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Production of dihaploids in durum wheat using *Imperata cylindrica* L. mediated chromosome elimination

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Abstract: The possibility of obtaining dihaploid durum wheat (*Triticum durum* L.) plants by interspecific hybridization with *Imperata cylindrica* L. was studied. Ten local varieties and 1 commercial durum wheat variety (*Triticum durum* L., 2n = 4x = 28; AABB) were used as maternal lines. *Imperata cylindrica* L. (2n = 2x = 20) ecotypes sampled from different districts of Hatay were used as the pollen source. Isolated embryos were placed in an MS medium for plant regeneration 14 days after the interspecific hybridization. The highest average seed setting, embryo formation, and dihaploid plant regeneration for the different hybrid combinations were determined as 31.1%, 15.5%, and 11.5%, respectively. Cytological observations revealed that all the regenerants had 2n = 2x = 14 chromosomes.

Key words: *Imperata cylindrica* L., interspecific cross, *Triticum durum* L., polyhaploid

1. Introduction

One of the most important problems of the pasta industry is the difficulty of obtaining high-quality durum wheat. The number of produced varieties that are capable of satisfying the quality requirements of the industry is quite limited. Therefore, breeding durum wheat varieties based on sector demand is a high priority. Doubled haploid techniques, which have become a routine part of durum wheat breeding programs, may assure great advantages for breeders because they provide pure recombinant lines in terms of desired characteristics from earlier generations. Haploid plants that have a single set of alleles at each locus are also very important in genetic and molecular studies. These plants save land and labor by obtaining completely homozygous doubled haploid lines via chromosome doubling. They also offer the possibility of selection in small-sized populations. Moreover, the haploid breeding approaches help eliminate deleterious mutations and weak plant types, which is useful in the generation of additional variability in the form of gametoclonal variation and in elimination of the dominant alleles that control undesirable traits (Kishore et al., 2011; Tayeng et al., 2012).

Various methods have been developed for producing both in vitro and in vivo haploids. The first report was by Barclay (1975) on in vivo haploid production via chromosome elimination with the interspecific hybridization of Chinese Spring × *Hordeum bulbosum*. This technique was a major breakthrough, but it was genotype-specific due to the presence of the dominant crossability inhibitor genes *Kr1* and *Kr2*, which are determined on the long arms of the 5B and 5A chromosomes of wheat and are also expressed in many wheat varieties (Lein, 1943; Laurie and Reymondie, 1991; Alfares et al., 2009). Maize pollen is not sensitive to the activity of these genes in wheat. Therefore, the interspecific hybridization of wheat × maize is not genotype-specific and has been used as an efficient chromosome elimination technique in obtaining haploid wheat (Laurie and Bennett, 1987). However, the unsynchronized flowering of these 2 species complicates the hybridization process. Chromosome elimination via *Triticum aestivum* L. × *Imperata cylindrica* L. interspecific hybridization, which was first reported by Chaudhary et al. (2005) and Pratap et al. (2005), is a relatively new and promising technique. *Imperata cylindrica* L. is actually a perennial invasive weed that synchronously blooms with wheat. Moreover, it is available under natural conditions wherever wheat is cultivated. Compared with other chromosome elimination techniques, including maize hybridization, this technique is much more efficient, practical, and economical (Pratap et al., 2005; Kishore et al., 2011). On the other hand, obtaining higher percentages of haploid regenerants from all the studied species proved

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that this technique, unlike the others, is not genotype-specific (Chaudhary et al., 2005). *Imperata cylindrica* L. mediated chromosome elimination has been applied to date only on the species *Triticum aestivum* L. (Chaudhary et al., 2005; Pratap et al., 2005; Komeda et al., 2007; Tayeng et al., 2012) and *Triticum aestivum* L. × *Secale cereale* L. hybrids (Kishore et al., 2011). Therefore, the objective of the present study was to evaluate the possibility of obtaining dihaploid durum wheat (*Triticum durum* L.) plants by interspecific hybridization with *Imperata cylindrica* L.

