International Journal of Plant Breeding ©2011 Global Science Books

Effect of Different Factors on Haploid Production through Embryo Rescue in Durum Wheat × Maize Crosses

Ayed Sourour[1*] • Slama-Ayed Olfa[1] • Jaime A. Teixeira da Silva[2] • Slim-Amara Hajer[1]

[1] Genetic and Plant Breeding Laboratory, Department of Agronomy and Biotechnology, National Agronomic Institute of Tunisia, 43, Avenue Charles Nicole, 1082 Tunis, Tunisia
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Ikenobe, Miki-cho, 761-0795, Japan

Corresponding author: ***ayedsourour@yahoo.fr**

ABSTRACT

Even though bread wheat haploids are being routinely produced by wheat × maize crosses, an efficient protocol for haploid production has been difficult to achieve in durum wheat. The objective of this study was to analyse the influence of various experimental factors on the production of embryos and haploid plants in durum wheat crossed with maize. Four Tunisian durum wheat genotypes (female parent), consisting of two local cultivars ('Jenah khotifa' and 'Biskri') and two improved varieties ('Karim' and 'Razzek'), were crossed with a maize genotype (Pioneer 37Y15) (male parent). After pollination, plant stems were either maintained *in situ* or cut 2-3 days after pollination near the base and kept in a solution of 2,4-dichlorophenoxyacetic acid (100 mg/l), sucrose (40 g/l) and sulphurous acid (8 ml/l). Twelve, 14, 16, 18, 20 and 22 days after pollination, embryos were excised from developed ovaries and cultured on either Murashige and Skoog (MS), MS/2 or Gamborg's (B5) media. By cutting the plant stems after pollination, better results in terms of developed ovaries (65.55%), embryos (13.62%) and regenerated haploid plants (10.50%) were obtained than if *in situ* plants (46.65, 10.85 and 7.71%) were used. The optimal stage for embryo rescue was 18 days for haploid plant formation (14.4%) while B5 medium resulted in significantly better haploid plant (13.17%) production than MS and MS/2 (6.92, 10.87%) following embryo culture.

Keywords: culture medium, durum wheat, embryo rescue, haploid plant regeneration, maize
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid

INTRODUCTION

The production of haploid-double haploids in self-pollinating crops such as wheat has proven to be a highly valuable tool for plant breeding, allowing the release of a high number of new cultivars. It also offers the quickest method for developing homozygous breeding lines within a few months.

Haploid wheat plants can be obtained from male or female gametic cells, including anther or isolated microspore culture and gynogenesis. However, low regeneration rate, high frequency of albino plants and dependence on the wheat genotype hinder the application of these techniques (Cistué *et al.* 2006; Slama-Ayed *et al.* 2010). Likewise, the crossability of *Triticum aestivum* x *Hordeum bulbosum* depends on the wheat allelic composition for the *Kr* genes responsible for the incompatibility between these two species because crossing wheat × *H. bulbosum*, as a method for haploid production, is confined to *Kr*-bearing varieties (Sitch and Snape 1987).

During the last 15 years the maize method emerged as an efficient technique for producing haploid wheat plants because no recalcitrant genotypes have been reported and thus avoiding the problem of albinism because all the haploids obtained are green (Laurie and Reymondie 1991; Amrani *et al*. 1993; Sarrafi *et al*. 1994). Laurie and Bennett (1988) cytologically examined embryos produced via this system and found that maize chromosomes are eliminated within the first few cell cycles following fertilization. As a result, the endosperm is absent and embryos fail to survive on the plant, thus artificial rescue of embryos is needed for the development of haploid plants (Laurie and Bennett 1989). Several investigations of haploid bread wheat production through maize × wheat crosses have since been reported (Riera-Lizarazu and Mujeeb-Kazi 1990; Laurie and Reymondie 1991; Kisana *et al*. 1993 Matzk and Mahn 1994; Ushiyama *et al.* 2007). However, similar success has not been achieved for durum wheat cultivars because the efficiency of haploid production is affected by many factors such as genotype, embryo rescue stage, nutritional and hormonal composition of culture medium (Almouslem *et al.* 1998; Cherkaoui *et al*. 2000).

In order to achieve an efficient protocol for *en-masse* haploid production, two experimental procedures (hybridization *in situ* and detached tillers) were compared in an attempt to improve responses in embryos and in haploid plant formation. The optimal stage and medium for embryo rescue were studied.

