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Dihaploid Induction in Tetraploid Durum Wheat (*Triticum durum* L.) Using Pollen of *Imperata cylindrica*

Harinder Kumar CHAUDHARY, Anima MAHATO, Vineeta KAILA, Shoukat Ahmad RATHER and Tisu TAYENG

Molecular Cytogenetics and Tissue Culture Laboratory, Department of Crop Improvement, CSK Himachal Pradesh Agricultural University, Palampur, India

Abstract

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Doubled haploidy breeding protocols have revolutionized the varietal development programmes in bread wheat, however, the protocols have not proved much efficient in durum wheat. Presently, the durum wheat × maize system is being widely followed for haploid induction but the frequency of haploid development is very poor which has limited its application in durum wheat improvement programmes. In order to formulate an efficient wide hybridization-mediated approach for haploid induction in durum wheat, different genotypes of durum wheat were subjected to hybridization with *Imperata cylindrica,* a wild perennial grass for the first time in this laboratory. The investigation was carried out for two seasons. During the 1st year, the investigation was carried out on one genotype only, Langdon, in order to notice the development of haploid embryos and the factors that influence the haploid induction frequency. The most important factor influencing the embryo formation frequency was found to be the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D). Upon pollinating the emasculated spikes of durum wheat genotypes, various concentrations of 2,4-D were injected into the uppermost internode of wheat culm for three consecutive days after pollination to find out the most responding concentration for haploid induction. During the next year, the protocol was applied to eight durum wheat genotypes. The frequency of haploid induction parameters varied with the durum wheat genotypes as well as 2,4-D concentration used. The mean pseudoseed and embryo formation frequency over all the genotypes ranged from 30.2 to 56.3% and 1.2 to 18.4%, respectively. The average embryo formation frequency over all the genotypes was found to be highest (18.4%) at 250 mg/l 2,4-D whereas it was superior for the genotypes WH 896 and Langdon over all the 2,4-D concentrations. At the most responding 2,4-D concentration (250 mg/l), the genotype A-9-30-1 exhibited the highest embryo formation frequency (32.1%). The ploidy status of the developed embryos was identified using cytological analysis carried out on the rootlets of the tissue culture generated plantlets.

Keywords: 2,4-dichlorophenoxyacetic acid (2,4-D); haploid induction; pseudoseed formation; wide hybridization

Doubled haploidy breeding opened new realms in the genetic upgradation endeavours by accelerating the development of homozygous lines and instant fixation of recombinants. Utility of haploids and doubled haploids in mutational, biotechnological and molecular genetic studies has enhanced the interest of various workers in the induction of haploids through various approaches. In wheat, several systems of haploid induction, *viz.* anther culture (Barnabas 2003) and chromosome elimination approaches like wheat × *Hordeum bulbosum* (Barclay 1975), wheat × maize (Laurie & Bennett 1986) and wheat × *Imperata cylindrica* system (Chaudhary *et al.* 2005), have emerged and are being utilized in different wheat improvement programmes. Among all these available systems of doubled haploid production in wheat, the *I. cylindrica*-mediated approach has shown its worth in terms of haploid embryo induction frequency not

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only in bread wheat but also in triticale × wheat and wheat × rye derivatives (Pratap *et al.* 2005; Kishore *et al.* 2011). The underlined mechanism behind all the intergeneric hybridization-mediated systems is the elimination of paternal chromosomes during the initial zygotic cell divisions (Komeda *et al.* 2007; Chaudhary *et al.* 2013).

After bread wheat, durum wheat (*Triticum durum*) is the second most important wheat species cultivated throughout the world. To speed up the durum wheat improvement programmes, a durum wheat × maize system has so far been exploited for haploid induction (Almouslem *et al.* 1998; Cherkaoui *et al.* 2000). However, the haploid embryo formation frequency of the system has been the major problem. Several attempts such as manipulation of environmental conditions (Ballesteros *et al.* 2003) and post-pollination hormonal applications (O'donoughue & Bennett 1994a) have been made to improve the efficiency of the system but there has been no significant enhancement in the haploid induction frequency. Keeping in view the success of *I. cylindrica* in bread wheat, triticale × wheat and wheat × rye derivatives in terms of haploid induction (Chaudhary 2008; Rather *et al.* 2014), the present investigation was carried out to find out its potential and efficiency of *I. cylindrica*-mediated haploid induction in durum wheat.

