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Comparative Pollination Efficiency of Freshly Harvested Pollen of *Imperata cylindrica* and *Zea mays* for Haploid Induction in Bread Wheat

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Pollen viability is among the critical factors, which determine the success of a doubled haploidy breeding programme, thus the present investigation was undertaken to assess the functional viability and relative longevity of the pollen from *Zea mays* and *Imperata cylindrica* for the chromosome elimination mediated approach of doubled haploidy breeding. Two wheat genotypes representing spring and winter ecotypes, namely DH 40 and Saptdhara were pollinated with freshly harvested pollen of two known potential haploid inducing sources, namely *Z. mays* (grown in polyhouse conditions) and *I. cylindrica* (wild grass) for 15 and 17 days, respectively, keeping five minutes interval between two subsequent pollinations. The results revealed a significant decrease in the viability of *Z. mays* pollen up to 95 percent in Saptdhara and 85 percent in DH 40 within one hour, whereas *Imperata* pollen was found to be functionally viable even at the end of experimentation period, showing high embryo formation in both the wheat parents. *I. cylindrica* exhibited higher frequencies for haploid induction parameters in both the wheat parents as compared to *Z. mays*. *I. cylindrica* pollen, being viable for relatively longer periods than *Z. mays* can hasten the haploid induction endeavours, thus may be a more efficient alternative to *Z. mays* for breeding programmes using doubled haploidy technique.

Keywords: pollen viability, *Imperata cylindrica, Zea mays,* haploid induction

Introduction

Wheat is the world's largest and most important food crop for direct human consumption and hence wheat improvement is paramount for feeding the ever-increasing population. Genetic enrichment as well as diversification has remained the mainstay of wheat improvement programmes across the world for sustainable crop improvement. Introduction of novel genes/alleles in wheat can be a stepping stone in the achievement of these goals. Wild relatives and related species of wheat offer a reservoir of novel genes but transfer of chromatin from them is a big challenge. The only practically feasible approach to introduce their chromatin into the wheat background is through wide hybridization of wheat and the alien donor. Generating diversity followed by stable heritability for a trait is the

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essential prerequisite of the crop improvement programmes. Traditionally, it may take up to seven years for stable integration and inheritance of alien genes in wheat but doubled haploidy breeding has reduced the time span to two years for obtaining a completely homozygous population. A dynamic phenomenon-chromosome elimination, occurring during the wide hybridization of wheat and its distant relatives has greatly facilitated haploid induction thus, hastening fruitful genetic introgressions. Among various chromosome elimination approaches, wheat × *Z. mays* system (Laurie and Bennett 1988) has been used worldwide for effective haploid induction. Recently wheat × *Imperata cylindrica* (Chaudhary et al. 2005) system has emerged as a highly efficient alternative for revolutionizing the doubled haploid production scenario. This system has responded to haploid induction significantly better than wheat × *Z. mays* system, not only in wheat × wheat hybrids (Chaudhary et al. 2005; 2013a and 2013b; Chaudhary 2008a; 2008b; 2009; 2010a; 2010b; 2012; 2013a and 2013b; Kaila et al. 2012; Rather 2012; Tayeng et al. 2012) but also been explored in triticale × wheat (Pratap et al. 2005; Pratap and Chaudhary 2007; Badiyal et al. 2014) and wheat × rye derivatives (Kishore et al. 2011) efficiently, where wheat × maize system failed altogether.

The success of any hybridization programme (especially wide hybridization) critically depends on the combining ability of gametes towards embryo formation which ultimately depends on the viability of the pollen and its compatibility with the female gametophyte (Bots and Mariani 2005). Even the success of chromosome elimination mediated doubled haploidy breeding is determined by the functional viability of the pollen. A rapid loss in pollen viability would greatly affect the efficiency of pollination and seed set. Among various methods for determining the viability of pollen (Dafni 1992; Kearns and Inouye 1993), artificially pollinating flowers and assessing seed production seems to be the most accurate means. Various studies in past have revealed that in favourable environmental conditions (high relative humidity, low temperature and cloudy day) maize pollen can survive up to 4 hours but in adverse conditions like low humidity, high temperature and sunny day, the complete loss of viability occurs in just half an hour (Luna et al. 2001; Roy et al. 1995; Aylor 2003; Muui et al. 2007). But all such studies were conducted either in the lab through staining or *in vitro* germination techniques (Roy et al. 1995; Dafni and Firmage 2000; Luna et al. 2001) or through seed set frequency in the corn fields (Muui et al. 2007).