MATERIALS AND METHODS

Donor plants and growth conditions

Four genotypes of durum wheat (*Triticum turgidum* ssp. Durum) were pollinated with a maize (*Zea mays* 2n=2x=20) genotype (Pioneer 37Y15) previously selected for its high response to wheat × maize crosses. Wheat was sown on two dates, first at the beginning of October 2010 and second, 15 days later. Maize was grown in 25-cm (diameter) clay pots at a density of 3 plants/pot in an unconditioned greenhouse at temperatures slightly warmer than ambient within the range of 20-30°C from January to April (2009/2010). Maize plants were fertilized once a week with water-soluble 20: 20: 20 NPK and irrigated daily with water. In order to obtain fresh maize pollen during the crossing period, maize genotypes were seeded every 10 days for 5 weeks starting 2 weeks before wheat was sown.

Emasculation and pollination of wheat spikes

Wheat flowers were emasculated 2-3 days before anthesis and, as

Received: 29 January, 2011. Accepted: 21 March, 2011.

Original Research Paper

Table 1 The tree different durum wheat embryo regeneration media used.

Media components	B5 (mg/l)	MS (mg/l)	MS/2 (mg/l)
Macro salts			
KNO_3 (potassium nitrate, Sigma)	2500	1900	950
$CaCl_2 \cdot 2H_2O$ (calcium chloride dihydride, Fisher Scientific Co., UK)	150	440	220
$MgSO_4 \cdot 7H_2O$ (magnesium sulfate, Chemi-Pharma, UK)	250	0	0
$(NH_4)_2 \cdot SO_4$(ammonium sulfate, Sigma)	134	0	0
$NH_4H_2PO_4$(ammonium sulfate monobasic, sigma)	150	0	0
NH_4NO_3 (ammonium nitrate, Sigma)	0	1650	825
Micro salts			
KI (potassium iodide, Sigma)	0.75	0.828	0.828
H_3BO_3 (boric acid, Fisher Scientific Co.)	3	6	6
$MnSO_4 \cdot 4H_2O$ (manganese sulfate, Fluka, UK)	10	16.88	16.88
$ZnSO_4 \cdot 4H_2O$ (zinc sulfate, Brix Worth-Northants, UK)	2	8.6	8.6
$Na_2MoO_4 \cdot 5H_2O$ (sodium molybdate, Siegfried Handel, UK)	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$ (copper sulfate (cryst pure, PARK, UK)	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$ (cobalt chloride, Fisher Scientific Co.)	0.025	0.025	0.025
Iron source			
Na_2 EDTA (ethylenediamine-tetraacetic acid, Sigma)	37.3	37.2	37.2
$FeSO_4 \cdot 7H_2O$ (ferric sulfate, NTL, UK)	27.8	27.8	27.8
Vitamins			
Nicotinic acid (Sigma)	1	0	0
Thiamine hydrochloride (Sigma)	10	0.5	0.5
Pyridoxine hydrochloride (Sigma)	1	0	0
Myo-inositol (Sigma)	100	0	0
Hormones			
2,4-D (Sigma-Aldrich)	0	0-1	0-1
Amino acid			
L-Asparagine (Sigma)	0	150	150
Others components			
Sucrose (Applichem, Germany)	20000	20000	20000
Agar (ICN, Biomedicals, France)	7500	6800	6800
pH	5.5	5.85	5 .85

2,4-D: 2,4-dichlorophenoxyacetic acid

soon as the stigmata became feathery, they were pollinated with fresh maize pollen. 24 hrs later, 0.5 ml of a 2,4- dichlorophenoxy-acetic acid (Sigma-Adrich) solution (100 mg/l) was injected with a syringe (20 ml) into the upper internode (Cherkaoui *et al.* 2000) and a drop of the same solution was placed on each pollinated floret.

In situ or cut plant stems

Two experimental procedures were tested:

1. *In situ* stems: The pollinated spikes remained *in situ* until embryo rescue (12 to 22 days after pollination).

2. Cut plant stems: Tillers were cut 2-3 days after pollination in the middle of the third internode and sprayed with insecticide (1 ml/l Zolone; Bayer Crop Sciences) and fungicide (2 ml/l Swing gold; BASF) solutions. Tillers with emasculated spikes were dipped in a solution of 2,4-D (100 mg/l), sucrose (40 g/l) and sulphurous acid (8 cm^3/l) (Inagaki *et al.* 1997) and kept in a growth room at about 25°C at a light intensity of 350–450 µE m^{-2} s^{-1} under a 16-h photoperiod at 80% relative humidity.

