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Doubled haploid production in advanced back cross generations and molecular cytogenetic characterization of rye chromatin in triticale × wheat derived doubled haploid lines

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Abstract: The rye genome has shown potential for improvement of bread wheat, where wheat-rye substitutions and translocations have been and are frequently used in resistance breeding. Crosses belongs to different generations viz., BC_1F_1, BC_1F_2, BC_1F_3, BC_1F_4 and BC_2F_3 of triticale × wheat derived were used for different haploid induction parameters using Gogon grass (*Imperata cylindrica)* as a pollen source. The percentage of pseudo seed formation ranged from 34.55% for BC_1F_2 to 63.77 for BC_1F_1 crosses, the haploid embryo formation ranges from 9.43% for BC_1F_1 to 30.2% for BC_1F_2, the haploid plant generation ranges from 19.36% for BC_1F_2 to 63.25% for BC_1F_1. Four doubled haploids were developed from ITSN 105/58 × VL 802 × VL 802 of BC_2F_3 underwent molecular cytogenetic analyses using the probes, viz., rye genomic rDNA, pSc 119 and pAs1. FISH and GISH analysis revealed an IBL.1RS translocation and substitution of 5R chromosome instead of the 5D chromosomes in these doubled haploids.

Abbreviations

FISH:	Fluorescence *in situ* hybridization
GISH:	Genotypic *in situ* hybridization
2,4-D:	2,4-dinitrophenylhydrazine
SSC:	Saline sodium citrate,
BSA:	Bovine serum albumin,
DAPI:	4',6-diamidino-2-phenylindole

Introduction

Among the food grain crops of the world, wheat (*Triticum aestivum* L. em Thell) is pre-eminent regarding its antiquity and importance as a food of humankind (Arjona *et al.*, 2020). There has been a tremendous increase in wheat production in India since the time of the Green Revolution. This has been possible with the introduction of the dwarf wheat genotypes that carrying the dwarfing genes viz. *Rht-B1b* and *Rht-D1b* from Norin-10-Brevor-14 background (Borner *et al.*, 1996) and the genes for photo-insensitivity from Mexican spring wheat. Wheat improvement has led the country's efforts to reach the status of self-sufficiency in food grain production (Rani and Mor, 2020). However, at present, production is affected by climate change and increase in pest and diseases infestation (Dar *et al.*, 2020; Wani *et al.*, 2020). To address this problem, new disease resistant genes need to be introgressed from cultivars/wild resources (Pietrusińska *et al.*, 2018; Klymiuk *et al.*, 2019). In the mountainous regions of India, wheat is generally grown under diverse and rainfed conditions (Dar *et al.*, 2020) Thus, drought becomes the major constraint, followed by frost stress and susceptibility to various diseases, which drastically reduce wheat production in these areas. Therefore, the breeding objectives must be essentially comprised of the development of high yielding varieties resistant to abiotic (drought and frost) and biotic (rusts and powdery mildew) stresses prevalent in this region. Winter wheat and rye are the important sources can be used for the transfer of resistance for biotic and abiotic stresses to spring wheat.

Triticale (× *Triticosecale* Wittmack) may be used as a bridging species to accomplish the transfer of the rye chromatin into the background of wheat. Because of rye