The uniqueness of the present research investigation lies in the assessment of pollen viability of maize through haploid induction parameters. As wheat × *I. cylindrica*-mediated approach is novel for the breeders worldwide, no studies regarding this aspect have been reported anywhere although Rather (2012) has reported that *I. cylindrica* pollen stored at –20 °C for one week can be effectively utilized for haploid induction.

Keeping all the above in view, the present investigation was designed to evaluate the functional viability and the relative longevity of freshly harvested *Z. mays* and *I. cylindrica* pollen. This will help to estimate the time for effective pollination for efficient haploid induction and thus accelerate the wheat improvement endeavours with increased precision and efficiency.

Materials and Methods

The present investigation was conducted at the Department of Crop Improvement, CSK HP Agricultural University, Palampur, India (32°5'46"N 76°32'43"E) during 2013–14. The maternal genotypes included spring wheat genotype, DH 40 (with parentage Saptdhara/HW 3024) and winter wheat genotype, Saptdhara [selection from Atou (Cappelle/Garnet)]. A north-west Himalayan leading Maize *(Z. mays*) composite, Bajaura Makka and a wildly growing perennial winter grass, cogon grass (*I. cylindrica*) were used as the pollen sources. Maize (*Z. mays*) was grown in the polyhouse in staggered manner for coinciding the timing of the wheat and flowering with that of Cogon grass (*I. cylindrica*) wildly growing on the bunds of wheat fields were selected as the pollen parents. The parental material was staggered sown with a 10 days interval to ensure regular availability of crossable material for enough replications of the experiment. Two rows of each genotype were sown with 1 m row length and 25 cm row × row distance following recommended package of practices. The wide hybridization programme using the two pollen sources i.e. maize and *I. cylindrica* was exercised following haploid induction protocol given by Laurie and Bennett (1986 and 1988) and Chaudhary et al. (2002 and 2005) during summer 2014. For embryo rescue, the amenities at Molecular Cytogenetics and Tissue Culture Lab, CSKHPAU, Palampur were utilized.

Manual emasculation was done three days before anthesis. Emasculated spikes were pollinated by freshly harvested *Z. mays* and *I. cylindrica* pollen simultaneously within one hour duration, keeping five minutes gap between two subsequent pollinations. It is pertinent to mention that the pollen of *Z. mays* and *I. cylindrica* was collected separately but used simultaneously to pollinate 13 spikes each of the wheat ecotypes ($13 \times 2 = 26$ spikes) for inducing haploids. The spikes were numbered 1 to 13 on butter paper bags as well as on the tags to specify the time. The pollination procedure was repeated for 32 days (15 days and 17 days, respectively for each ecotype depending on anthesis). Pollination with the freshly harvested pollen of each pollen source was used as control and numbered 0. Additionally, five spikes of each wheat genotype were left unpollinated. 2,4-D (100 ppm) was injected at the uppermost internode 24 hours after pollination and repeated for two consecutive days. The pseudoseeds were harvested 18–20 days after pollination and embryo carrying seeds were screened by putting them against light source. Pseudoseeds were excised under strict aseptic conditions and embryos were cultured on MS medium supplemented with 0.5 mg/L kinetin, 20 mg/L each of L-arginine, L-cysteine and L-leucine, 30 g/L sucrose and 8 g/L agar agar. The cultured embryos were incubated in the dark at 20 ± 2 °C for regeneration. The regenerated plantlets were then placed in the growth room at 20 ± 2 °C with 10/14 hours light/dark regime until they developed sufficiently. At 3–5 leaf stage the haploid plants were subjected to colchicine treatment (0.05% colchicine solution +1.5% DMSO) for five hours and transplanted into pots.