Embryo rescue and culture

Twelve to 22 days after emasculation, pollinated maize spikes were removed from plants. Caryopses were removed from these spikes and sterilized with 12% bleach for 10 min and washed 3 times with sterilized water. On a sterile working surface, embryos were removed from caryopses with the aid of a X20 stereoscope. Forceps were flamed before and between uses. Excised embryos were placed, with the scutellum in contact with the medium and the radicle and shoot apex facing up, on one of three media: MS (Murashige and Skoog 1962), MS/2 or B5 (Gamborg *et al.* 1968) supplemented with 20 g/l sucrose and 7 g/l agar (**Table 1**). Petri dishes containing embryos were maintained in the dark at about 25°C. Haploid wheat seedlings at the three-leaf stage (1-2 cm) were transferred to a bottle (3 plants/pot) with the same medium but at a light intensity of 350–450 µE m^{-2} s^{-1} under a 16-h photoperiod.

Transfer of plants to soil

Plants were transferred when they reached a height of 2-3 cm in 9-cm pots (3 plants/pot) to a mixture of sand and peat (1: 2) sterilized by autoclaving at 120°C for 20 min. To prevent plants from water stress they were covered with glass caps for about 1 week in a lighted growth room at 25°C in a 16-h photoperiod and irrigated daily with Hoagland's solution (FAO 1984) for hardening roots and shoots. When plants initiated vigorous growth and roots overgrow the soil, they were transferred into bigger pots (25 cm) with a mixture of sand and peat (1: 2). The number of plants that developed was counted one month after embryo rescue.

Data analyses

Three traits were measured:

Frequency of developed ovaries = (number of seeds set/total florets pollinated) × 100;

Frequency of embryos formed = (number of embryos formed/total florets pollinated) × 100;

Frequency of haploid plants = (total of plant regenerated/total florets pollinated) × 100.

Statistical analysis

In this study a complete randomized block design was employed. Data were analysed by analysis of variance (ANOVA) and means were compared by Fisher's least significant difference (LSD) at 5% using SPSS v. 10.0 statistical software.

RESULTS AND DISCUSSION

Comparison of *in situ* or cut plant stems

Two experimental procedures were used: either plant stems were maintained *in situ* until embryo rescue 18 days later or they were cut 2-3 days after pollination and placed in a growth room in a solution containing 2,4-D (100 mg/l), sucrose (40 g/l) and sulphurous acid (8 cm^3/l). Results showed that cut plants developed significantly more ovaries (66.35%), embryos (13.62%) and regenerated haploid plants (12.75%) than *in situ* plants (29.15, 6.35 and 5.5%) (**Fig. 1**). Best embryo formation and haploid plant yield were observed in genotype 'Jeneh Khotifa', 10.40 and 10.8% for *in situ* plants and 17.4 and 16.20% for cut plants, respectively. Cutting the plant stems resulted in higher haploid plant production because detached tillers were cultured in a growth chamber which allowed optimum control of environmental growth conditions during embryo development. However, *in situ* plants were not controlled and great variation between the number of days until the production of caryopses was observed. Cherkaoui *et al.* (2000) also demonstrated that in durum wheat × maize crosses, cut plants gave 2-3 times more embryos (18.8%) and regenerated haploid plants (8.9%) than *in situ* plants (11.7, 4.1%).

A significantly higher percentage of embryos and plants obtained by the cut plant stem procedure could also be due to the high concentration of 2,4-D in solution, which might favour embryo survival and development. Other results such as those of Riera-Lizarazu *et al.* (1992) or Inagaki

