Production of haploid wheat plants from wheat (*Triticum aestivum* L.) x maize (*Zea mays* L.) cross system

Raj K Niroula[1*], Hari P Bimb[1], Dhruva B Thapa[2], Bindeswor P Sah[1] and Sanothos Nayak[1]

[1] Biotechnology Unit, Nepal Agricultural Research Council, Khumaltar, Lalitpur, NEPAL
[2] Agriculture Botany Division, Nepal Agricultural Research Council, Khumaltar, Lalitpur, NEPAL
* For correspondence, email: rkn27st@yahoo.com

The present study was carried out taking single F_1 wheat and four maize varieties, viz. Arun-1, Arun-2, Khumal Yellow and Rampur Composite, to determine the efficiency and influence of maize genotypes on various parameters of haploid formation. Wheat spikelets were hand pollinated with freshly collected maize pollen, and 1 ml of 100 ppm 2,4-D was immediately injected on the uppermost internode. Twenty-four hours after 2,4-D injection, the cups of the florets were filled with the same solution of 2,4-D for two more consecutive days. Seventeen days after pollination, the embryos were excised and cultured in half-strength MS basal medium supplemented with 30 g/l sucrose, and 7 g/l agar. The cultured embryos were maintained at 25°C with 16/8 hours light/darkness after treating in the dark for seven days at 4°C and incubation in the dark for seven days at 25°C. Application of 2,4-D after pollination was found to be essential to the recovery of culturable size of embryos. The significant effect of maize genotypes on frequency of ovary development, embryo formation and haploid plant per pollinated floret was observed. The mean percentages of embryo formation and haploid plants per pollinated floret varied from 5.17 to 21.45 and 0.96 to 10.15, respectively, depending upon the maize varieties used. The highest frequency of embryo recovery and plant per floret was found when wheat F_1 was pollinated with Arun-2 followed by Arun-1 and Khumal Yellow. It is suggested that the production of dihaploids (DHs) in wheat can be enhanced by using more responsive maize genotypes as pollinators.

Key words: 2,4-D, caryopsis, floret, haploid embryo, wheat x maize cross

Wheat is one of the most important life-supporting cereals in Nepal and ranks third in terms of area and production. The aim of the Nepalese wheat breeding program is to produce high-yielding wheat varieties with enhanced adaptability and shorter growth period to fit into the rice-wheat cropping system. In Nepal, however, the development of a homozygous wheat cultivar can take up to 14 years. In other countries the time needed to reach homozygosity has been markedly reduced through the adoption of haplodiplodization (HD) technique (Baenziger et al. 2001). Since the discovery of haploid plants from *Datura inoxia* (Guha and Maheshwari 1964), HD based on gamete selection is considered the fastest means of cultivar development. This technique not only substantially reduces the time required to attain absolute homozygosity, but also increases many fold the selection efficiency of crop breeding (Choo et al. 1985). In conventional plant breeding, the chances of obtaining truly homozygous lines are rare and most selections contain some heterozygous loci (Baenziger et al. 2001), markedly reducing the precision of selection. For successful and cost-effective use in a breeding program, a HD system should fulfill three criteria (Snape et al. 1986): i) easy and consistent production of large numbers of dihaploids (doubled haploid from polyploid species) from all genotypes, ii) Dihaploids (DHs) should be genetically normal and stable, and iii) DHs should contain a random sample of the parental gametes.

In wheat, haploid/dihaploid plants can be produced either through anther/microspore culture (Patel et al. 2004, Liang et al. 1987) or intergeneric crossing of wheat with barley (Barclay 1975), maize (Laurie and Bennet 1988) and various other grasses belonging to the Gramineae (Pratap et al. 2005). Intergeneric crosses between wheat and maize followed by elimination of the genome of maize has been considered an efficient method for the induction of haploid zygotic embryos and subsequent haploid and dihaploid plants (Suenaga and Nakajima 1989, Inagaki 1997). The maize-mediated haploid production system for wheat has shown to be less genotype dependent and more efficient and simple than wheat x *Hordeum bulbosum* crosses (Suenaga 1994) or anther culture (Sadasivaiah et al. 1999, Bitsch et al. 1998, Kisana et al. 1993). The *Hordeum bulbosum* technique in wheat is constrained by the presence of incompatible genes (Kr_1 and Kr_2) situated on the 5A and 5B wheat chromosomes that markedly reduce the crossability between wheat and *H. bulbosum* (Falk and Kasha 1981). Nonetheless, maize pollen appears to be insensitive to