Observations were recorded with respect to total number of florets pollinated, number of pseudoseeds, number of embryo carrying pseudoseeds and regeneration of embryos in each cross using the formulae given below:

$$\text{Pseudoseed formation frequency (\%)} = \frac{\text{Number of pseudoseeds formed}}{\text{Total number of florets polinated}} \times 100$$

$$\text{Embryo formation frequency (\%)} = \frac{\text{Number of pseudoseeds carrying embryos}}{\text{Total number of pseudoseeds formed}} \times 100$$

$$\text{Embryo regeneration frequency (\%)} = \frac{\text{Number of haploid plantlets developed}}{\text{Total number of embryos cultured}} \times 100$$

The significance of difference for haploid induction parameters, viz. pseudoseed formation, embryo formation and embryo regeneration frequencies was analyzed using Student's *t*-test. Variance among the embryo formation frequency has been analyzed using completely randomized block design (Cochran and Cox, 1960).

Results

The present investigation was focused on the estimation of relative longevity of pollen of *Z. mays* and *I. cylindrica* and their role in efficient haploid induction. The viability of freshly harvested pollen from respective male parents was studied in the field conditions for one hour after collection. A total of 780 spikes of each wheat genotype were utilized in the experiment during the summer, 2014.

The pseudoseed formation in the spring wheat genotype, DH 40, ranged from 64.42 to 76.08 percent with an average of 68.98 percent, when pollinated with *Z. mays*. Similar was the scenario with the winter wheat genotype, Saptdhara, where the pseudoseeds formed in the range 42.68 to 49.96 percent with an average of 46.98 percent on pollination with *Z. mays*. With *I. cylindrica,* both the genotypes exhibited an average of 72.85 and 51.76 percent pseudoseed formation with their ranges falling between 67.69–78.40 and 47.62–57.52 percent, respectively. No particular pattern in the pseudoseed formation was noticed in both wheat genotypes. It has been advocated by many researchers that pseudoseed formation is dependent on the auxin treatment (Brazauskas et al. 2005; Pratap and Chaudhary 2012). For investigating the effect of growth hormone 2,4-D simultaneously, five spikes each in case of Saptdhara and DH 40 were left unpollinated and injected with 2,4-D as per the standard procedure. All the spikes produced imbibed pseudoseeds which confirmed the earlier observations.

Table 1. Analysis of variance for embryo formation

Cross	df	EMS (df)	MS
DH 40 × Maize	12	19.56 (156)	51.59*
DH 40 × *Imperata cylindrica*	12	90.19 (156)	48.60
Saptdhara × Maize	12	8.40 (208)	227.67*
Saptdhara × *Imperata cylindrica*	12	107.53 (208)	145.32

*P ≤ 0.05.

Table 2. Effect of time period on frequencies of various haploid induction parameters in bread wheat ecotypes pollinated by *I. cylindrica* and maize during 2013–14