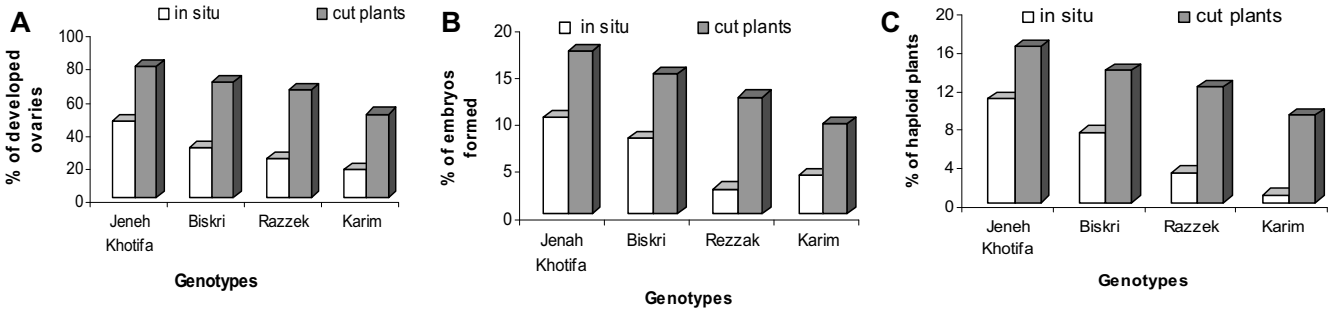

Fig. 1 Comparison of frequencies of developed ovaries, embryo production and plant haploid regeneration for *in situ* and cut plant stems in durum wheat × maize crosses. (**A**) Percentage of developed ovaries; (**B**) Percentage of embryos formed; (**C**) Percentage of haploid plants.

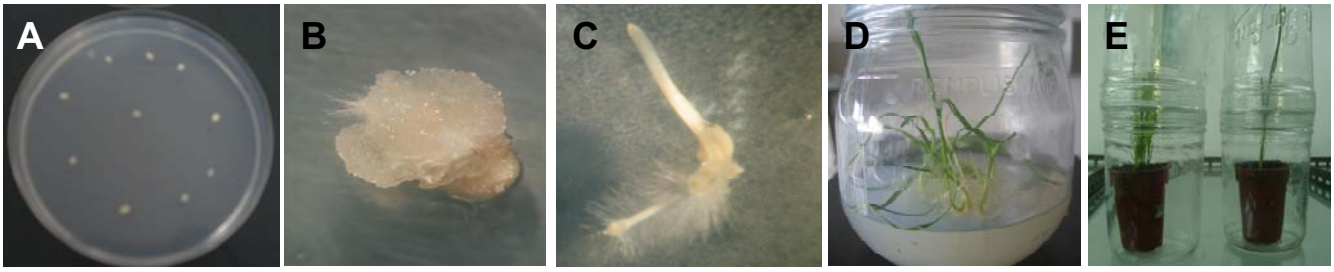

Fig. 2 Haploid durum wheat embryo and its development after durum wheat × maize cross. (**A**) Rescue of embryo in culture; (**B, C**) germinated embryo respectively after 1 and 2 weeks of culture; (**D**) haploid plant after 6 weeks; (**E**) haploid plant transferred to a pot.

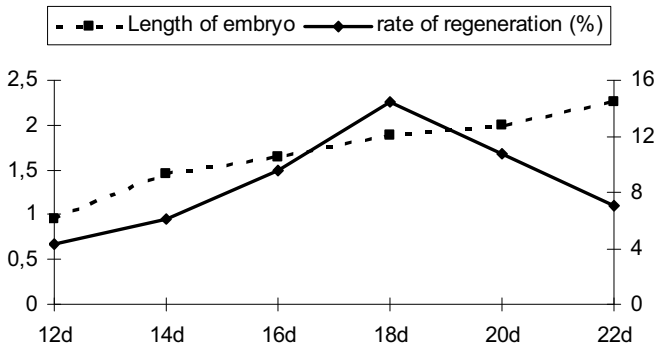

Fig. 3 Comparison of duration after pollination (12-22 days) on percentage haploid plant regeneration.

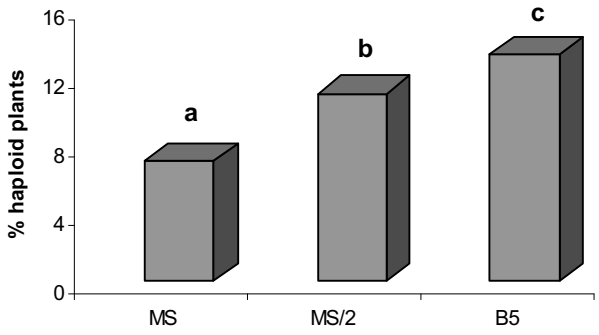

Fig. 4 Effect of embryo rescue media after 18 days (wheat genotypes grouped, cut plant stems). Values with different lower-case letters are significantly different at $P = 0.05$ according to the F-test (n = 4).

(1997) confirm the positive effect of 2,4-D on both embryo formation and plant regeneration in durum wheat × maize crosses. According to Laurie and Reymondie (1991), 2,4-D treatment can induce ovary enlargement and enhance the survival and development of haploid embryos in hexaploid wheat to a stage that enables their culture onto nutrient media.