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chromosome complement, triticales have many agronomic attributes not found in wheat (Merker, 1984). To isolate promising recombinants from the segregating populations, careful selection of parents is required on the part of breeders for triticale × wheat hybridization programs. To achieve faster desirable results, doubled haploid breeding has a significant advantage over conventional breeding, where it leads to the production of completely homozygous plants in just a single step (1 year). In contrast, the conventional breeding approach takes about 7–8 years to isolate stable lines from the crosses. Bread wheat has been improved using the rye genome as a potential resource, where wheat-rye translocations and substitutions have been and are commonly used in resistance breeding (Rabinovich, 1998), and the 1B/1R wheat-rye translocation is incorporated in most of the wheat currently grown around the world (Heslop-Horrison *et al.*, 1990). Doubled haploid breeding helps to attain homozygous populations from the triticale × wheat hybrids and their backcrosses following chromosome elimination approach through the use of *Imperata cylindrica*-mediated systems. It is further required to enhance the precision and efficiency of selection amongst the newly developed wheat doubled haploids to identify alien chromatin/genes introgressed with minimal introgression of undesirable genes. The molecular cytogenetic approach gives various powerful and novel tools such as genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) which can help in the physical mapping of introgressed chromatin/genes into wheat genome. The present investigation envisages the development of triticale × wheat-derived doubled haploids involving triticale and elite wheat lines following intergeneric hybridization with *I. cylindrica* and identification and characterization of rye chromatin introgressed into wheat genome (developed through DH breeding) through FISH and further isolation of wheat like doubled haploids having the desired rye chromatin with minimal undesirable genes.

Materials and Methods

Plant material

Triticale × wheat (Tab. 1) derived populations of different backcross generations used for doubled haploid production following chromosome elimination approach by using *Imperata cylindrica* pollen and further utilized to detect rye chromatin introgression using GISH and FISH techniques. The present work was carried out in the Molecular Cytogenetics Laboratory of Department of Crop Improvement, CSK HP Agricultural University, Palampur, H.P, India.

Wide hybridization procedure

Emasculation was done three days before anthesis by removing the anthers manually. The next day, fresh pollen from *I. cylindrica* was collected and applied to the feathery stigma of the emasculated spikes. On third day, the spikes were injected with a 2,4-D solution of 250 mg/L concentration (Pratap and Chaudhary, 2007) at the base of the uppermost internode using a syringe fitted with a fine hypodermic disposable needle. Petroleum jelly (Vaseline-Hindustan Lever, Ltd.) was used for sealing the injection holes. The injections were repeated for two more consecutive days to ensure proper seed and embryo formation. Murashige and Skoog medium (Murashige and Skoog, 1962) was used to rescue haploid embryos. This medium was supplemented with 0.5 mg/L kinetin, 150 mg/L glutamine, 20 mg/L each arginine, cysteine and leucine, and solidified agar. The pollinated spikes were harvested from the tiller base after 18–20 days of pollination. The embryo carrying seeds were identified using the technique of Bains *et al.* (1998). The embryos were removed under strict aseptic conditions and placed on the culture medium in the test tubes. Cultured immature embryos were given cold treatment at 4°C temperature in dark for first 24 h. After that, they were incubated in the dark in the Plant Growth Chamber at 25 ± 1°C for regeneration for about a week till the roots and shoots initiated. The regenerated plantlets were then transferred to the other section of the Plant Growth Chamber at 25 ± 1°C with 10/14 h light/dark profile for plants' proper development. The haploid plantlets were transferred to rooting medium for profuse rooting, then potted in soil mixture for hardening and later treated with 0.1% colchicine solution for chromosome doubling. The haploid plantlets were treated with colchicine at three to five tiller stages according to the method given by Inagaki (1985) with slight modifications. Each haploid plant's crown was submerged in a 0.1% colchicine solution supplemented with 1.5% dimethyl sulphoxide at 20°C for 5 h. The treated plants were kept in the running tap water for 20 min, then potted in soil and maintained in the cage house up to maturity.

Recording of observations

Observations were recorded with respect to haploid induction traits on per cent basis as follows:

$$\textit{Pseudo seed formation frequency} = \frac{\textit{Number of pseudo seeds formed}}{\textit{Total number of florets pollinated}} \times 100$$

$$\textit{Embryo formation frequency} = \frac{\textit{Number of pseudo seeds carrying embryo}}{\textit{Total number of pseudo seeds formed}} \times 100$$

$$\textit{Haploid plantlet regeneration frequency} = \frac{\textit{Number of haploid plantlets developed}}{\textit{Total number of embryos cultured}} \times 100$$

These data were transformed to definite value using Arcsine transformation. The significant difference for various haploid induction parameters, namely pseudo seed formation, embryo formation and haploid regeneration frequencies was analysed by simple *t*-test.