2. Materials and methods

Genotypes that were selected and self-pollinated from the local durum wheat (*Triticum durum* L., 2n = 4x = 28; AABB) landrace populations from a previous study (obtained from the GAP International Agricultural Research and Education Center, Diyarbakır, Turkey) and a commercial variety were used as maternal lines. The pollen source was *Imperata cylindrica* L. (2n = 2x = 20) ecotypes that were sampled from different districts of Hatay (Antakya, Alahan village, and Samandağ). Five pods, each of them including 4 maternal durum wheat plants for each genotype, were grown in an uncontrolled net-house. Maternal planting was replicated 2 times at a 15-day interval. The interspecific hybridization process and preliminary preparations for durum wheat × *I. cylindrica* were conducted according to the standard protocol determined by Chaudhary et al. (2005). The wheat spikes were emasculated before anthesis. Thus, the bottom and upper smaller spikelets and central florets of each spikelet were removed, and the anthers were isolated from the remaining florets. Two days after emasculation, the tops of the glumes were intersected to expose the stigma. The pollen viability test of tetrazolium, as described by Norton (1966), was applied to the mixed collected pollen from the naturally occurring cogongrass populations (for each ecotype) before its application on the stigmas with a soft brush. Next, 1 mg L^{-1} of 2,4-D of the 100 mg L^{-1} stock solution was injected into the uppermost internodes or sprayed onto the pollinated spikes 24 h, 48 h, and 72 h after hybridization to stimulate the seed setting. The spikes were harvested 14 days after pollination, and the seeds were examined for the presence of embryos. Embryos were found and cultured on an MS nutrient medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 100 mg of myo-Inositol, 0.1 mg L^{-1} thiamine HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.5 mg L^{-1} kinetin, 400 mg L^{-1} glutamine, 20 mg L^{-1} L-arginine, 20 mg L^{-1} L-cysteine, and 20 mg L^{-1} L-leucine. The medium's pH was adjusted to 5.8 and solidified with 8 g L^{-1} agarose. The glass culture tubes with embryos were incubated at 4 °C overnight to activate the stress before transfer to 20 ± 2 °C in a 10-h light/14-h dark photoperiod regime until regeneration (Kishore et al., 2011). The regenerated plantlets were then transferred to a liquid MS medium with the same ingredients as the nutrient medium to improve the root growth. The number of somatic chromosomes was determined in 10 well-dispersed cells of root tips for both the plantlets that were in acclimatization and the parental lines. Data were recorded as percentages for the seed set, embryo formation, and plant regeneration. Data transformations using arcsine $\sqrt{x + 0.001}$ or $\sqrt{x + 0.5}$ were applied to normalize the proportional data, and SPSS 16 was used for the statistical analyses.

3. Results

Observations on the haploid induction efficiency of *I. cylindrica* mediated chromosome elimination in durum wheat showed promising results (Figure 1). The calculated average results of the haploid induction parameters of seed setting, embryo formation, and embryo regeneration for each hybrid combination were compared (Table). Interspecific hybridization between local durum wheat varieties and *I. cylindrica* resulted in seed formation for all combinations, with the exception of the Antakya ecotype. However, different seed setting rates were obtained among the genotypes. The effect of the *I. cylindrica* ecotypes on seed setting was also varied. The highest seed setting was obtained from the Kurtalan genotype at an average rate of 12.7% for all hybrid combinations; this rate was actualized at 31.1% for the Kurtalan × Samandağ hybridization. In addition, the rate of 24.3% for the Diyarbakır-81 × Samandağ hybrid combination was statistically similar to the above-mentioned rate of seed setting. Among the *I. cylindrica* ecotypes, Samandağ was determined to be the most effective generator of haploid seeds from durum wheat genotypes. The embryo formation was significantly varied in different hybrid combinations. Both the maternal lines and the paternal ecotypes affected the embryo formation. While the highest embryo formation was observed for the Şırnak durum wheat genotype, with an average of 8.2%, the highest for the Şırnak × Samandağ hybrid combination was 15.5%. The effect of the *I. cylindrica* ecotypes on the embryo formation showed differences across genotypes. Although Antakya was the worst ecotype with respect to embryo formation, it was the best pollinator for the Menceki genotype, with an average of 6.2%. Similarly, the Alahan ecotype was the best stimulator for the durum genotype Minaret. The Samandağ *I. cylindrica* ecotype formed 7.3% embryos, which was the average of all hybrid combinations. The highest number of regenerated plantlets was observed for the durum genotype Kurtalan. It was 5.4% proportionally as an overall average of each of the 3 hybrid combinations. However, it was 11.5% for the hybrid combination of Kurtalan × Samandağ. Although the Samandağ ecotype may seem to produce the best results of all the hybrid combinations with respect to