S. No.	Time (min)	Pseudoseed formation				Embryo formation				Embryo regeneration			
		DH 40		Saptdhara		DH 40		Saptdhara		DH 40		Saptdhara	
		IC	M	IC	M	IC	M	IC	M	IC	M	IC	M
1	0	78.40* (401)	64.85* (327)	51.50 (287)	47.47 (265)	5.04 (20)	1.31 (4)	18.10* (52)	8.02* (21)	83.33* (17)	80.00* (3)	78.87 (41)	69.23 (15)
2	5	71.53 (363)	65.96 (333)	49.62 (274)	45.79 (255)	5.04 (18)	1.56* (5)	17.56 (48)	8.63* (22)	68.75 (13)	60.00* (3)	84.91* (41)	72.73 (16)
3	10	73.25 (373)	64.66* (331)	56.25 (311)	46.23 (257)	3.54 (13)	0.62 (2)	12.42 (39)	6.56* (17)	72.73 (10)	50.00 (1)	79.31 (31)	81.82* (14)
4	15	76.22* (388)	68.39 (347)	57.52 (321)	42.68 (240)	4.77 (19)	1.80* (6)	18.99* (61)	3.56 (9)	60.00 (11)	50.00 (3)	62.50* (38)	69.23 (6)
5	20	70.41 (354)	67.63 (347)	53.95 (302)	45.13 (250)	2.98 (11)	1.15 (4)	13.52 (41)	4.75 (12)	66.67 (7)	50.00 (2)	80.65 (33)	63.16 (8)
6	25	69.31* (347)	65.96 (334)	51.40 (286)	49.96 (279)	3.06 (11)	1.03 (3)	13.28 (38)	4.86 (14)	50.00* (5)	50.00 (2)	82.86* (32)	61.54 (8)
7	30	69.87 (353)	71.30 (362)	51.95 (288)	47.16 (265)	4.01 (14)	1.12 (4)	12.41 (36)	4.79 (13)	66.67 (9)	33.33 (1)	68.92* (25)	55.56 (7)
8	35	67.69* (345)	64.42* (326)	53.37 (299)	49.21 (274)	5.28 (18)	1.33 (4)	12.83 (38)	4.25 (12)	64.29 (12)	40.00 (2)	62.92* (24)	61.11 (7)
9	40	70.71 (360)	72.67* (373)	51.41 (286)	48.93 (273)	6.21 (22)	1.24 (5)	10.67* (30)	3.15 (9)	72.22 (16)	25.00* (1)	76.39 (23)	61.54 (5)
10	45	77.78* (394)	76.08* (390)	47.62 (263)	48.54 (269)	4.70 (19)	0.28* (1)	11.49 (30)	3.67 (10)	64.29 (12)	100.00* (1)	79.63 (24)	55.56 (5)
11	50	76.99 (387)	69.97 (357)	47.88 (269)	44.68 (250)	2.94 (11)	0.04* (0)	16.67 (45)	1.85 (5)	71.43 (8)	0 (0)	67.24* (30)	25.00* (1)
12	55	69.74 (354)	74.17* (376)	48.84 (269)	48.31 (268)	4.33 (15)	1.35 (5)	14.81 (40)	2.60 (7)	63.64 (10)	20.00* (1)	76.27 (30)	60.00 (4)
13	60	75.20 (381)	70.65 (360)	51.53 (287)	46.65 (260)	5.28 (20)	0.43* (2)	17.30 (50)	0.45* (1)	70.00 (14)	100.00* (2)	73.61 (37)	0 (0)
	Mean SE±	72.85 1.01	68.98 1.07	51.76 0.83	46.98 0.57	4.40 0.29	1.02 0.14	14.62 0.77	4.40 0.64	67.23 2.15	50.64 8.16	74.93 2.04	56.65 5.96

IC = *Imperata cylindrica*; M = Maize; Figures in parentheses represent the numbers obtained. *P≤0.05.

Analysis of variance for embryo formation has revealed a significant deviation from mean in wheat × maize combinations whereas, no such variation has been observed in wheat × *I. cylindrica* crosses (Table 1). While analyzing the embryo formation in different pollination regimes in the wheat genotypes under experimentation, a significant decrease of 97.77 percent (from 1.80 to 0.04 percent) was observed in DH 40 when pollinated with *Z. mays* pollen after 50 minutes of its collection (Table 2). The embryo formation in the spikes of DH 40 pollinated with maize ranged from 0.04 to 1.80 percent with an average of 1.02 percent. Similarly in Saptdhara, a significant decline of 95 percent (8.63 to 0.45 percent) was revealed during the entire observation period with an average of 4.40 percent. When *I. cylindrica* was used to pollinate the spikes of DH 40, embryo formation frequency exhibited non-significant deviation from the performance obtained with the freshly harvested pollen. It revealed decrease from 5.04 to 2.98 percent in first 20 minutes after which the frequency followed a trend of increase up to 6.21 percent in next 20 minutes, decrease up to 2.91 percent in next 10 min and again increase to 5.28 percent in last 10 minutes. Similar curved pattern of increase and decrease in embryo formation was depicted by Saptdhara in the second half of the experimentation period. The average embryo formation frequency in the wheat genotypes, DH 40 and Saptdhara was found to be significantly more (4.40 and 14.62 percent, respectively) than that of maize (1.02 and 4.40 percent, respectively) when pollinated with *I. cylindrica* (Fig. 1).