Molecular cytogenetics

Genomic and fluorescence in situ hybridization procedure

Four doubled haploid lines derived from (ITSN 105/58 × VL 802) × VL 802 crosses of BC_2F_3 generation of the present investigations were utilized in this study for molecular cytogenetic analysis following genomic *in situ* hybridization (GISH), and Fluorescence *in situ* hybridization (FISH)

TABLE 1

Wheat and triticale lines used in the present investigation

S.No.	Genotype	Parentage	Source
	Wheat		
1	HPW 89	INTERMEDIO RODI/HD 2248	CSKHPKV, Palampur
2	HPW 155	BT 2549/FATH	-do-
3	HPW42	VEE'S'/4/PVN'S'/CBB//CNO'S'/3/JAR/ORZ'S'	-do-
4	DH 776	Pnfjoumee × HPW 143	-do-
5	W5	Selection Local Potia	DHY
6	VL 802	CPAN3018/CPA N 3004//PBW65	VPKAS, Almora
7	HS 396	–	
8	RL-14-1	–	
	Triticale		
1	TL 1210	CINNAMON/RAJ 821//IN 19-Turkey 602/3/AYMC	PAU, Ludhiana
2	TL 2920	PBW 189/WHITE RYE/JNIT 128	-do-
3	TL 2900	–	-do-
4	TL1217	–	-do-
5	ITSN 65	–	-do-
6	ITSN 105/58	–	-do-
	Imperata cylindrica	Wildly growing weedy grass in the surroundings of Experimental Fields at Palampur	-do-

approaches (Yamamoto and Mukai, 1989; Mukai *et al*., 1993) to identify and characterize the introgressed rye chromatin and isolate wheat like recombinants with less undesirable genes. Molecular Probes (Tab. 2) viz., Genomic probe of rye, Ribosomal DNA probe (pTa 71) and repetitive DNA sequences probes (pSc119 and pAs1) were used to detect and characterize the alien introgressions (Yamamoto and Mukai, 1989). All the probes were labelled with the haptens viz., biotin-16-dUTP (Vitamin H) and digoxigenin-11-dUTP (Steroid) following the nick translation protocol given by (Maniatis *et al*., 1975). Detection of the labelled sites was executed by the fluorophores viz., fluorescein-conjugated streptavidin and rhodamine-conjugated anti-digoxigenin.

Hybridization signals were detected with an Olympus fluorescence microscope equipped with a filter for FITC (fluorescein isothiocyanate), a filter for rhodamine and a triple-band filter set for FITC, DAPI (4',6-diamidino-2-phenylindole) and rhodamine. Images were captured using an Olympus CCD (charge-coupled device) camera.

TABLE 2

Molecular probes

S. No.	Probe	Source
1.	Rye Genome	Total rye genome DNA from Himalayan collection
2.	pTa 71	45S rDNA from *Triticum aestivum*
3.	pSc 119	*Secale cereale*
4.	pAs 1	*Aegilops squarrosa*

Statistical Analysis

The data were transformed to definite value using Arcsine transformation (Warton and Hui, 2011). The significant difference for various haploid induction parameters, namely pseudo seed formation, embryo formation and haploid regeneration frequencies was analysed by simple *t*-test.

Results

Doubled haploid breeding

The pseudo seed formation data are provided below (Tab. 3). The data were transferred from percentage to absolute value for easy analysis using arcsine transformation. The pseudo seed formation was lower in the crosses of BC_1F_2, whereas higher in the crosses of BC_1F_4 generations. Variation in the pseudo seed formation might be due to genotype specificity in the crosses. The embryo formation was lower in the crosses of BC_1F_1, whereas higher in the crosses of BC_2F_3 generations. It may happen because all the pseudo seeds might not be containing the haploid embryo due to the chromosomal disharmony between the triticale × wheat derivatives and *Imperata cylindrica*. Although successful wheat haploid formation after crossing with *I. cylindrica* has been reported through cytological evidence of fertilization of parental gametes and further complete elimination of *I. cylindrica* chromosomes. The haploid plant regeneration was lower in the crosses of BC_1F_3 and higher in the crosses of BC_1F_1 generations. The recovery of doubled haploids was less after colchicine treatment due to the mortality of haploid plants. The cross (ITSN 105/58 × VL 802) × VL 802 × VL 802 of BC_2F_3 generation was yielded four doubled