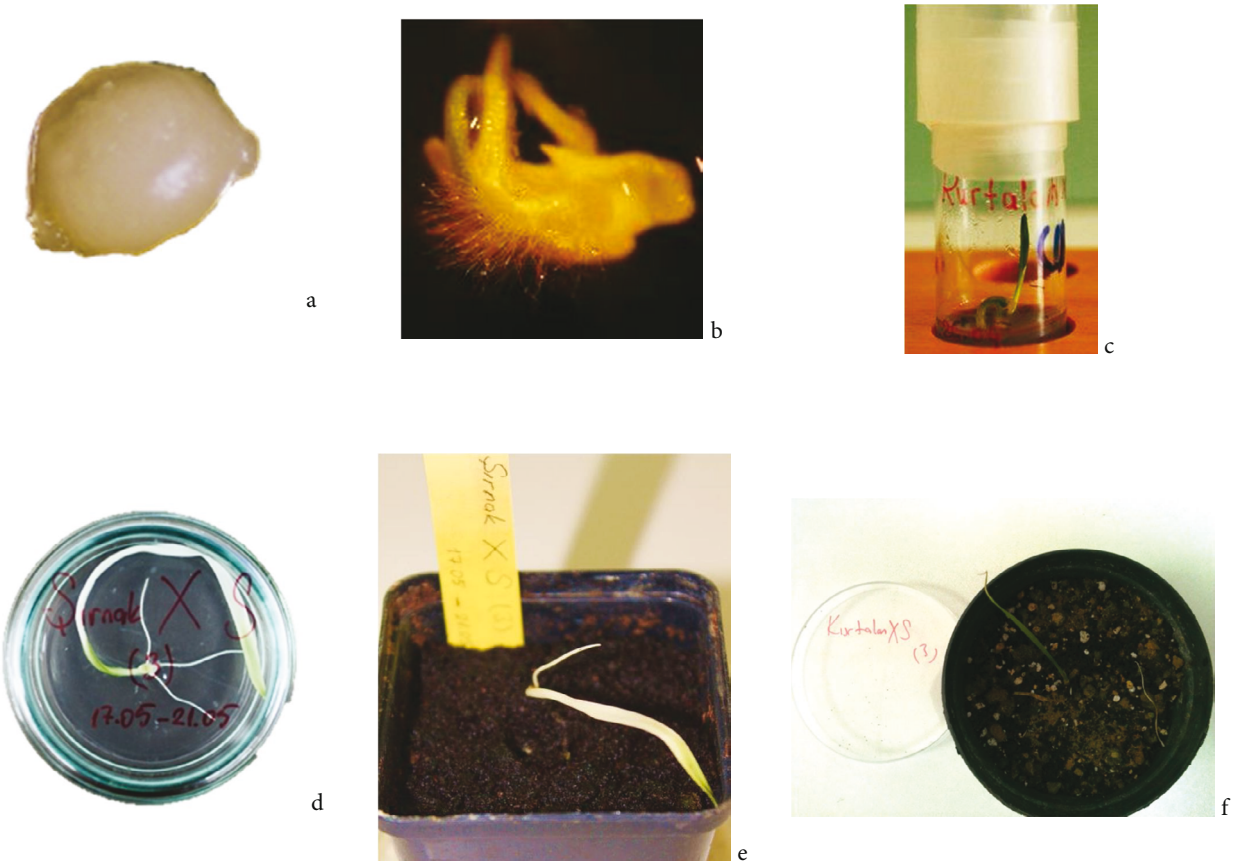

Figure 1. Durum wheat × *I. cylindrica* derived dihaploid embryo formation and plant regeneration: **a)** isolated embryo 14–16 days after hybridization; **b)** dihaploid embryo regeneration in MS medium (20 days after incubation); **c, d)** the rooted regenerants (30 days after incubation); **e, f)** the plantlets in acclimatization.

plant regeneration, it is obvious that the Alahan ecotype should not be ignored for some durum genotypes, such as Diyarbakır-81.

An injection of 2,4-D solution in order to stimulate the seed setting rate yielded 40.4% seed setting, while the spraying method yielded 33.3% seed setting. This difference, however, was not statistically significant.