Himalayan Journal of Sciences 4(6): 65–69, 2007
Available online at www.himjsci.com
Copyright©2007 by Himalayan Association for the Advancement of Science

Table 1. Effect of four maize genotypes on ovary development, embryo formation and subsequent haploid plant regeneration

Maize genotypes	F_1 Wheat (Acc #7103/WK 1204)				
	No. of florets pollinated	No. of developed ovaries	No. of embryos formed	No. of embryos culture	Haploid plants/ florets pollinated
Khumal Yellow	116	52 (44.37) [a]	22 (18.91) [a]	15	7 (6.04) [a]
Rampur Composite	91	28 (30.65) [b]	5 (5.17) [b]	4	1 (0.96) [b]
Arun-1	102	36 (35.18) [a]	19 (18.51) [a]	16	7 (6.81) [a]
Arun-2	111	49 (44.32) [a]	24 (21.45) [a]	18	11 (10.15)[a]
Total	420	165 (38.631)	70 (16.01)	53	26 (6.00)
Coefficient of variation (%)	-	8.82	15.31	-	25.22

Figures in parentheses indicate the original mean percentage of well-developed ovaries (ovaries/pollinated floret), embryo formation (embryos/pollinated floret) and haploid plant formation (seedlings/pollinated florets). Original means within parentheses followed by the same letter are not significantly different at α =0.05

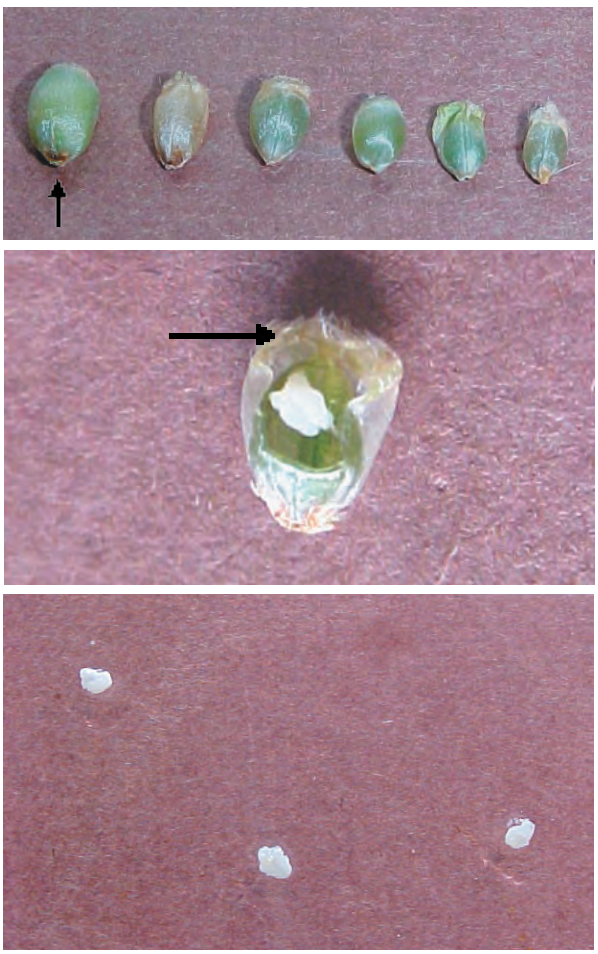

Plate 1. a) wheat caryopses obtained following selfing (arrow) and wheat x maize intergeneric crosses, b) seventeen-day old haploid floating embryo in watery embryo sac (arrow), c) variations in size and shape of embryos

these crossability limiting factors, and pollen can be taken from a wide range of maize germplasm (Suenaga 1994). Moreover, the sexual route of dihaploid production systems in wheat is free from tissue culture associated variations and the problem of albinism in regenerants. Haploid embryo induction and subsequent plant regeneration from wheat x maize crosses have been greatly improved by manipulating factors affecting the overall efficiency of this system (Gracia-llamas et al. 2004, Campbell et al. 1998, Suenaga et al. 1997). Some researchers have reported that this method is also significantly affected by both wheat and maize genotypes used in crossing programme (Berzonsky et al. 2003, Chaudhary et al. 2002, Verma et al. 1999). As a result some of the combinations yielded better than others.

Although this technique may be useful in more rapidly breeding elite wheat cultivars, Nepal has not yet tested its efficacy as compared to other methods. Therefore, as a part of the breeding efforts, this study was carried out to standardize the technique of wheat × maize method suitable for the Nepalese environment, and to study the effect of maize genotypes on haploid embryo formation and plantlet regeneration.