Optimal stage for embryo rescue

In this experiment, immature durum wheat embryos obtained from crosses with maize were extracted from ovaries 12 to 22 days after pollination and were successfully regenerated to plants within 3 weeks after incubation (**Fig. 2**). The percentage of haploid embryos which subsequently germinated varied significantly according to the number of days between pollination and embryo rescue. The best yield in haploid plant formation (14.4%) was obtained at the 18-day stage (**Fig. 3**). At this stage, embryos were approximately 1.99 mm long. The dissection of caryopses 12 days after pollination showed that most of them contained a very small (< 0.9 mm) embryo. Thus, the regeneration of embryos, when placed in culture medium, was low (4.3%). Beyond 18 days, the percentage of plants was reduced over time to 7.1% at 22 days. According to Inagaki and Snape (1982) and Cherkaoui *et al.* (2000), this reduction in the percentage of plants might have been due to dehydration of the embryos and the degeneration of the endosperm.

The embryo stage of durum wheat crossed with maize selected by Sarrafi *et al.* (1994) and Saidi *et al.* (1998) was 12-15 days. However, Savaskan *et al.* (1997) reported that 18 to 21 days after pollination was the optimal stage when the embryo was well structured and vigorous. In none of these studies was 2,4-D applied.

Effect of rescue medium for embryos on plant yield

Rescued embryos were placed, 18 days after pollination, on one of three media (MS, MS/2 or B5), which varied only in salt composition and concentration. An equivalent number of embryos on B5 and MS/2 resulted in significantly more haploid plants (13.17 and 10.87%) than MS (6.92%) (**Fig. 4**). Besides, haploid embryos germinated significantly earlier on B5 than on MS/2 and MS medium without any callus phase (**Fig. 5**). These results agree with those reported by Cherkaoui *et al.* (2000), who found that B5 medium gave positive results because it contained a higher level of magnesium, but lower levels of calcium and three micronutrients (boric acid, zinc and manganese) than MS and MS/2. The superiority of MS/2 medium with half-strength macronutrients compared with MS seemed to show that 12-18 day old embryos did not need a particularly rich medium for germination to occur. Comeau *et al.* (1992) suggested that a richer medium with important amino acid component for the rescue of smaller and less differentiated embryos is

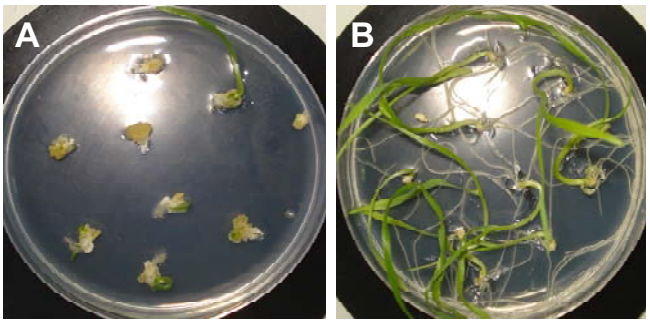

Fig. 5 Germination of durum wheat embryo after durum wheat and maize cross on MS and B5 after 3 weeks in culture. (**A**) Germination of embryo on MS medium; (**B**) germination of embryo on B5 medium.

necessary. These results contradict those reported by Kammholz *et al.* (1996), who indicated that there were no significant differences between three media (MS, MS/2 and B5) on the percentage of haploid plants in a wheat × maize cross. The differences between the two studies could be due to the different plant material used.

CONCLUSIONS

The results of this study have been useful for improving wheat × maize crosses for durum wheat haploid plant production, with a particular emphasis on the choice of embryo rescue stage and culture conditions for haploid embryo germination and growth *in vitro*. This protocol allowed us to obtain a high percentage of haploid plants (13.1%) from most of the genotypes. The efficiencies described are very promising, suggesting that this technique could be a system of choice for haploid production of durum wheat in the future.

