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Evaluation of Pollen Samples of Inducer Line with Different Techniques and Pollen Size Effect on Haploid Seed Formation in Maize

Gülizar PINAR[1*], Fatih KAHRIMAN[1]
[1]Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Field Crops, Çanakkale
[*]Sorumlu Yazar (Corresponding author): glzr.meliss@gmail.com

Abstract
There is an urgent need to evaluate and optimize use of different sized pollen grains of inducer lines to increase haploid seed production in maize breeding programs. Therefore, this study aimed to evaluate the effect of pollen size on haploid seed production in maize under greenhouse conditions to characterize pollen samples taken from an inducer maize line by morphological, molecular and spectrophotometric measurements. Three donors and one inducer line were used as the experimental material. The pollen samples of inducer line were collected and filtered into two different sizes (50µ and 100µ) using a special vacuum filtration device. Morphological measurements based on image analysis were made, spectral data were collected in Near Infrared Reflectance (NIR) and Raman spectroscopy devices. The protein bands were examined with SDS-PAGE analysis on the collected pollen and seed samples. Haploid induction rates of the seed samples obtained after pollination of donor materials with sized-pollen samples were determined. The results of the study indicated spectral and molecular differences in the pollen samples separated by 50 and 100µ filters. Haploid induction rate (HIR) values ranged 7.40% -18.9% in the used donors, however higher haploid seed formation was observed in small-sized pollen samples compared to the others.

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1. Introduction

In vivo haploid technique is one of the methods widely used in commercial maize breeding programs (Mitiku, 2022). This technique facilitates in rapid production of haploid plants with significant benefits to breeders and shorten the breeding period from 6-7 generations to 2-3 generations providing a 100% homozygosity rate in the inbred lines (Gilles et al., 2017; Vanous et al., 2017). The first report was made by Chase (1969) on the development of doubled haploid lines based on the maternal haploid technique in maize breeding and genetic programs (Jacquier et al., 2021). The natural occurrence of haploid maize plants was a very frequency (0.1%); subsequently, the Stock-6 line was developed with a commercially viable haploid induction rate (2-3%), which represented the basis of all inducer lines developed later (Chaikam et al., 2019). While the HIR value was around 1-2% in the first inducer-developed lines, that increased up to 8-15% in the new generation inducer lines (Uliana et al., 2020).

The haploid induction rate (HIR) is defined as the ability of an inducer to form haploid seeds in the donor material. The main focus of all studies on this technique is directly or indirectly devoted to increasing haploid seed formation from donor materials. *In vivo* doubled haploid technique can be applied maternally and paternally. The maternal haploid method is widely preferred because of higher success rates in maize breeding programs (Liu et al., 2016; Chidzanga et al., 2017). In the maternal haploid technique, the inducer lines are used as a pollen source and induction of hybridization is performed by pollinating the donors with pollen collected from the inducer parent (Chaikam et al., 2019).

Several studies on the investigation of pollen characteristics by plant species have been reported in the literature (Joujeh et al., 2019; Liu et al., 2023). Most of these studies focus on issues such as the determination of allergen effects of pollens and evaluations of pollen size for interspecific comparisons. Studies on comparing pollen samples of different species by creating spectral libraries have also been published (Pereira et al., 2021). Pollen fingerprints based on spectral data are considered a practical technique in this context. Similarly, comparative analyses of pollen samples in different plant species with the help of molecular techniques, has also been reported using molecular analyses with more reliable results (Bell et al., 2016). Similar studies have been made in maize, but no study has been performed to understand the pollen properties of inducer maize lines. Some studies indicate that the pollen of haploid plants, (not directly in inducer lines), are smaller in diameter compared to the diameter of diploid plants, and these pollens can be separated by spectral measurements (Wang et al., 2018).

Pollen is considered an important vector in the induction of hybridization and haploid formation. Different approaches have focused on the development of new inducers or the development of new techniques to separate haploid-diploid seed samples (Geiger et al., 2009). Studies on the determination of pollen properties of inducer lines based on cytogenetic analysis are also performed (Qiu et al., 2014). However, pollen studies in inducer lines are limited when compared to other topics *in vivo* doubled haploid techniques. No literature is available on haploid induction rates, when pollen samples are separated into different sizes for use for pollination.