TABLE 3

Performance of various crosses in respect of different haploid induction parameters in triticale × wheat derivatives through *Imperata cylindrica*-system in BC_1F_1 to BC_1F_4 and BC_2F_3 generations

S. No.	Generation/Crosses	(Triticale × wheat) derivatives × *Imperata cylindrica*			
		No. of florets pollinated	sf (%)	ef (%)	hpr (%)
	BC_1F_1				
1	ITSN 65 × HPW 155 × HPW 155	386	46.88 (206)	20.41* (25)	50.00 (15)
2	ITSN 65 × HPW 89 × HPW 89	152	47.87 (82)	18.97* (9)	31.10 (3)
3	TL 1210 × W 5 × W 5	311	49.85 (183)	16.27 (14)	42.11 (9)
4	TL 1217 × HPW 42 × HPW 42	182	55.22* (123)	21.17* (16)	51.89* (10)
5	TL 2920 × DH 776 × DH 776	348	63.77* (280)	15.03 (19)	49.33 (11)
6	TL 2920 × HS 396 × HS 396	366	43.33 (173)	9.43 (5)	42.75 (2)
7	TL 2920 × W 5 × W5	130	59.33* (96)	17.62 (9)	63.25* (7)
	Mean		52.32	16.99	47.20
	SE(m)±		1.09	0.56	1.47
	BC_1F_2				
1	ITSN 105/58 × HS 396 × HS 396	1742	34.55 (582)	12.91 (28)	19.36 (3)
2	TL 2900 × VL802 × VL 802	209	38.36 (80)	30.21 (21)	65.96 (17)
	Mean		36.36	21.56	42.66
	SE(m)±		0.38	1.62	4.36
	BC_1F_3				
1	ITSN 105/58 × VL 802 × VL 802	1338	46.9 (715)	17.37 (65)	38.60 (25)
2	ITSN 105/58 × HPW 89 × HPW 89	1400	51.24 (850)	17.27 (75)	46.22 (39)
	Mean		49.07	17.82	42.41
	SE(m)±		0.35	0.01	0.61
	BC_1F_4				
1	ITSN 105/58 × VL 802 × VL 802	288	56.24* (200)	25.68* (38)	45.00 (19)
2	TL 2900 × VL 802 × VL 802	2481	56.65* (1730)	19.79 (198)	23.76 (31)
3	TL 2920 × VL 802 × VL 802	76	48.31 (42)	14.87 (3)	56.08* (2)
	Mean		53.73	20.11	41.61
	SE(m)		0.53	0.61	1.85
	BC_2F_3				
1	ITSN 105/58 × VL 802 × VL 802 × VL 802	6463	42.54 (2955)	29.51 (718)	26.61 (144)
2	ITSN105/58 × RL-14-1 × RL-14-1 × RL-14-1	1397	43.14 (653)	27.77 (142)	51.77 (88)
	Mean		42.84	28.64	39.19
	SE(m)		0.03	0.08	1.16