Materials and methods

F_1 wheat seeds derived from the cross between a landrace, Acc. No. 7103 (early but low yielder), and WK-1204 (yellow rust resistant, high yielder, but late) were planted in five earthen pots; three seeds per pot at a time, and repeatedly planted four times at six day intervals and grown in natural condition during the 2004–05 wheat growing season at Khumaltar, Nepal. Four staggered plantings of each maize cultivar (viz. Arun-1, Arun-2, Khumal Yellow and Rampur Composite) were made 10 days after the first wheat sowing at seven day intervals in order to insure an adequate source of pollen and to synchronize the flowering time of maize with wheat. Each maize genotype was planted in six plastic buckets (35 x 25 cm^2) at a time and grown in a glasshouse. During winter, the glasshouse was illuminated in the morning and evening for four hours to enhance the length of photoperiod. The wheat plants were thinned to a single plant per pot at the two-three leaf stage. Once the wheat heads were ready for emasculation, four healthy pots containing single plants were selected and five of the spikes from each plant were hand-emasculated one or two days prior to anthesis. The emasculated spikes were covered with 5 x 12 cm plastic bags until pollination.

The experiment was carried out in completely randomized design with four replications; each spike was considered a replication. One to two days after emasculation the spikes of wheat were hand-pollinated with freshly collected maize pollen from each cultivar; the plastic bags were then replaced with glassine bags. One ml of 100 ppm 2,4-D was immediately injected on the uppermost internode of wheat with a one-ml capacity hypodermic insulin syringe following the procedure of Suenaga and Nakajima (1989). The pore was sealed with vaseline to prevent leakage. Twenty-four hours after 2,4-D injection, the cups of the pollinated florets were filled with the same concentration of 2,4-D; this was repeated twice on two consecutive days. Once the application of 2,4-D completed, the whole pollinated spikes were covered with glassine bag until the embryo harvest. The extra spike from each pot was treated only with distilled water as a control. Seventeen days after pollination, the spikes were harvested and the number of intact, non-selfed florets from each replication was counted and recorded as the number of florets pollinated. Well-developed caryopses were removed from the florets, sterilized in 70% ethanol for 30 seconds, briefly rinsed in sterile distilled water, and then sterilized for 15 minutes in 1% sodium hypochlorite. Finally, the caryopses were again rinsed three times with sterile distilled water. The embryos were aseptically extracted under a stereomicroscope. Small and poorly developed embryos were counted to determine the total embryos formed, but they were not cultured: only well developed embryos longer than 0.5 mm were cultured aseptically on to 70 mm petri plates containing a half-strength MS (Murashige and Skoog 1962) basal medium supplemented with 0.5 mg/l nicotinic acid, 0.1mg/l thiamine HCl, 0.5 mg/l pyridoxine HCl, 2 mg/l glycine, 30g/l sucrose, and 7 g/l agar as gelling agent. The cultured embryos were kept at 4°C for seven days in the dark and then transferred to an incubation room for the next seven days at 25±1°C in dark. After incubation, the cultured embryos were kept in a temperature-controlled chamber for regeneration at 25±1°C with alternating periods of 16 hours light and 8 hours dark. When the plantlets reached the two three-leaf stage, they were hardened and transferred into soil and then maintained in a temperature-controlled chamber as in the off-season nursery. The traits for analysis included: percentage of swollen caryopses containing florets, percentage of embryos formed (number of embryos divided by number of florets pollinated), and frequency of haploid plants per floret (number of regenerated seedling compared to total number of pollinated florets).

Depending on the nature of data in respective parameters, the percentage values were transformed into arcsine $\sqrt{x}$ and square root function $(x + ½)^{0.5}$ in order to normalize the distribution before analysis of variance (Gomez and Gomez 1984). The control data was excluded from statistical analysis. Duncan's Multiple Range Test (DMRT) was used for comparing the mean effect of maize genotypes on three parameters using MSTATC (version 1.3, 1989). The ploidy level of regenerated plantlets was determined by counting the chromosomes in root tip cells, using the standard acetocarmine squashing technique.