The aims of this study was to characterize the maize pollen size samples of the inducer lines with different techniques, and to investigate their potential effects on the haploid seed formation in maize.

2. Materials and Methods

2.1. Plant material

Three donor genotypes and one inducer line were used in the study. The donor materials consisted of two inbred lines and their F1 hybrids, which were formed by hybridizing inbred lines. The inducer line, CIM2GTAIL-P2, was obtained from the International Wheat and Maize Research Center (CIMMYT).

2.1.1. Experimental conditions

Plant materials were grown under greenhouse conditions at Çanakkale Onsekiz Mart University (ÇOMÜ) Agricultural Farm, Plant Production Research and Application Unit, in Turkey (long: 26.4°N and lat. 40.1°E). The genotypes were sown as 30 pots in field soil/ manure mixture (3/1 ratio) prepared in 18-liter pots. To meet the light need in greenhouse conditions, PlantGrow led lighting system with 16 hours of light/8 hours' dark photoperiod was used. The pots were drip-irrigated as needed and fertilized with a total of 180 kg ha^{-1} nitrogen and 80 kg ha^{-1} phosphorus. The donor materials were prevented from self-pollination, by removing the tassels, and the protecting ears' silks by covering with in-shoot bags. The pollen samples were collected from the inducer lines with a special vacuum assembly containing PE filters with a diameter of 50-100, and 125 µ. The 125 µ filter was used to separate the anthers and other coarse particles from the pollen grains, to separate the pollen samples into two classes.

2.2. Pollen characterization

2.2.1. Morphological characterization

TTC (Triphenyl Tetrazolium Chloride) staining and image analysis were used to characterize pollen samples of different sizes. Therefore, three imaging slides were prepared for each group of pollen samples. For slide preparation, 1-2 drops of 1% TTC solution were dropped on the slide. Thereafter, the pollen samples were sprinkled with a brush and kept in the dark for 2 hours at room temperature (RT). The images were taken under a digital microscope with 10× magnification. The images of the calibration slides (Motic, San Antonio, TX, USA) were taken at the same magnification to make morphological measurements of the images taken from the pollen samples. These images were transferred to the ImageJ program (Schneider et al., 2012) to determine the size, viability, and minimum-maximum values of the pollen samples by using the particle analysis method.

2.2.2. Spectral characterization

Fifty milligrams of pollen samples were placed in the transflectance cup of the NIR (Near Infrared Reflectance) device (Spectrastar 2400D, USA) to collect the spectrum of 1200-2400 nm at each nanometer. These spectra were taken from 3 samples depending on the sample groups and recorded as jdx files. Thereafter, these were converted to Excel format and shown graphically by taking sample means in each group according to their filter size.

To collect Raman spectra from pollens, one hundred milligrams of the pollen samples were visualized using a 532 nm laser after grating (600 g mm^{-1} BLZ: 500 nm) in a Raman spectroscopy device (Witec Alpha 300 RA, Germany). The measurements were made using a 20× objective, taking the integration time of 0.5 s and accumulation as 10in the imaging process.

2.2.3. SDS-PAGE of pollen samples

Total protein extraction from pollen samples for SDS-PAGE analysis was performed according to the method proposed by Iqbal et al. (2014). 50 mg samples of different sizes were weighed into separate tubes with 3 replications for this purpose, by adding 950 ml of extraction