haploids. The pseudo seed formation data are provided below (Tab. 3). The data were transferred from percentage to absolute value for easy analysis using Arcsine transformation. The pseudo seed formation was lower in the crosses of BC_1F_2, whereas higher in the crosses of BC_1F_4 generations. Variation in the pseudo seed formation might be due to genotype specificity in the crosses. The embryo formation was lower in the crosses of BC_1F_1, whereas higher in the crosses of BC_2F_3 generations. This may happen because all the pseudo seeds might not be containing the haploid embryo due to the chromosomal disharmony between the triticale × wheat derivatives and *Imperata cylindrica*. Although successful wheat haploid formation after crossing with *I. cylindrica* has been reported through cytological evidence as the elimination of complete set of *I. cylindrica* chromosomes. The haploid plant regeneration was lower in the crosses of BC_1F_3 and higher in the crosses of BC_1F_1 generations. The recovery of doubled haploids was less after colchicine treatment due to mortality of haploid plants. The cross (ITSN 105/58 × VL 802) × VL 802 × VL 802 of BC_2F_3 generation was yielded four doubled haploids.

FISH and GISH analysis in triticale × wheat-derived doubled haploids

The four doubled haploid lines TWDH 1, TWDH 2, TWDH 4 and TWDH 5 (Figs. 1–4) derived from the BC_2F_3s of ITSN

FIGURE 1. Spike and seeds of the doubled haploid, TWDH 1.

FIGURE 2. Spike and seeds of the doubled haploid, TWDH 2.

105/58 × VL 802 × VL 802 were also subjected to molecular cytogenetic analysis using probes viz., rye genomic rDNA, pSc119 and pAs1 (Tab. 4). The lines TWDH 1, TWDH 2 (Figs. 5 and 6), and TWDH 4 were possessing 1BL.1RS translocation. The photographic plates of spikes and seeds of TWDHs are given in Figs. 1 to 4. The GISH analysis revealed that 1RS replaced the translocation chromosome's short arms due to its obvious satellite. The line TWDH 1 (Fig. 2), apart from 1BL.1RS translocation, showed 5D (5R) chromosome substitution and the line TWDH 5 exhibited 5D (5R) chromosome substitution.

Discussion

Pseudo seed formation

The good triticale response to *Imperata cylindrica* induced haploid induction has also been reported in the few studies carried out earlier. Kishore *et al.* (2011) had reported 21.3%

FIGURE 3. Spike and seeds of the doubled haploid, TWDH 4.

FIGURE 4. Spike and seeds of the doubled haploid, TWDH 5.

pseudo seed formation in BC_1F_1 and 46.8% in BC_1F_2, and Pratap and Chaudhary (2007) had reported similar results. Chaudhary *et al.* (2015) findings of enhanced production of pseudo seed (30.2 to 56.3%) were also corroborated with the present investigation. The successful seed set obtained in this investigation (62.79%) was close to the value reported by Matzk and Mahn (1994) with 12 wheat genotypes. Percent of seed set represents the efficiency of emasculation and pollination procedures. The lack of embryos in the F_1s of triticale × wheat in the current study indicated that either fertilization did not take place or embryo development was stalled at an early stage. The problem seemed to be accentuated due to the chromosomal imbalance in the gametes produced by triticale × wheat F1s. With the idea of improving chromosomal balance in the gametes of

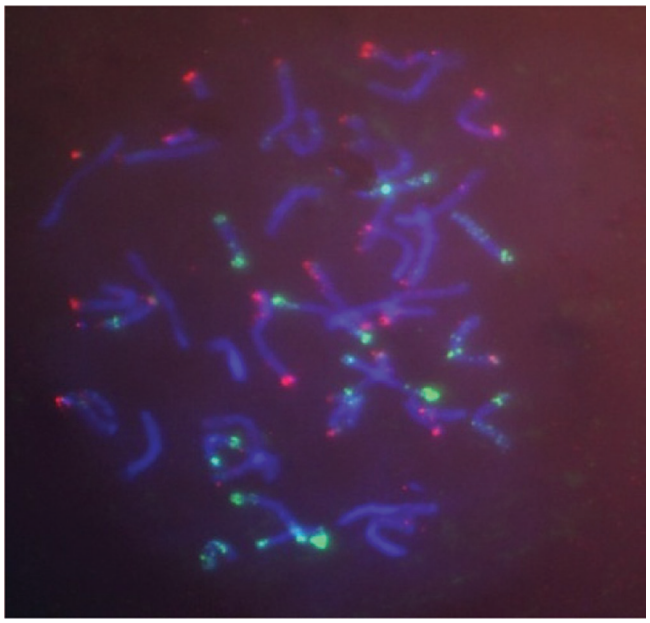

FIGURE 5. Detection of 1BL· 1RS translocation in triticale × wheat derived doubled haploid bread wheat line, TWDH 2 with the probe Bio: pAs1 (green), Dig: pSc 119 (red).