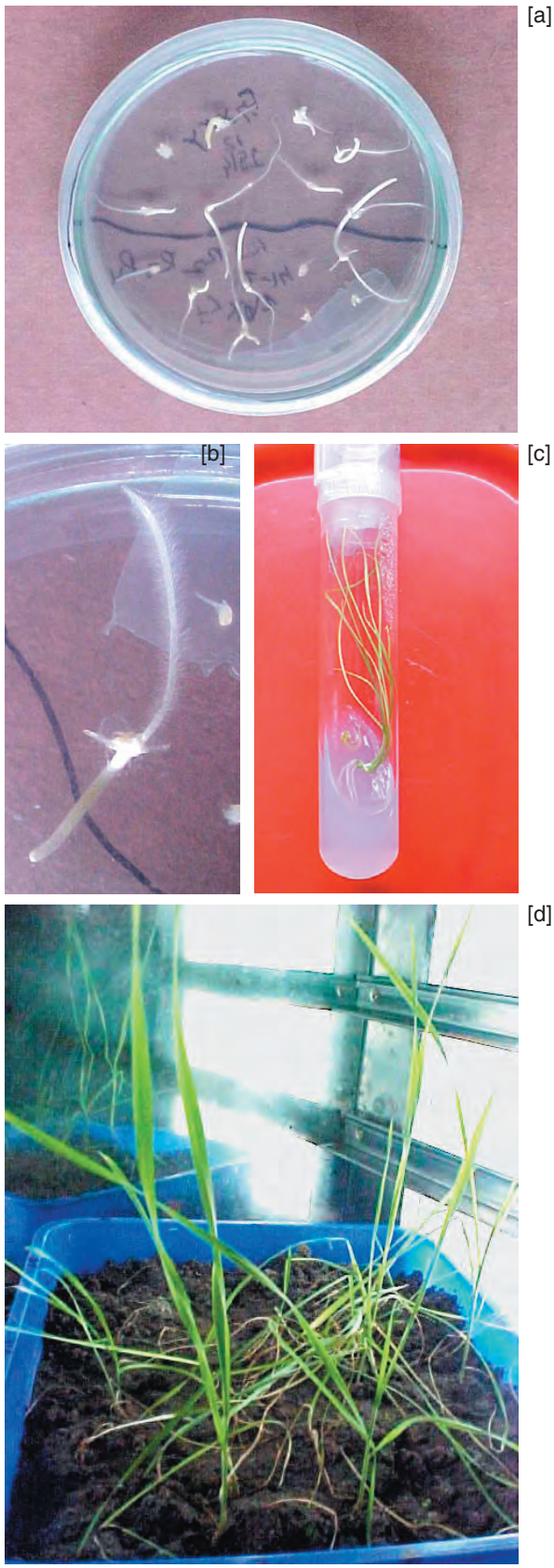

Plate 2. a–b) germinating embryos ten days after incubation, c) plantlet regeneration after 4 weeks, wheat x Arun-2, d) established haploid plants obtained from wheat x Arun-2 crosses

Results and discussion

In the present study the protocol for maize pollen mediated haploid production in wheat was standardized to existing laboratory environments. Pollination of wheat florets by maize pollen following 100 ppm 2,4-D treatment proved effective in producing haploid embryos. Application of 2,4-D after pollination was found to be essential to the recovery of embryos of culturable size via the wheat x maize system (**Plate 1c**). Each cross combination produced many haploid embryos (**Table 1**). The embryos obtained through this system were observed floating in watery embryo sacs (**Plate 1b**), from which they were rescued and placed on the culture medium (**Plate 2, a–c**). Lack of endosperm in the developed caryopsis after harvest served as the initial criterion for identifying haploid embryos. Altogether twenty-six haploid plants were successfully produced by pollinating 420 wheat florets with four maize genotypes (**Table 1, Plate 2d**). We were able to demonstrate the highly significant effect of maize genotypes on ovary development, embryo formation and florets per plant. Among pollen sources, the highest mean percentage of well developed ovaries (44.37%) was recorded for Khumal Yellow (**Plate 1a**), whereas Rampur Composite showed the lowest (30.65%). Similarly, the mean percentage of embryo formation varied from 5.17 – 21.45, depending on the maize variety (**Table 1**). The highest percentage of embryo recovery was found when wheat genotype was pollinated with Arun-2. Among the four maize varieties tested, Rampur Composite showed the lowest overall results in the tested parameters. The ratio of haploid plants to florets pollinated ranged from 0.96–10.15% with an average of 6.0%. The plantlets, when transplanted into soil, grew into green haploid plants. The haploid status (n = 3x = 21) of these plants was confirmed by their somatic chromosome counts.