MATERIAL AND METHODS

The present investigation was carried out for two seasons. During the 1st season, a preliminary study was carried out on one genotype, Langdon. The emasculated spikes of the genotype were pollinated with *I. cylindrica* pollen. The pollinated spikes were injected with various concentrations of 2,4-D ranging from 100 to 300 mg/l. The injections were given to the upper most internode of the culm for three consecutive days at a 24-h interval. The spikes were harvested at 13-15 days after pollination and the pseudoseeds were screened for embryos against a light source (Bains *et al.* 1998) and washed using Tween-20 detergent for 1 to 2 min under tap water to remove dirt. The surface of the embryo carrying pseudoseeds was sterilized using 0.1% $HgCl_2$ for 3–5 min, followed by rinsing twice with autoclaved distilled water for 1 min.

The embryos rescued by dissecting the pseudoseeds using forceps in laminar air flow were then cultured on standardized artificial MS media (Murashige & Skoog 1962) supplemented with essential amino acids, viz. 0.5 mg/l kinetin, 150 mg/l glutamine and 20 mg/l each of L-arginine, L-cystine and L-leucine under aseptic conditions. The culture embryos were subjected to cold treatment at 4°C for 24 h immediately after embryo rescue followed by incubation at 20 ± 2°C under dark conditions till shoot initiation. The regenerated plantlets were placed under control temperature (20 ± 2°C) and relative humidity (75%) in a growth chamber and then transferred to a rooting MS medium; liquid rooting medium consisted of half strength MS salts, 1 mg/l naphthalene-3-acetic acid (NAA) and 1 mg/l indole-3-butyric acid (IBA) for profuse rooting and at the appropriate time for root development. The roots of the regenerated plantlets were used to prepare metaphase spreads for counting the somatic chromosome number of the plants (Singh 2003). The haploid plantlets with properly developed roots were shifted to a potting mixture prepared by mixing soil, sand and compost at the ratio of 2:1:1. The potting mixture was sterilized by autoclaving it at 15 lb/in^2 pressure for 15 min at a temperature of 121°C.

In order to check the reproducibility of the protocol on a number of durum wheat genotypes, the experiment was repeated for the 2nd season and the pollinated durum wheat spikes were injected with different 2,4-D concentrations. Observations were recorded with respect to various haploid induction parameters, viz. pseudoseed formation, embryo formation and haploid regeneration in each durum wheat genotype at each 2,4-D concentration. The information was used to generate data with respect to pseudoseed formation frequency (number of pseudoseeds obtained per 100 wheat florets pollinated with *I. cylindrica*), embryo formation frequency (number of embryo-carrying seeds per 100 florets pollinated) and haploid regeneration frequency (number of green haploid plantlets developed per 100 florets pollinated). The data obtained from ten spikes per genotype per 2,4-D concentration in each haploid induction parameter was recorded as per the completely randomized design and subjected to analysis of variance after arcsine transformations (Gomez & Gomez 1984).

RESULTS

Upon hybridization of *T. durum* cv. Langdon with *I. cylindrica* during the first season, pseudoseed formation was observed at all concentrations of 2,4-D

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Table 1. Performance of *Imperata cylindrica* in *Triticum durum* cv. Langdon in terms of haploid induction during the preliminary study using different concentrations of 2,4-D

2,4-D concentration (mg/l)	Florets pollinated	Pseudoseed formation frequency	Embryo formation frequency
		(%)	
100	108	45.23	2.16
150	105	59.21	5.91
200	114	64.11	7.26
250	124	70.33	14.36
300	96	75.06	12.99

applied in the form of injection. The average percent pseudoseed formation frequency increased from 45.23 at 100 mg/l 2,4-D to 75.06 at 300 mg/l 2,4-D concentration (Table 1). The number of embryos developed per 100 florets pollinated was found to be influenced by the concentration of 2,4-D injection. The percent embryo formation obtained in the Langdon cultivar of durum wheat ranged from 2.16 at 100 mg/l 2,4-D to 14.36 at 250 mg/l 2,4-D concentration (Table 1). The cytological spreads of the meristematic root tips derived from regenerated plantlets made it evident that the plants were haploids and carried only 14 out of 28 chromosomes (Figure 1).

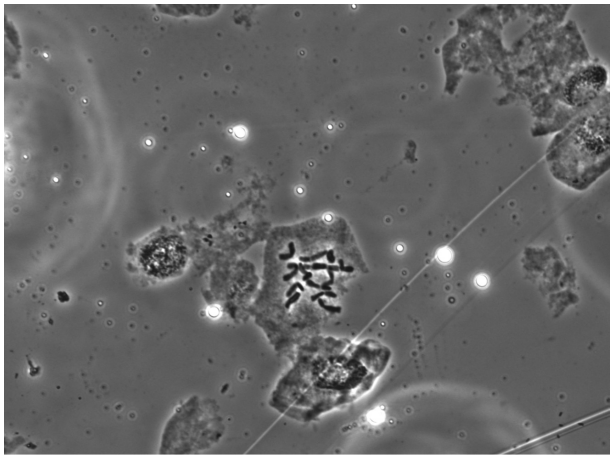

Figure 1. Metaphase spread of haploid durum wheat plantlet (n = 14)