Maximum regeneration was observed in the embryos obtained after the pollination of wheat parents with freshly harvested pollen (control) of both the male parents, *Z. mays* and *I. cylindrica* (Table 2). Moreover, embryos obtained in Saptdhara showed maximum regeneration even after 25 minutes of collection of *I. cylindrica* pollen. A variation from zero to 100 percent and zero to 81.22 percent germination was observed in embryos obtained from spikes of DH 40 and Saptdhara, respectively when pollinated with *Z. mays*.

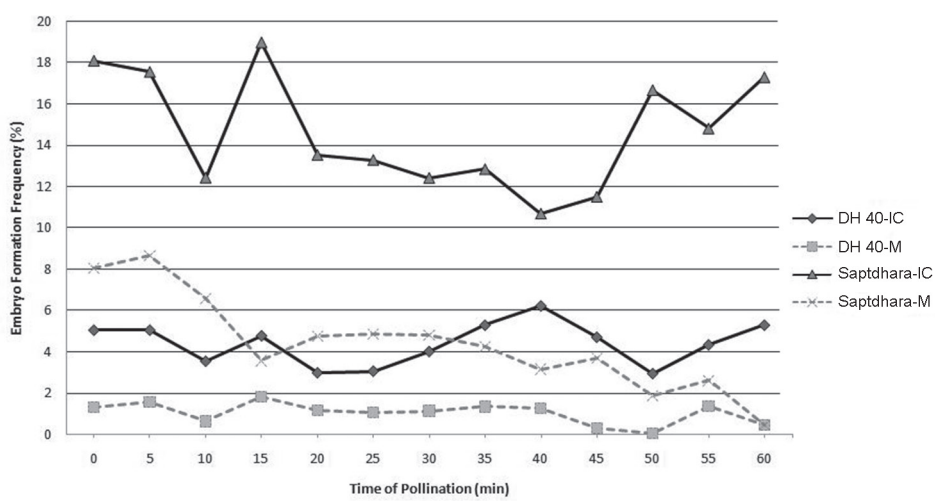

Figure 1. Effect of time period on frequency of haploid embryo formation in bread wheat ecotypes pollinated by maize and *I. cylindrica* during 2013–2014 (IC = *Imperata cylindrica*; M = Maize)

Similarly, with *I. cylindrica,* the germination percentage ranged from 50.00 to 83.33 percent in DH 40 and 62.50 to 84.91 percent in Saptdhara. It was found that in both the wheat genotypes, the embryos obtained after pollination with *Z. mays* recorded minimum regeneration frequencies of zero whereas in that of *I. cylindrica* minimum regeneration frequencies were between 50–60 percent. A great amount of variation in the regeneration was observed in embryos obtained after pollination with maize which can be attributed to lesser number of embryos. On an average, the regeneration frequency was found to be higher when the wheat ecotypes were pollinated with *I. cylindrica* than *Z. mays*.

Discussion

The success of chromosome elimination approach of doubled haploidy breeding is critically influenced by the quality of pollen used, which is, measured by pollen viability. In the present investigation, wheat × *Z. mays* and wheat × *I. cylindrica* systems were explored for the assessment of relative pollen viability of *Z. mays* and *I. cylindrica.* There are various methods devised by researchers to estimate the loss in the viability of pollen after dehiscence from anther but the most appropriate method is to study the seed set in artificially pollinated florets. The field studies are comparatively more reliable than the staining or germination experiments as these take all the factors into account whether environmental or genotype specific. Pollen viability estimates in the field conditions could result in more precise estimation of the outcome of wide hybridization assuming that pollen which remains viable for longer periods of time would have more efficient haploid induction.