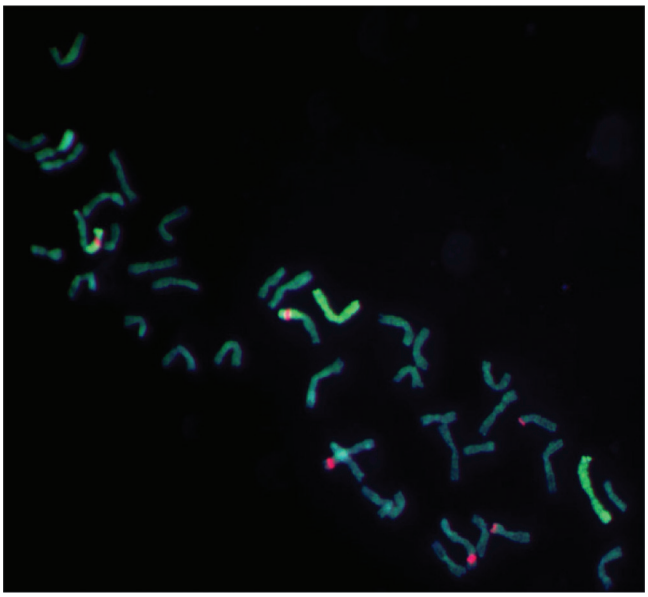

FIGURE 6. Detection of 1 BL· 1 RS translocation and 5D (5R) substitution in triticale × wheat derived doubled haploid bread wheat line, TWDH 1 with the probe Bio: rye genomic (green), Dig: rDNA (red).

triticale × wheat crosses, the F_1s were advanced to the subsequent generations by backcrossing to wheat or selfing. Advanced generations (BC_1F_1, BC_1F_2, BC_1F_3, BC_1F_4 and BC_2F_3) were used for attempting crosses with maize employing 250 ppm 2, 4-D hormonal treatment. This kind of hormonal modification was used by Pratap *et al.* (2004); Chaudhary *et al.* (2002) with 20% pseudo seed production. However, variation was observed even within a group of lines derived from the same triticale × wheat cross where some lines had very low to no response to maize-mediated induction. Again, a probable reason may be the imbalanced chromosome complement of these lines. These results are in correspondence with the previous studies by Gill *et al.* (2008). They reported low seed set and no embryo development in F_1 lines crossed with maize. Overall, the approach has high feasibility as a rapid technique for generating chromosome transfers between triticale and wheat. Successful seed formation was reported by Pratap *et al.* (2005) in all the 15 triticale × wheat hybrids fertilized with maize, *Imperata cylindrica*, pearl millet, sorghum pollen which suggests that these species effect fertilization in triticale × wheat hybrids, thereby stimulating seed formation. Similar studies were conducted by Mahato and Chaudhary (2015) involving seven diverse durum wheat genotypes and using two composite varieties of Himalayan maize, viz., Bajaura Makka and Early Composite and a wild grass, *I. cylindrica*, as pollen sources. Their result showed that *I. cylindrica* performed better for haploid induction in durum wheat over maize in terms of pseudo seed formation (46.93%), embryo formation (38.06%), haploid regeneration (40.42%) and haploid formation efficiency (7.44%). Authors opined the use of durum wheat × *I. cylindrica* as a superior technique over the maize-mediated system, and its large-scale use could open a new horizon in the sphere of durum wheat doubled haploid breeding programme.