While repeating the investigation during the next season over eight different durum wheat genotypes, haploid embryos were developed in all the genotypes with variable frequency. The analysis of variance of the data revealed significant differences between durum wheat genotypes and 2,4-D concentration for pseudoseed formation, embryo formation and haploid regeneration frequency whereas the genotype × 2,4-D interaction exhibited significant differences for pseudoseed and embryo formation frequency only (Table 2). The mean pseudoseed formation frequency for different 2,4-D concentrations over all the genotypes varied from 30. 2 to 56.3%. The mean embryo formation and haploid regeneration frequency over all the genotypes varied from 1.2% to 18.4% and 0% to 0.119%, respectively (Table 3). The mean pseudoseed formation frequency was significantly superior at 300 mg/l 2,4-D concentration whereas the mean embryo formation (18.4%) and haploid regeneration frequency (0.119%) were significantly superior at 0.025% 2,4-D concentration used for injection. At the most optimum 2,4-D concentration (250 mg/l), the haploid embryo formation and haploid regeneration frequency of durum wheat genotypes varied from 6.4% to 32.1% and 0.04 to 0.195, respectively (Table 3). The mean pseudoseed

Table 2. Analysis of variance for different haploid induction parameters in durum wheat × *Imperata cylindrica* crosses (mean sum of squares)

Source of variation	df	Pseudoseed formation frequency	Embryo formation frequency	Haploid regeneration frequency
Genotypes	7	1594*	649*	2.54*
2,4-D concentration	4	3931*	5847*	16.20*
Genotype × 2,4-D concentration	28	238*	205*	0.57
Error	360	60	23	0.78

*Significant at P = 0.05; df – degrees of freedom

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Table 3. Pseudoseed and embryo formation frequency obtained in eight different durum wheat genotypes after pollination with *I. cylindrica* using various concentrations of 2,4-D (mg/l) as post-pollination hormonal treatment

Genotype	Pseudoseed formation frequency					Embryo formation frequency					Haploid regeneration frequency				
	100	150	200	250	300	mean	100	150	200	250	300	mean	100	150	200	250	300	mean
WH 896	50.6	52.3	53.7	54.0	54.8	53.08^{a}	5.4	7.3	5.7	15.2	8.9	8.50^{b}	0	0.014	0.038	0.105	0.057	0.042^{a}
Langdon	54.2	56.2	58.9	59.1	60.1	57.70^{a}	3.1	5.2	8.9	13.1	8.1	7.68^{b}	0	0.007	0.057	0.097	0.067	0.045^{a}
A-9-30-1	13.1	31.7	38.5	54.4	60.5	39.64^{c}	0	0	10	32.1	5.8	9.58^{a}	0	0	0.056	0.195	0.036	0.057^{a}
HI 8498	33.3	38.1	37.0	42.7	57.1	41.64^{b}	0	0	0	12.5	5.4	3.58^{e}	0	0	0	0.083	0.028	0.022^{c}
PDW 233	30.9	31.8	33.6	50.6	58.0	40.98^{c}	0.7	0.7	2.3	20.4	4.5	5.72^{cd}	0	0	0.011	0.131	0.017	0.031^{abc}
PDW 291	14.3	21.5	25.9	40.0	40.8	28.50^{d}	0	0	2.8	16.6	3.9	4.66^{d}	0	0	0.009	0.133	0.022	0.032^{abc}
PDW 314	22.2	24.0	36.9	61.3	64.7	41.82^{c}	0	0	3.1	30.5	5.5	7.82^{bc}	0	0	0.003	0.169	0.028	0.04^{bc}
WHD 943	22.6	23.7	30.0	52.9	54.8	36.80^{c}	0	0	1.9	6.4	4.3	2.52^{e}	0	0	0	0.040	0.016	0.011^{c}
Mean	30.2^{e}	34.9^{d}	39.3^{c}	51.8^{b}	56.3^{a}		1.2^{d}	1.7^{d}	4.3^{c}	18.4^{a}	5.8^{b}		0^{d}	0.002^{d}	0.021^{c}	0.119^{a}	0.033^{b}	
CD (5%)			2.41			3.05			1.5			1.90			0.27			0.035

Statistical superiority (based on CD values) is represented by the order of alphabets and the same alphabets represent that the values are statistically at par within a particular character; CD – critical difference

Figure2. Durum wheat × *Imperata cylindrica*-derived embryo regenerating on the culture media

Figure3. Haploid seedlings obtained from durum wheat × *Imperata cylindrica* cross

formation, embryo formation and haploid regeneration frequency of different genotypes over all the 2,4-D concentrations ranged from 36.80% to 57.70%, 2.52% to 9.58% and 0.011% to 0.057%, respectively. The mean pseudoseed formation frequency over all the 2,4-D concentrations was significantly highest for genotypes Langdon (57.70%) and WH 896 (53.08%) whereas the genotype A-9-30-1 exhibited the significantly superior mean embryo formation frequency (9.58%). The mean haploid regeneration frequency was significantly highest for A-9-30-1 (0.057%), Langdon (0.045%) and WH 896 (0.042%) (Table 3). The regenerating embryo and green haploid seedlings obtained from durum wheat × *I. cylindrica* crosses are depicted in Figures 2 and 3, respectively.