A failure of normal caryopsis and endosperm development was also reported in wheat when pollinated with several genera of grasses (Pratap et al. 2005, Chaudhary et al. 2005). With the post-pollination application of 2,4-D, however, ovary tissues enlarge as in normal caryopsis development, appear turgid, but are filled with liquid; within such caryopses embryos may or may not be found (Suenaga and Nakajima 1989). In our study also, when 2,4-D was not applied after pollination, the caryopses failed to grow due to the lack of endosperm and most of them were shrunken and collapsed within 9 to 14 days after pollination (data not shown). The present results are consistent with other studies that have shown significant influence of maize genotypes on percent of embryo formation and on ratio of haploid plants per pollinated floret (Zhang et al. 1996, Suenaga 1994, Verma et al. 1999, Karanja et al. 2002). Karanja et al. (2002) obtained 8.53–19.34% embryos per floret when wheat was pollinated with six maize genotypes using similar method except that wheat was grown in green house. Likewise Suenaga (1994) also obtained a varying rate of embryo formation efficiency (1.6–36%) when a single wheat genotype, Fukoho-komugi, was pollinated with 52 diverse maize genotypes. Based on analysis of variance, this study also showed highly significant effect of maize genotypes on frequency of embryo and subsequent plant formation. Among three better maize genotypes, the response of Arun-2 was the best for haploid plant regeneration and plants per floret pollinated. Using this combination, 11 plants were successfully produced from 111 pollinated florets (**Table 1**). This figure was found consistent with the results of Sadasivaiah et al. (1999) who reported an average of 6.29 plant per 100 florets pollinated. The present study also clearly indicated that a high incidence of swollen ovaries does not always lead to a high yield of culturable embryos; this was the case with Rampur Composite (**Table 1**), where only five embryos were obtained out of 28 developed caryopses. This might have been due to the use of 2,4-D without maize pollen fertilization.

The larger number of embryos of culturable size is one of the crucial factors that determine the germination and post-germination efficiency of wheat x maize system. The number of embryos of culturable size can be increased through the judicious application of an auxin source such as Dicamba, alone or in combination with 2,4-D (Gracia-llamas et al. 2004). The number of embryos can also be improved by fine-tuning environmental factors such as temperature regime (Knox et al. 2000, Campbell et al. 1998) and relative humidity (Ballesteros et al. 2003), and by using the middle portion of the spikelet (Martins-Lopes et al. 2001) during pollination. Moreover the efficiency of this system is also influenced by other factors such as timing and technique of hormone manipulation, age of embryo to be cultured and *in vitro* conditions (Kaushik et al. 2004). The slight discrepancy between the results reported in this study and those of previous studies might be attributable to differences in wheat and maize genotypes and their interactions that influenced the rate of embryo recovery and subsequent plantlet formation (Chaudhary et al. 2002, Verma et al. 1999). Therefore, selection of better responsive maize genotypes such as Arun-2, Arun-1, and Khumal Yellow would seem to offer a likelihood of higher rates of haploid wheat induction. In summary, wheat x maize system was found to be simple and efficient and can be used as alternative to other systems of haploid induction in wheat.

Acknowledgements

The authors are grateful to K Suenaga, Japan International Research Center for Agricultural Sciences, 1–2 Ohashi, Tsukuba, Ibaraki 305, Japan for his assistance in providing some of the valuable research papers.

References

Baenziger S, KM Kim and K Haliloglu. 2001. Wheat in vitro breeding. In: Bonjean AP and WJ Angus (eds), *The world wheat book: A history of wheat breeding*, Intercept, Limagrain, New York. p 979–1000

Ballesteros J, C Garcia-llamas, MC Ramirez and A Martin. 2003. Low relative humidity increases haploid production in durum wheat x maize crosses. *Plant Breeding* **122**: 276–278

Barclay IR. 1975. High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. *Nature* **256**: 410–411

Berzonsky WA, SL Kleven and GD Leach. 2003. The effects of parthenogenesis on wheat embryo formation and haploid production with and without maize pollination. Euphytica 133: 285–290

Bitsch C, S Groger and T Lelley. 1998. Effect of parental genotypes on haploid embryo and plantlet formation in wheat x maize crosses. *Euphytica* **103**: 319–323

Campbell AW, WB Griffin, AJ Conner, JS Rowarth and DJ Burritt. 1998. The effects of temperature and light intensity on embryo numbers in wheat doubled haploid production through wheat x maize crosses. *Annals of Botany* **82**: 29–33

Chaudhary HK, GS Sethi, S Singh, A Pratap and S Sharma. 2005. Efficient haploid induction in wheat by using pollen of *Imperata cylindrica*. *Plant Breeding* **124**: 96–98