REFERENCES

Almouslem AB, Jauhar PP, Peterson TS, Bommineni VR, Rao MB (1998) Haploid durum wheat production via hybridization with maize. *Crop Science Society of America* **38**, 1080-1087

Amrani N, Sarrafi A, Allibert G (1993) Genetic variability for haploid production in crosses between tetraploid and hexaploid wheats with maize. *Plant Breeding* **110**, 123-128

Cherkaoui S, Chlyah A, Chlyah H (2000) Durum wheat x maize crosses for haploid wheat production: Influence parental genotypes and various experimental factors. *Plant Breeding* **119**, 31-36

Cistué L, Soriano M, Castillo AM, Vallès MP, Sanz J, Echavarri MB (2006) Production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. *Plant Cell Reports* **25**, 257-264

Comeau AP, Nadeau A, Plourde R, Simard O, Maès O, Kelley S, Harper L, Lettre J, Landry B, St. Pierre CA (1992) Media for the *in ovulo* culture of proembryos of wheat and wheat-derived interspecific hybrids or haploids. *Plant Science* **81**, 117-125

FAO (1984) Fertilizer and plant nutrition guide, *Bulletin 9*, Rome, 191 pp

Gamborg OL, Eveleigh DE (1968) Culture methods and detection of glucanases in suspension cultures of wheat and barley. *Canadian Journal of Biochemistry* **46**, 417-421

Inagaki MN (1997) Technical advances in wheat haploid production using ultra-wide crosses. *JIRCAS Journal* **4**, 51-62

Inagaki MN, Nagamine T, Mujeeb-Kazi A (1997) Use of pollen storage and detached tiller culture in wheat polyhaploid production through wide crosses. *Cereal Research Communications* **25**, 7-13

Inagaki MN, Snape JW (1982) Frequencies of haploid production in Japanese wheat varieties crossed with tetraploid *Hordeum bulbosum* L. *Japanese Journal of Breeding* **32**, 41-47

Kisana NS, Nkongolo KK, Quick JS, Johnson DL (1993) Production of doubled haploids by anther culture and wheat × maize method in a wheat breeding programme. *Plant Breeding* **110**, 96-102

Laurie DA, Raymondie S (1991) Factors affecting fertilization frequency in cross of *Tritieum aestivum* cv. 'Highbury' × *Zea mays* cv. 'Seneca 60'. *Plant Breeding* **103**, 133-140

Laurie DA, Bennett MD (1988) Chromosome behaviour in wheat × maize, wheat × sorghum crosses. *Proceeding of Kew Chromosome Conference* **3**, 167-177

Laurie DA, Bennett MD (1989) The timing of chromosome elimination in hexaploid wheat × maize crosses. *Genome* **32**, 953-661

Lefebre D, Devaux P (1996) Doubled haploids of wheat from wheat × maize crosses: Genotypic influence, fertility and inheritance of the 1BL-1RS chromosomes. *Theoretical and Applied Genetics* **93**, 1267-1273

Matzk F, Mahn A (1994) Improved techniques for haploid production in wheat using chromosome elimination. *Plant Breeding* **113**, 125-129

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

Riera-Lizarazu O, Mujeeb-Kazi A (1990) Maize (*Zea mays* L.) mediated wheat (*Triticum aestivum*) polyhaploid production using various crossing methods. *Cereal Research Communications* **18**, 339-343

Riera-Lizarazu O, Mujeeb-Kazi A, William MDHM (1992) Maize (*Zea mays* L.) mediated polyhaploid production in some Triticeae using a detached tiller method. *Journal of Genetics and Breeding* **46**, 335-346

Saidi N, Chlyah O, Chlyah H (1998) Production of green haploid durum wheat plants by pollination of wheat with maize. *Canadian Journal of Botany* **76**, 652-656

Sarrafi A, Amrani N, Alibert G (1994) Haploid regeneration from tetraploid wheat using maize pollen. *Genome* **37**, 176-178

Savaskan S, Ellabrouk C, Fis LJ, Snape JW (1997) Doubled haploid production in Turkish durum wheats using crosses with maize. *Plant Breeding* **116**, 299-301

Sitch LA, Snape JW (1987) Factors affecting haploid production in wheat using the *Hordeum bulbosum* system. 1. Genotype and environmental effect on pollen grain germination, pollen tube growth and frequency of fertilization. *Euphytica* **36**, 483-496

Slama Ayed O, De Buyser J, Picard E, Trifa Y, Slim Amara H (2010) Effect of pre-treatment on isolated microspores culture ability in durum wheat (*Triticum turgidum* subsp. *durum* Desf.). *Journal of Plant Breeding and Crop Science* **2**, 30-38

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

Suenaga K, Tamaki M, Nakajima K (1991) Influence of wheat (*Triticum aestivum*) and maize (*Zea mays*) genotypes on haploid wheat production in crosses between wheat and maize. *Bulletin of the National Institute of Agrobiological Resources* **6**, 131-142

Ushiyama T, Kuwabara T, Yoshida T (2007) Effects of various phytohormones on haploid wheat production in wheat x maize crosses. *Plant Production Science* **10**, 36-41