Embryo formation

Very few investigations have been reported to produce Doubled Haploid (DH) on triticale × wheat cross using *I. cylindrica*. Several technical problems affect the haploid embryo production efficiency. The genotypes pollinated with *I. cylindrica* pollen coupled with the post-pollination treatments efficiently produced haploids and doubled haploids. However, in cross combinations, the frequency of both haploid embryo development and DH production varied considerably. *Imperata cylindrica* was crossable with all the triticale × wheat-derived F_1 hybrids, as shown by pseudo seed formation and embryo formation in all wheat genotypes. These hybrids' parental lines did not carry recessive crossability alleles *kr1* and *kr2* except for variety C 306. Thus, the wheat × maize system, as demonstrated earlier (Singh *et al.*, 2005; Bakos *et al.*, 2005), is independent of crossability alleles *kr1* and *kr2*. Similarly, *I. cylindrica* also independent of crossability problem observed by Jamwal *et al.* (2016) in triticale × wheat recombinants used for DH production. Many possible reasons for the effect of the environment on haploid embryo recovery have been reported in other similar investigation (Pienaar *et al.*, 1996). The variation in glasshouse condition could affect the pollen viability and wheat fertilization. Similarly, environment influenced durum wheat embryo survival in a genotypically dependent manner (Donoughue and Bennett, 1994). As reported, the performance of cultivar 'Rampton Rivet' for embryo recovery was significantly better in a 20°C growth room than in an unheated glasshouse as compared to cultivars 'Wakona' and 'Chinese Spring'. Campbell *et al.* (1998) evaluated the effects of temperature and light intensity on wheat genotypes crossed with maize pollen and showed that both could significantly affect haploid embryo numbers. They also reported that the light intensity of 1000 μmol/m^2 s produced the greatest number of embryos (38% of florets pollinated) compared to the optimal temperature (22/17°C) embryo recovery. Overall germination

TABLE 4

Molecular cytogenetic analysis of DH lines derived from triticale × wheat advanced generations

Sr. No.	Line Name	Probe used	Ch No.	Result obtained
1.	TWDH 1	Bio: rye genomic,pAs1 Dig: rDNA, pSc 119	42	1BL.1RS translocation 5D(5R) substitution
2.	TWDH 2	-do-	42	1BL.1RS translocation
3.	TWDH 4	-do-	42	-do-
4.	TWDH 5	Bio: rye genomic; pSc 119 Dig: pAs1,r DNA	42	1BL.1RS translocation 5D(5R) substitution

rates were still low (~40% of all embryos) irrespective of variety. Low germination rates (43%) of wheat × maize cross-derived embryos were consistently observed (Inagaki and Tahir, 1990). Similarly, the non-development of embryos into plants in this investigation appeared due to vitrification or hyperhydricity. Badiyal *et al.* (2016) study also corroborated with present investigation for the haploid embryo formation using *I. cylindrica* as a pollen source. The chromosome elimination study (Komeda *et al.*, 2007) indicated that *I. cylindrica* chromosomes were eliminated during the first mitotic cell division, whereas maize chromosomes were eliminated after two to three cell divisions. This gives a clear picture of the haploid nature of embryos obtained.

Haploid plant regeneration

The present investigation of plant regeneration ranged from 19.36% to 65.69% for different triticale × wheat-derived generations. One of the factors limiting the further development of a germinated embryo to a plantlet is the embryos' deficiency in one of their polarity (poles) to obtain shoot or root induction. In such cases, meristems are not properly formed (Lefebvre and Devaux, 1996). More florets can be pollinated for getting more haploid plants. Earlier investigations have revealed that spikelet positions (lower, middle, and upper) determined the success ratios for embryo initiation (Martins-Lopes *et al.*, 2001). However, Bitsch *et al.* (1998) previously observed that embryo initiation was found to be distributed evenly all over the wheat spike. Similar results were obtained by Tayeng *et al.* (2012) in wheat × wheat recombinants using *I. cylindrica* as a pollen source. In a normal wheat × maize crossing process, 25–30 florets are generally pollinated per spike, and the synchronization of flowering decides the increase in the number of florets to be pollinated. Better space planting of the wheat plants might increase the number of spikes for pollination. Similar results of efficient haploid induction by *I. cylindrica* were obtained by Rather *et al.* (2014) for 21 F_1 wheat crosses assessed for their haploid induction efficiency when crossed with four Indian and one Japanese accession of *I. cylindrica*. Authors concluded that both wheat and *I. cylindrica* genotypes influenced haploid induction and the accession Ic-Aru, performed better as a pollen source.