Chaudhary HK, S Singh and GS Sethi. 2002. Interactive influence of wheat and maize genotypes on the induction of haploids in winter x spring hexaploid wheat hybrids. *Journal of Genetics and Breeding* **56**: 259–266

Choo TM, E Reinbergs and KJ Kasha. 1985. Use of haploids in breeding barley. *Plant Breeding Reviews* **3**: 219–261

Falk DE and KJ Kasha. 1981. Comparison of the crossability of rye (*Secale cereal*) and *H. bulbosum* onto wheat (*Triticum aestivum*). *Canadian Journal of Genetics and Cytology* **23**: 81–88

Garcia-llamas C, A Martin and J Bllesteros. 2004. Differences among auxin treatments on haploid production in durum wheat x maize crosses. *Plant Cell Reports* **23**: 46–49

Gomez KA and AA Gomez. 1984. *Statistical procedures for agricultural research*. 2nd edition. A Willey-Interscience Publication, John Wiley and Sons, Singapore. p 1–294

Guha S and SC Maheshwari. 1964. In vitro production of embryos from anther of *Datura*. *Nature* **204**: 497

Inagaki MN. 1997. Technical advances in wheat haploid production using ultra-wide crosses. *Japan International Research Center for Agricultural Sciences Journal* **4**: 51–62

Karanja L, M Kinyua and J Malinga. 2002. Development of a doubled haploid system for wheat through wheat x maize crosses in Kenya. *African Crop Science Journal* **10**: 311–316

Kaushik N, M Sirohi and VK Khanna. 2004. Influence of age of the embryo and method of hormone application on haploid embryo formation in wheat x maize crosses, In: T Fischer, N Turner, J Angus, L McIntyre, M Robertson, A Borrell, and D Lloyd (eds), *New directions for a diverse planet,* Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, 26 Sep1 Oct 2004. www.cropscience.org.au. Access date: 12th July 2005

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

Knox RE, JM Clarke and RM Depauw. 2000. Dicamba and growth condition effects on doubled haploid production in durum wheat crossed with maize. *Plant Breeding* **119**: 289–298

Laurie DA and MD Bennett. 1988. The production of haploid plants from wheat x maize crosses. *Theoretical and Applied Genetics* **76**: 393–397

Liang GH, A Xu and H Tang. 1987. Direct generation of wheat haploids via anther culture. *Crop Science* **27**: 336–339

Martins-Lopes PF, H Guedes-Pinto, O. Pinto-Carnide and J Snape. 2001. The effect of spikelet position on the success frequencies of wheat haploid production using the maize cross system. *Euphytica* **121**: 265–271

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

Patel M, NL Darvey, DR Marshall and JO Berry. 2004. Optimization of culture conditions for improved plant regeneration efficiency from wheat microspore culture. *Euphytica* **140**: 197–204

Pratap A, GS Sethi and HK Chaudhary. 2005. Relative efficiency of different Gramineae genera for haploid induction in triticale and triticale x wheat hybrids through the chromosome elimination technique. *Plant Breeding* **124**: 147–153

Sadasivaiah RS, BR Orshinsky and G. Korzub. 1999. Production of wheat haploids using anther culture and wheat x maize hybridization technique. *Cereal Research Communication* **27**: 33–40

Snape JW, E Simpson and BB Parker. 1986. Criteria for the selection and use of doubled haploid systems in cereal breeding programmes. In: W Horn, CJ Jensen, W Odenbach and O Schieder (eds), *Genetic manipulation in plant breeding*. Walter de Gruyter, Berlin. p 217–229

Suenaga K and K Nakajima. 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, AR Morshedi and NL Darvey. 1997. Haploid production of Australian wheat (*Triticum aestivum* L.) cultivars through wheat x maize (*Zea mays* L.) crosses. *Australian Journal of Agricultural Research* **48**: 1207–1211

Suenaga K. 1994. Doubled haploid system using the intergeneric crosses between wheat (*Triticum aestivum*) and maize (*Zea mays*). *Bulletin of National Institute of Agrobiological Resource* **9**: 83–139

Verma V, NS Bains, GS Mangat, GS Nanda, SS Gosal and K Singh. 1999. Maize genotypes show striking differences for induction and regeneration of haploid wheat embryos in the wheat x maize systems. *Crop Science* **39**: 1722–1727

Zhang J, B Friebe, WJ Raupp, SA Harrison and BS Gill. 1996. Wheat embryogenesis and haploid production in wheat x maize hybrids. *Euphytica* **90**: 315–324