Cytological observations revealed that all the regenerants had n = 2x = 14 chromosomes (Figure 2). No differences were determined within the ploidy levels of *I. cylindrica* ecotypes, and 3 of them were fixed as diploid (2n = 2x = 20) (Figure 3). The largest *Imperata cylindrica* chromosome was much smaller than the smallest wheat chromosome and therefore more difficult to determine (compare Figures 2 and 3).

4. Discussion

Several techniques have been employed to generate haploids in wheat, yet they come with significant problems that prevent the extensive usage of these methods in wheat breeding (Fadel and Wenzel, 1990; Lashermes et al., 1991). Chromosome elimination mediated techniques are the methods of choice for haploid production in cereals (Almouslem et al., 1998). Despite several attempts to explain uniparental chromosome elimination, the molecular basis is still not clear. One theory posits that centromeres from the 2 parent species interact unequally with the mitotic spindle as a result of inactivation of centromeric histone CENH3, causing selective chromosome loss (Komeda et al., 2007; Ravi and Chan, 2010; Sanei et al., 2011). The combination of parental species determines the complete or incomplete chromosome elimination. Interspecific hybrids with *Imperata cylindrica* result in the production of maternal haploids by complete loss of paternal chromosomes. The technique is a new, practical, and more effective haploidization method for cereals (Chaudhary et al., 2005; Pratap et al., 2005; Kishore et al., 2011). The effectiveness of the technique depends mostly on hybridization practice and in vivo conditions. The adverse temperatures that were detected (around 30 to 35 °C) for several days during the course of this study (involving the stages of anthesis, hybridization, embryo formation, and development) could have negatively affected its outcomes. The desiccation of the maternal stigma due to emasculation

Table. The efficiency of *I. cylindrica* mediated chromosome elimination technique with respect to seed set, dihaploid embryo formation, and plant regeneration in crosses with durum wheat genotypes.

Durum wheat genotypes	*Imperata* ecotypes									Florets pollinated
	Alahan	Antakya	Samandağ	Alahan	Antakya	Samandağ	Alahan	Antakya	Samandağ	
	Seed setting %			Embryo formation %			Regeneration %			
Şırnak	14.6[+](15.75) b–d[*]	0.0(0.57) d	20.0(21.28) a–c	9.1(11.22) a–c	0.0(0.57) c	15.5(17.68) a	0.0(0.57) c	0.0(0.57) c	6.1(6.74) b	276
Havrani	2.5(4.42) cd	0.0(0.57) d	2.7(4.56) cd	3.8(4.1) bc	0.0(0.57) c	1.1(2.3) c	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	96
Karadere	4.5(5.17) cd	0.0(0.57) d	11.1(12.44) b–d	4.9(6.0) bc	0.0(0.57) c	8.6(10.7) a–c	0.0(0.57) c	0.0(0.57) c	2.5(3.37) bc	139
Kurtalan	7.1(8.26) b–d	0.0(0.57) d	31.1(32.25) a	4.2(6.4) bc	0.0(0.57) c	13.1(15.6) ab	4.6(4.61) bc	0.0(0.57) c	11.5(11.44) a	338
Menceki	4.4(6.74) cd	8.6(8.33) b–d	3.4(3.92) cd	2.3(4.1) c	6.3(7.0) a–c	2.7(3.4) c	0.0(0.57) c	0.0(0.57) c	4.6(4.61) bc	142
Devedişi	6.8(6.99) b–d	0.0(0.57) d	8.6(8.39) b–d	4.2(4.3) bc	0.0(0.57) c	4.1(5.6) bc	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	64
Minaret	8.3(8.96) b–d	0.0(0.57) d	12.6(12.11) b–d	6.8(7.3) a–c	0.0(0.57) c	5.2(7.4) bc	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	148
Hacıhalil	13.4(11.20) b–d	0.0(0.57) d	8.2(7.02) b–d	4.7(6.0) bc	0.0(0.57) c	5.8(5.3) bc	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	64
Bağıcak	3.2(4.95) cd	0.0(0.57) d	7.6(7.66) b–d	3.8(4.1) bc	0.0(0.57) c	3.7(4.1) bc	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	102
Karakılçık	4.6(4.61) cd	6.8(5.97) b–d	10.0(10.33) b–d	1.5(2.7) c	3.2(3.8) c	7.7(8.9) a–c	0.0(0.57) c	0.0(0.57) c	0.0(0.57) c	112
Diyarbakır-81	12.7(13.32) b–d	0.0(0.57) d	24.3(24.33) ab	6.9(9.4) a–c	0.0(0.57) c	13.3(17.2) ab	4.6(4.61) bc	0.0(0.57) c	3.1(4.89) bc	248
Mean	7.5(8.26) b[**]	1.4(1.77) c	12.7(13.12) a	4.7(6.00) b	0.9(1.45) c	7.3(8.96) a	0.8(1.30) ab	0.0(0.57) b	2.5(3.13) a	