buffer (62.5 mM Tris–HCl (pH 6.8), 2.3% SDS, 5% 2-ME, 10% glycerol, 0.1% bromophenol blue) in each tube. The samples were then vortexed and shaked for 1 hour at RT. These samples were centrifuged at +4°C using 10000 g and the supernatants were taken into clean tubes. Stacking gel (4%) and 12% resolving gel (12%) were prepared for the electrophoresis of samples. The gels were allowed polymerization for about 1 hour. 10× running buffer (30 g Trisbase, 144 g glycine, 10 g SDS and 1000 ml distilled water) was diluted and used in the gel tank to carry out the electrophoresis. 300 µL of the upper phase of the samples was taken into Eppendorf tubes and 100 µL of 4× loading buffer (4 ml glycerol, 2 ml mercaptoethanol, 1.2 g SDS, 5 ml 4×stacking solution, 0.03 g bromophenol) was added. The samples were vortexed and kept in a hot water bath at 95 °C for 5 minutes. 15 µL of each sample was loaded into wells on top of gels to load protein samples. A prestained protein ladder (Sigma, Germany) was also loaded in the first and last of the wells to facilitate molecular size calculations. The samples and standards were run at 100 V until complete separation and bromophenol blue dye reached the bottom of the gels. After this process, the gels were left in a shaker overnight with a mixture solution of 60 g Trichloroacetic acid (TCA), 1 g Brilliant Blue, and 25 mL ethanol with distilled water to 500 mL. The images of the gels were recorded using a flatbed scanner. The gel images were loaded into the Gel Analyzer 19.1 software (www.gelanalyzer.com) then the seven bands of molecular standard (25 kDa, 35 kDa, 55 kDa, 70 kDa, 100 kDa, 130 kDa, 250 kDa) were defined. The relative mobility (Rf) values of the samples were calculated according to the Rf values of the bands obtained from the used molecular weight markers.

2.3. Seed evaluations

2.3.1. Determination of haploid induction rates

Ear samples obtained from induction crosses were separately shelled. The samples were evaluated as haploid or diploid by visibly according to anthocyanin pigmentation in the endosperm/embryo regions as suggested by Chaikam at al. (2012). HIR values were determined by the ratio of haploid seeds to the total number of seeds in each group.

2.3.2. SDS-PAGE of seed samples

Three to five kernel samples obtained from induction of hybridization with pollen samples of 50 and 100µ pollen size were ground. 50 mg of the ground samples were weighed into Eppendorf tubes and the extraction buffer was placed on them and shaken at RT for 1 hour. SDS-PAGE analyses were performed according to the methods described in the analysis of pollen samples.

2.3.3. Statistical analysis

Data were analyzed in the R software. The averages obtained from the pollen size were compared with the Welch test. The differences among the means were shown graphically using the *ggstatpplot* of R software.

3. Results and Discussion

3.1. Morphological characterization of pollen samples

The results for morphological measurements of pollen samples of the inducer line are shown in Figure 1. The mean diameter, minimum, and maximum diameter values of 50µ pollen samples were calculated as 87.4 µm, 58.92 µm, and 90.8 µm, respectively. The respective values for the 100µ sample group were 101.2 µm, 88.6 µm, and 100.3 µm. The average values for morphological measurements were significantly different across the pollen size

(Figure 1). Maize pollen is large compared to other cereal species and its average size varies between 70-100 µm (Fonseca et al., 2002). Maize pollen has an oval shape and a smooth outer membrane. There are no specific reports on the pollen characteristics of inducer lines. The minimum and maximum of the pollen diameter obtained in our study are outside the limits specified in the literature. This may be due to the method of measurement used in this study. Fonseca et al. (2002) measured the pollen size using electron microscopy while image analysis was used in the current study. Indeed, previous studies revealed that pollen diameter measurements for the same species could vary based on the microscopy technique used (Pospiech et al., 2021). Also, pollen morphology measurements are affected by the calculation method, and they can vary for the same species even in the same microscopy method used (Soares et al., 2017). On the other hand, due to vacuum filtration, pollen morphology may have changed in a way that could have affected the morphological measurements. To the best of our knowledge, there is no comparative study for different pollen collection methods on the pollen size measurement in previous literature. It is known that, the collection method of pollen samples has significant effects on the number of collected pollens and a high amount of pollen can be collected under vacuum filtration (Wizenberg et al., 2020). We did not apply any measurement about the amount of collected pollen, but accumulation the high amount of pollen grains on filters could be due to adverse effect of vacuum.

