004).

a) Genotypes

Wheat hybrids	Pollinated florets (nº)	Caryopses obtained (%)	Induced embryos (%)	Haploid plantlets** (%)
Hyb 1	392	79.3 c	8.4 d	15.4 b
Hyb 2	667	73.2 b	2.9 b	28.6 b
Hyb 3	340	74.7 b	6.7 c	23.5 b
Hyb 4	46	71.7 b	0.0 a	00.0 a
Hyb 5	194	65.5 a	0.0 a	00.0 a
Hyb 6	136	70.6 b	1.0 b	00.0 a
Total	1775	73.7	4.4	20.7

b) Embryo rescue date

DAP†	Pollinated florets (nº)	Caryopses obtained (%)	Induced embryos (%)	Haploid plantlets** (%)
15	849	70.6 a	6.5 b	20.5 a
21	926	76.7 b	2.7 a	21.1 a
Total	1775	73.7	4.4	20,7

Hyb-1 (PI-E x CC); Hyb-2 (DE-I x CC); Hyb-3 (DE-I x PI-E); Hyb-4 (PI-F x PI-E);Hyb-5 (PI-IV x CC); Hyb-6 (PI-IV x DE-I);
PI-E: ProInta Elite, CC: Cooperación Calquín, DE-I: Don Ernesto Inta, PI-F: ProInta Federal, PI-IV: ProInta Isla Verde.
** base on morphological phenotypes
†Days Alter Pollination
Different letters indicate significant differences (p <= 0.05)

time for embryo rescue was 12-15 days, while for Kaushik *et al.* (2004) the optimum time of rescue was 17-19 days after pollination. In this first assay, under our experimental conditions, the right time for embryo rescue (the one that allows recovering of the higher percentage of embryos) was 15 days after pollination. It is also important to note that most of the investigations about wide hybridization were carried out in greenhouses under controlled environmental conditions. Our work was conducted under field conditions and, in this first assay, out of the wheat growing season. The low percentage of induced embryos observed, compared with those observed in other studies, may be attributed to this fact (Sirohi *et al.*, 2004).

Winter Assay - From 31 pollinated spikes 57 induced embryos were recovered (approximately 2 embryos per spike). Four of the hybrid combinations produced caryopses and embryos. The frequencies of seed production ranged between 81.2% and 92.3% but no significant differences were observed between the hybrids, while the percentage of recovered embryos was significantly different among the hybrids, ranged between 1.4 and 7.7% (Table 3a) which demonstrates once again the influence of the maternal genotype (wheat) on the formation of haploid embryos. Taking into account only the days from the pollination up to the rescue of the embryos, the percentage of caryopses recovered was signicantly higher in spikes were harvested 15 days after pollination (86.8%) than those harvested at day 21 (80.1%); but in this second assay the percentage of induced embryos was signicantly higher at day 22 (9.5%) than at day 15 (3.2%) (Table 3b), in contrast with the results of the summer assay.

Analyzing summer and winter assays together, without considering either genotypes or days elapsed from the pollination up to the rescue of the embryos, both contingency and logistic regression tests showed that the percentage of caryopses obtained was significantly higher when the assay was conducted during the winter season (Table 4). Regarding to the percentage of embryos recovered and haploid plantlets regenerated, no differences were observed between summer and winter, but the percentage of haploid seedlings was 33.3% higher in the winter assay. It is well known that some environmental factors, such as the temperature, influence the frequency of haploid embryos formation, affecting the egg fertilization and the embryonic development (Matzk & Mahn, 1994; Viscarra Torrico, 2001); as a consequence the thermal fluctuations reduce notably the percentage of haploid embryos recovered (Riera-Lizarazu & Mujeeb-Kazi, 1990). Moreover, in the winter assay the wheat plants grew and developed in their natural growing

Table 4: Percentages of caryopses, embryos and haploid plantlets obtained in for two assays (summer and winter)

Assay	Caryopses obtained (%)	Induced embryos (%)	Haploid plantlets (%)
Summer	73.6 a	4.4 a	20.7 a
Winter	84.8 b	5.3 a	31.6 a

*days passed from the first pollination.