The present investigation revealed encouraging trends for all the haploid induction parameters under consideration. The pseudoseed formation frequency did not show any particular pattern during the five-minute interval regime of entire experimentation period which depicts that the time of pollination does not affect the rate of pseudoseed formation. Thus, it can be inferred from the results that pseudoseed formation is not influenced by pollen viability directly. Moreover, production of imbibed pseudoseeds in unpollinated spikes confirmed the dependency of this aspect on the growth hormone treatment i.e. 2,4-D. The findings are in agreement with the earlier reports (Suenaga and Nakajima 1989; Laurie and O'Donoghue 1994; Brazauskas et al. 2005; Pratap and Chaudhary 2012) which support the role of auxins in seed imbibition through directed flow of solutes.

A direct relation was observed between the time of pollination and the frequency of embryo formation which facilitated this parameter to be utilized as the measure of pollen viability. The embryo formation frequency was found to be highest in both the pollinators when the wheat spikes were pollinated with the fresh pollen (control). The embryo formation frequency decreased significantly with increase in pollination time with *Z. mays* after 45 minutes which depicted loss of viability in pollen. The present findings with respect to embryo formation frequency in spikes pollinated with *Z. mays* are in concordance with the earlier reports where all viability of *Z. mays* pollen was lost within 50 minutes (Luna et al. 2001; Aylor 2004; Muui et al. 2007). Exposure to the environment was associated with a progressive loss of water from the pollen leading to loss in embryo formation

(Aylor 2003). The embryo formation frequency with *I. cylindrica* showed a gradual dip and rise pattern in embryo formation throughout the experimentation period with an elevation at the end. This revealed that the pollen of *I. cylindrica* was still viable at the end of the time of experiment and could have been used for more time for embryo formation. Such results are quite encouraging for enhancing the efficiency of the existing haploid induction system. The difference in the embryo formation frequency between DH 40 and Saptdhara depicts the role of genotypes and their interaction with the pollen and environment in embryo formation which is in agreement with the findings by Rather et al. (2013) and Badiyal et al. (2014) who showed the role of genotypes and their interaction with environment on the embryo formation frequency. No field studies on the pollen viability of *I. cylindrica* are available in literature to compare with the present investigation.

As embryo regeneration is an important parameter to study the variability and stability among the haploids, the genetics of haploids can be studied only if the haploid embryos are regenerated into plants. But embryo regeneration depends on the number of embryos formed, culture conditions, media onto which the embryos are cultured and the mechanical handling. Hence, deviation in embryo regeneration is not directly affected by pollen viability. Further, a great amount of variation in embryo regeneration in *Z. mays* pollinated spikes may be due to less number of embryos. The results are in agreement to the study conducted by Khan and Perveen (2014) which revealed that the genotypic interaction present between wheat and *Z. mays* significantly influences the recovery of haploid embryo formation and plant regeneration.

As evident from the study, *I. cylindrica* performed better than *Z. mays* towards all haploid induction parameters in wheat ecotypes. The observations in the present research investigation completely comply with the earlier reports (Chaudhary et al. 2005; Chaudhary 2008a; Pratap et al. 2005; 2006; Kishore et al. 2011). Moreover, a significant decline in the pollen viability of *Z. mays* may greatly diminish effective pollen flow with longevity thus being a crucial factor in pollination efficiency in haploidy breeding. *I. cylindrica* is a wildly growing grass near the wheat fields. It has been shown in studies pertaining to pollen viability of grasses that their pollen remain viable for very long duration, sometimes up to 48 hours (Pacini et al. 1997) after dehiscence from anthers. Further, it has been revealed by the researchers that pollen viability of grasses is not influenced over the range of temperature often encountered during the pollination season, while excessively high temperature during this period may negatively influence pollen viability (Wang et al. 2004). The present report also sustains the aforesaid statements as the pollen of *I. cylindrica* was functionally viable even at the end of one hour.

Conclusively, the present investigation established wheat × *I. cylindrica* system as highly efficient alternative to the existing wheat × *Z. mays* system of haploid induction. As the time for assessment of pollen viability in the experiment was less in view of potential of this wild grass, further studies in this direction with wider time duration can open new horizons towards enhancement of precision in wheat improvement programmes.

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