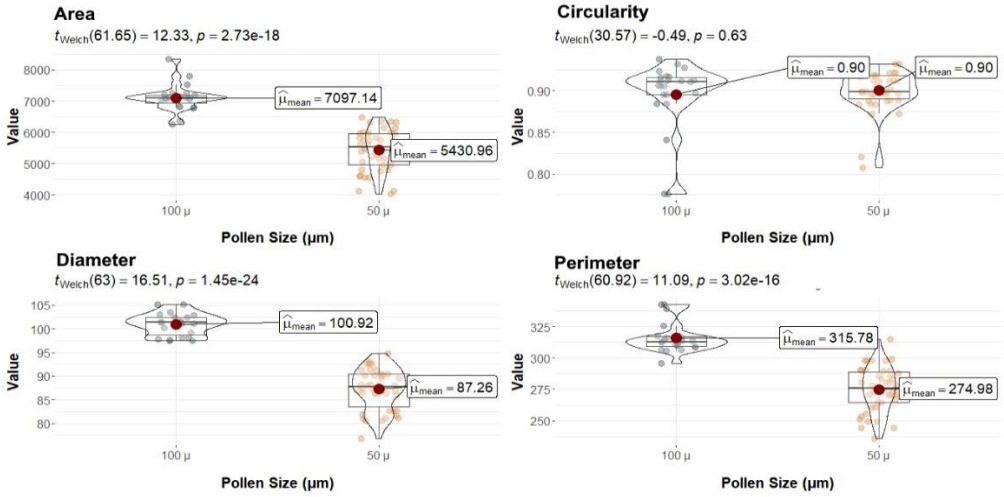

Figure 1. Boxplots for morphological measurements of pollen samples collected from inducer line with vacuum filtration using 50 and 100µ filters

3.2. Spectral characterization of pollen samples

According to the NIR and Raman measurement results, there are significant differences in the spectral data of pollen samples of different sizes (Figure 2). In NIR measurements, it is evident that the reflectance values of 100µ pollen samples between 1400-2400 nm are higher compared to those observed in the other group, while the reflectance values were lower than those between 1200-1400 nm. A similar situation was observed in the Raman measurements, with an average intensity of 100µ pollen samples; which was found higher compared to 50µ samples lying between 95-2500 cm-1 (Figure 2).

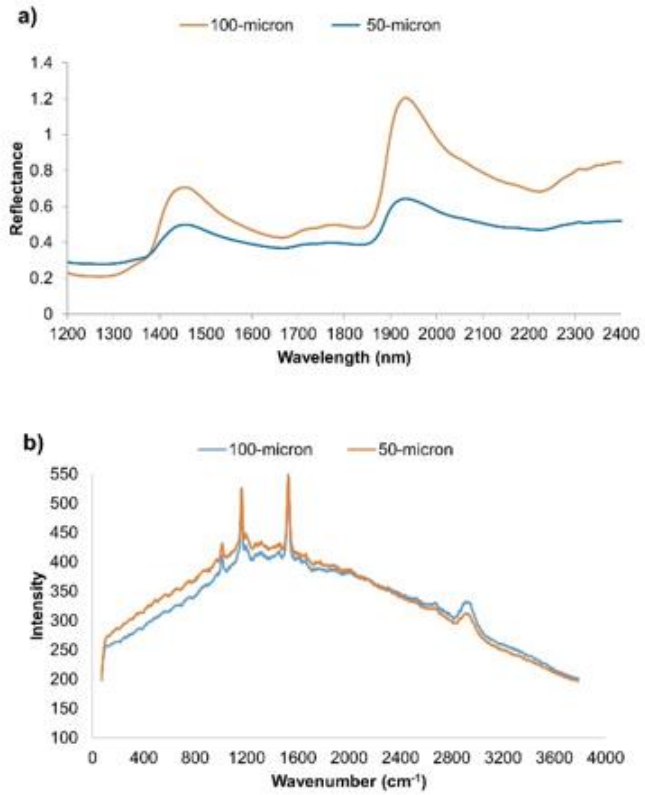

Figure 2. NIR (a) and Raman (b) spectral plot of maize pollen samples with different size