Table 3–Number of pollinated florets and percentages of caryopses, embryos and presumably haploid plantlets obtained over all four hybrid combinations (a) and two rescue dates (b), winter assay (2005)

a) Genotypes

Wheat hybrids	Pollinated florets (nº)	Caryopses obtained (%)	Induced embryos (%)	Haploid plantlets** (%)
Hyb-7	366	81.2 a	3.4 b	20.0 b
Hyb-8	200	84.5 a	3.0 b	40.0 b
Hyb-9	78	92.3 a	1.4 a	00.0 a
Hyb-10	618	85.8 a	7.7 c	34.1 b
Total	1262	84.8	5.3	31.6

b) Embryo rescue date

DAP†	Pollinated florets (nº)	Caryopses obtained (%)	Induced embryos (%)	Haploid plantlets** (%)
15	817	86.8 b	3.2 a	39.1 a
21	448	80.1 a	9.5 b	26.5 a
Total	1262	84.6	5.3	31.6

Hyb-7: (PI-E x CC) x (DE-I x CC);
Hyb-8 [(PI-E x CC) x (CC x PI-F)] x [(PI-E x CC) x (PI-IV x PI-E)];
Hyb-9 [(DE-I x CC) x (CC x PI-F)] x [(DE-I x CC) x (PI-IV x PI-E)];
Hyb-10 [(CC x PI-F) x (PI-IV x PI-E)] x [(PI-E x CC) x (PI-IV x PI-E)]
PI-E: ProInta Elite, CC: Cooperación Calquín, DE-I: Don Ernesto Inta, PI-F: ProInta Federal, PI-IV: ProInta Isla Verde.
Different letters indicate significant differences (p <= 0.05)
** base on morphological phenotypes
†Days Alter Pollination

season while in summer they were exposed to environmental stressful conditions (mainly high temperatures). Thus the lower efficiency observed in the summer assay can be attributed to the stress caused by the high temperatures registered.

With respect to the chromosome number, there were no suitable metaphase plates for chromosome counting; the few cells in division had chromosomes in agglomeration and/or in different planes, difficulting the counting. Therefore, it was not possible to determine the chromosome number of the seedlings. However, morphological characteristics, such as small grain size and the absence of a well-developed endosperm, allow us to identify haploid embryos and plantlets derived from them, even when it is not possible to perform chromosome counts. Both characteristics were observed in all harvested caryopses from which embryos were recovered. These results agree with those obtained by Viscarra Torrico (2001), who confirmed the haploid chromosome number in bread wheat seedlings from small caryopsis without endosperm.

In synthesis, by means of the application of the technology of interspecific hybridization the obtainment of presumably haploid plants was achieved successfully in 6 of the 10 hybrids of bread wheat that were used in the crossing experiment. Regardless of the sowing season, the genotypes and the harvest dates, 69.4% over 3037 pollinated flowers gave place to the formation of caryopses, 5.4% of which developed into embryos and 26.1 % of the recovered embryos developed into presumably haploid plantlets. In agreement to the the bibliography, the range of obtained caryopses ranged between 8.4 and 94.8% (Mehtá & Angra, 2000; Martins-Lopes *et al.*, 2001), whereas the ranges of recovery of embryos and haploid plantlets ranged among 6.5 – 45.2% (Sirohi *et al*, 2004; Suenaga *et al.*, 1997) and 23.3 – 83.6% (Polci *et al.*, 2005; Suenaga *et al.*, 1997) respectively.

With regard to the percentage of haploid plants regeneration (calculated over all pollinated flowers) the range observed for bread wheat ranged among 0.3 and 10.1% (Riera-Lizarazu *et al.*, 1992; Lefebvre & Devaux, 1996; Bistch *et al.*, 1998; Verma *et al.*, 1999; Mehtá & Angra, 2000; Jobet *et al.*, 2003; Biesaga-Koscielniak *et al.*, 2003; Kaushik *et al.*, 2004; Chaudhary *et al.*, 2005; Polci *et al.*, 2005) being in our work of 1%.

Taking into account that our assays were carried out under field conditions, both in summer and in winter season, it is important bear this information in mind at the moment of projecting results, that is to say depending on the season in which the material is cultivated it will be possible to programme the crop in such a way to assure the major rate of recovery of embryos.

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