*Means with the same letter for each durum wheat × *I. cylindrica* hybrid combination in the same column are not significantly different by Duncan's multiple range test at $P \leq 0.05$ level.
**Means with the same letter for each *I. cylindrica* ecotype in the same row are not significantly different by least significant difference test at $P \leq 0.05$.[+]Transformed values.

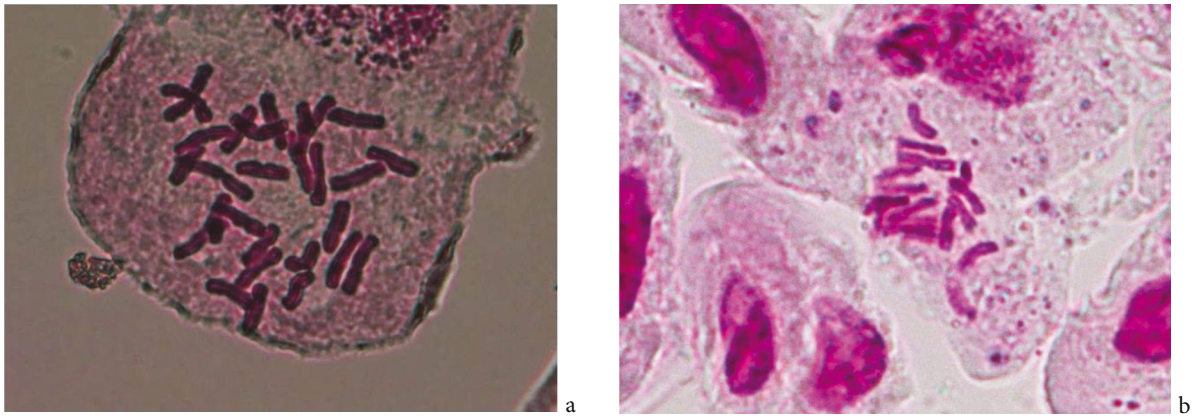

Figure 2. Cytological investigations of the maternal tetraploid durum wheat (genotype Kurtalan) (2n = 4x = 28) **(a)** and regenerated dihaploid plantlets (genotype Kurtalan) (2n = 2x =14) **(b)** obtained from the interspecific hybridization of wheat × *I. cylindrica* derived embryos.

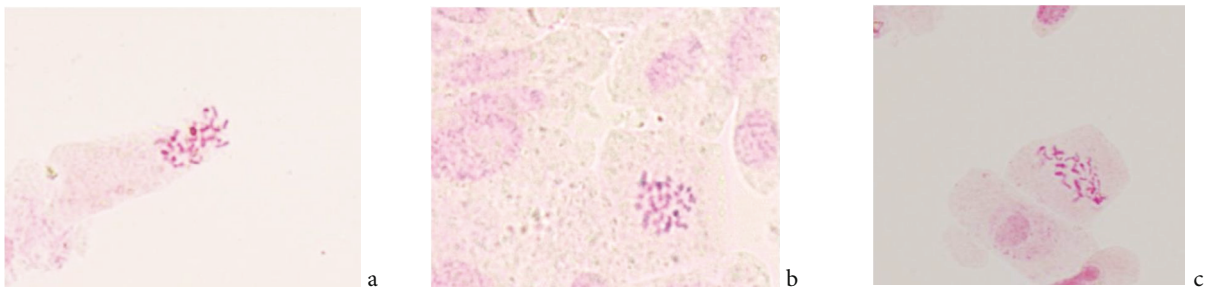

Figure 3. Cytological investigations of the *I. cylindrica* ecotypes: **a)** Samandağ, **b)** Antakya, **c)** Alahan (2n = 2x = 20).