DISCUSSION

Durum wheat is the second most important type of wheat cultivated in the wheat growing regions of the world. The genetic upgradation programmes of this important type of wheat can be accelerated to a great

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extent by achieving instant homozygosity through the production of haploids and doubled haploids. Haploid production in durum wheat will also play a vital role in producing transgenic plants through direct gene transfers. So far, the maize-mediated system of haploid production has been commonly followed to accelerate durum wheat crop improvement programmes but the frequency of haploid production is quite low (O'Donoughue & Bennett 1994a, b). In the present investigation, *I. cylindrica* was used as a pollen source for producing haploids in durum wheat when the development of haploids was observed in all durum wheat genotypes, however the frequency of haploid induction parameters was influenced by the genetic constitution as well as 2,4-D concentration. Moreover, the genotype × 2,4-D interactions were also significant for all the haploid induction parameters (pseudoseed formation and embryo formation frequency). While working on the efficiency of haploid induction in durum wheat by utilizing the wheat × maize system, Almouslem *et al*. (1998) also reported that besides genotypic specificity, haploid production is greatly affected by different 2,4-D treatment doses. In the present investigation utilizing *I. cylindrica* as a pollen source, the mean pseudoseed formation frequency over all the durum wheat genotypes increased with the increase in 2,4-D concentration, however, no such a trend was noticed in embryo formation frequency. The data pertaining to the embryo formation frequency of both seasons revealed that 250 mg/l 2,4-D is the optimum concentration to be applied as a post-pollination hormonal application. As far as the mean haploid regeneration frequency over all the genotypes is concerned, it was significantly superior at 250 mg/l 2,4-D. The increased concentration of 2,4-D beyond 250 mg/l reduced the regeneration of haploid embryos and induced the callus formation of cultured embryos on the media.

Although the frequency of haploid induction parameters varied with genotypes, the average embryo formation and haploid regeneration frequency over all the genotypes were significantly superior at 250 mg/l 2,4-D concentration. Moreover, all the genotypes produced haploids when pollinated with *I. cylindrica* pollen, indicating genotype non-specificity of the protocol. The average haploid embryo formation frequency of the durum wheat × *I. cylindrica* system recorded during the investigation carried over 8 genotypes of durum wheat is 18.4%, which is very commendable when compared with other haploid induction approaches in durum wheat. Inagaki and Hush (1998) reported that durum wheat × pearl millet crosses produced pseudoseed formation at the level of 7.2–23.7% and haploid embryos 2.1–6.4%. They attributed it to the poor crossability of durum wheat with pearl millet due to the absence of D genome in durum wheat. Ballestros *et al*. (2003) reported pseudoseed formation and embryo formation frequency of 15.2% and 5%, respectively, at the most in durum wheat × maize crosses when the relative humidity was ameliorated towards the higher side as well. Similarly, the efficiency of maize-mediated haploid induction was also reported to be as low as 13.7% in Turkish durum wheat genotypes (Savaskan *et al*. 1997). 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. The present investigation indicates that the *I. cylindrica*-mediated chromosome elimination technique is quite successful also for durum wheat. Conclusively, it can be stated that the utilization of this widely distributed natural grass, *I. cylindrica*, as pollen a source for the induction of haploids in durum wheat will be quite useful for speeding up durum wheat improvement programmes via accelerating the development of novel durum wheat cultivars, fixation of desirable recombinants and quick development of doubled haploid (DH) mapping populations. Moreover, the induction of dihaploid durum wheat with *I. cylindrica*-mediated chromosome elimination technique is more efficient and cost-effective than maize, *H. bulbosum* and pearl millet systems. The synchronization of *I. cylindrica* flowering with that of durum wheat and the natural abundance of paternal fresh pollen around the maternal parent makes the *I. cylindrica*-mediated system more advantageous.

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Corresponding author:

Shoukat Ahmad Rather, MSc., CSK Himachal Pradesh Agricultural University, Department of Crop Improvement, Molecular Cytogenetics and Tissue Culture Laboratory, 176062 Palampur, India; e-mail: shoukat.pbg@gmail