FISH and GISH analysis in triticale × wheat-derived doubled haploids

In the present investigation, doubled haploid line TWDH2 possessed IBL.IRS translocation, amber colour seed and good spike type and the line TWDH1 showed 1BL. 1 RS translocation and 5R (5D) substitution. A similar result was obtained by Carvalho *et al.* (2009) and Efremova *et al.* (2014) in wheat × rye crosses using fluorescent *in situ* hybridization (FISH) performed with genomic DNA probes for genomic *in situ* hybridization (GISH) from rye. Among the 55 plants, a wheat-rye translocation was detected in one plant after GISH. Recombinant chromosomes were identified using probes pTa71 and pSc119.2. Badaeva *et al.* (2002) reported that the repetitive DNA probe pAs1 was not only hybridized well with D-genome chromosomes of wheat but was also successfully used in the identification of specific chromosomes having different colour bands. In our investigation, the identification of D chromosomes present in the line TWDH2 was made using the probe pAs1. Tan *et al.* (2009) used FISH and suggested that line 15-3-2 possessed all 14 D-genome chromosomes and chromosome 5U, concluding that line 15-3-2 was a new synthetic wheat – *Aegilops biuncialis* partial amphiploid, and could be used to transfer the disease resistance genes to wheat. Georgiev (2008) reported that after GISH, it was obtained that the mutant forms *K1* and *K2* of *Triticum aestivum* carried the 1B/1R chromosome translocation. Altuntepe and Jauhar (2001) study of GISH in haploid plants derived from durum substitution lines also supports the current investigation results. An *et al.* (2015) developed WR49-1 using sequential GISH (genomic *in situ* hybridization), mc-FISH (multicolour fluorescence *in situ* hybridization), mc-GISH (multicolour GISH) and EST (expressed sequence tag)-based marker analysis, WR49-1 proved to be a new wheat-rye 6R disomic addition line. Yang *et al.* (2016) observed that the newly developed wheat-rye addition line N9436B possessed two rye chromosomes. A similar result of wheat-rye translocation T1RS.1BL was obtained by Ren *et al.* (2017) from the progeny of the crossing of the wheat cultivar Mianyang11-1 and a Chinese local rye variety, Weining. Two novel translocation lines were identified by molecular cytogenetic analysis and the results also revealed that the pSc119.2 signals of 5AL were absent in both lines along with the pSc119.2 signals of 4AL of RT828-11. Liu *et al.* (2017) was also observed one 1B(1R) substitution line and five 1BL.1RS whole-arm translocation lines. Most of the recombinant lines were associated with important alien chromatin translocation like 1BL/1RS, substitutions 1R (1D), 5R (5D) and combination of both, i.e., 1BL/1RS + 5R (5D) and in some cases presence of more than 4 rye chromosomes (Jeberson *et al.*, 2021).

Conclusions

Very few investigations have been conducted using *Imperata cylindrica* as a pollen sources for crossing different generations of triticale × wheat derivatives. Therefore, from the above study, it can be inferred that *I. cylindrica* is a useful pollen source for efficient doubled haploid production in triticale × wheat advanced generations. Doubled haploids possessing an alien introgression of rye chromatin were identified through the molecular cytogenetic analysis viz., FISH and GISH technique, which are efficient techniques for detecting alien addition, translocation, and substitution lines. The developed doubled haploid lines can be released as varieties for use in future wheat breeding programmes.

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