and the intersection of glumes were obvious in some of the maternal florets. That is why Moradi et al. (2009) recommend emasculating the florets without intersecting the glumes. Due to unfavorable conditions, the waiting period for embryo maturity was shortened to 14 days in the present study. Likewise, Khan et al. (2012) reported that the optimum embryo rescue time after interspecific hybridization depends on the genotype, temperature, and humidity. The seed set was thus actualized below those rates in the reports of Chaudhary et al. (2005), with a 51.6% seed set for soft wheat × *I. cylindrica*. On the other hand, our findings were very similar to the results of interspecific crosses of [*Triticum aestivum* L. × *Secale cereale* L.] × *I. cylindrica* recorded by Kishore et al. (2011). Species and protocol differences may account for the variations. The uncontrolled growth conditions prevented the collection of sufficient amounts of paternal pollen, especially for the naturally occurring Antakya ecotype. This resulted in fewer or no pollinated florets and seed settings for some hybrid combinations with the mentioned ecotype (Table).

Our experience showed that the application of 2,4-D by spraying to stimulate seed setting was not an appropriate method because of the possibility of overdose. Detached tiller culture in a nutrient medium consisting of both 2,4-D and $AgNO_3$ a few days after hybridization is a more efficient method (Almouslem et al., 1998; Khan et al., 2012). Komeda et al. (2007), suggest that the *I. cylindrica* chromosomes were completely eliminated from the nuclei in the first cell division, probably due to a lack of functional kinetochore proteins. Therefore, early intervention in the hybrid florets with a detached tiller culture would have also introduced more healthy haploid embryos.

A high percentage of viable paternal pollen was revealed in a sufficient number of embryo formations in some hybrid combinations. These rates are in harmony with the outcomes of soft wheat × *I. cylindrica* hybrids introduced by Chaudhary et al. (2005) and the findings of Pratap et al. (2005) for [triticale × triticale] × *I. cylindrica* hybrids. Our plant regeneration results were not as promising as the seed setting and embryo formation results. As mentioned above, the *Imperata* system is not genotype-specific. No variation was recorded previously with respect to haploid plant regeneration arising out of the ecotype or ploidal variation of *I. cylindrica*. Thus, the lower percentages, especially from combinations with the Antakya ecotype, may be due to laboratory errors. The *Imperata* technique is one of the

examples of complete loss of paternal kinetochore function in interspecific hybrids, inducing missegregation of uniparental chromosomes and paternal genome elimination (Komeda et al., 2007). Nevertheless, the regeneration rate of the Kurtalan × Samandağ hybrid combination was higher than many other reported rates in durum wheat × maize interspecific crosses (Almouslem et al., 1998; Inagaki and Tahir, 1992; Inagaki and Hash, 1998). Similarly, Pratap et al. (2005) and Kishore et al. (2011) stated that the *I. cylindrica* mediated chromosome elimination technique is more efficient than the maize system for soft wheat. Our findings indicate that this statement applies to durum wheat, as well. It should be noted that previous wheat × maize interspecific crosses were usually done in a controlled environment. On the other hand, Almouslem et al. (1998) argued that most of the haploidy techniques are convenient for the production of haploids of soft wheat, but are not successful with tetraploid durum wheat. Our results show that the *I. cylindrica* mediated chromosome elimination technique is, in fact, quite successful for durum wheat. As with all other techniques, however, the most critical stages are acclimatization and chromosome doubling. Significant plantlet losses occurred throughout this study in both stages, but the losses after colchicine treatment were remarkable. Tayeng et al. (2012) also reported on the lethal effect of colchicine treatment on haploid plantlets obtained by chromosome elimination techniques.

In conclusion, the present investigation clearly indicates that the induction of dihaploid durum wheat with *I. cylindrica* mediated chromosome elimination techniques is more efficient and cost-effective than both the maize and *H. bulbosum* systems. The time synchronization with respect to the blooming of these 2 species and the natural abundance of paternal fresh pollen around the maternal durum wheat at the required time makes the *I. cylindrica* mediated system more advantageous. The problems with the other methods, as discussed here and in previous reports, are readily resolved. In fact, the failure of some hybrid combinations in the present study was not related to the *I. cylindrica* system. The obtained data from most of the hybrid combinations indicate that the system is effective in producing dihaploid embryos from most durum wheat genotypes by means of different *I. cylindrica* ecotypes. The *I. cylindrica* mediated system is not genotype-specific and therefore this new and promising approach is open to large-scale production for commercial utilization and useful for improving populations for gene mapping or other molecular and breeding studies.

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