The results obtained from the spectral measurements are related to the differences in the biochemical structure of the measured samples. The effect of moisture and dry matter content of biological samples on the results of spectral measurements taken with NIR devices is well known (Williams et al., 2016). The higher average spectra in 100µ samples obtained from the NIR instrument in most parts of the scanning range is related to the development status of the pollen cells in these samples. 50µ pollen samples, which were small in diameter, are in the early stages of pollen development. Unlike the NIR spectral data, the 100 and 50µ samples showed variable changes between the intervals scanned with the Raman spectrometer (Figure 2). Pollen characterization using Raman spectra has been widely used in the scientific literature (Mondol et al., 2019; Kendel et al., 2020). It is reported that 1900-800 cm^{-1} and 2000-500 cm^{-1}regions are associated with the results of the analysis of the chemical composition of aromatic substances in the pollen wall. Bujang et al. (2021) found that the major biochemical components in mature maize pollen are carbohydrates (44%), water content (23%), proteins (17%), fiber (9%) and ash (4%), as well as some minerals, microelements, phenolics and flavonoids.

In current study, four peaks (1013 cm^{-1}, 1159 cm^{-1}, 1524 cm^{-1} and 2929 cm^{-1}) were visible in the scanned spectral range in Raman measurement. The regions associated with biochemical components in NIR and Raman scans differed according to pollen size.

3.3. SDS-PAGE analysis of pollen samples

SDS-PAGE analysis results of pollen samples are presented in Table 1.

Table 1. Protein band profiles of 50µ and100µ maize pollen samples of inducer line

Molecular Weight	50 µ	100 µ
36	+	+
38	+	+
40	+	+
43	+	+
46	+	+
49	+	+
52	+	-
55	-	+
60	+	+
62	-	+
66	-	+
68	+	-
73	-	+
75	+	+
83	-	+
85	-	+
95	-	+
99	-	+

The number and intensities of bands showed significant differences in pollen samples of different sizes. The total number of bands was higher in the 100µ group (18 bands) than in the 50µ pollen samples (10 bands). The molecular weights of detected bands were calculated between 36-75 kDa in 50µ samples, and 36-99 kDa in 100µ samples. Significant differences were observed among pollen samples in SDS-PAGE analysis. This difference in the presence and intensities of the bands in the gel analyses support the results obtained in the spectral measurements. Maize pollen is large in size when compared to other cereal species. Proteins are ranked at the third position among the biochemical compounds found in pollen and are generally considered to have low protein content (Bujang et al., 2021; Radev et al., 2018). The differences between 50µ pollen grains samples and 100µ pollen grains samples (Table 2) could be associated with less protein content in small diameter pollen grains samples.

After the generative period, the pollen undergoes different phases from the meiocyte stage to the tricellular formation in the development stages (V8 to V17) of maize plant (Begcy et al., 2017). The filtration of pollen samples was allowed for the small-diameter pollens at the development stage in the current study. Smaller pollen grains are expected to have small quantities of biochemical compounds due to their early maturity stages. This situation could have affected the number and intensity of bands detected in SDS-PAGE analyses. Yu et al. (2014) reported that there are a total of 24 different proteins in terms of the diversity of pollen and pistil proteins of W22 and its isogenic line. 18 different protein bands were detected and the presence of 15 different proteins was observed in this study. The proteins with molecular weights of 66kDa, 62kDa, 60kDa, 55kDa, 49kDa, 43kDadetected in this study were the same as reported by Yu et al. (2014). The molecular weights of the remaining proteins were different, and this difference can be attributed to different inducer lines used in the two studies.

3.4. Assessment of HIR values and SDS-PAGE analysis of seed samples of different pollen size

The number of haploid and diploid seeds varied among the seed samples obtained after pollination with samples of different sized pollen grains (Table 2).

Table 2. The number of putative haploids, diploid and cross seeds pollinated by 50 and 100µ pollen samples in each induction cross

Induction Cross	Pollen Size	Number of Putative Haploids	Number of Diploid Seeds	Number of Cross Seeds
B73× CIM2GTAIL-P2	100 µ	22	143	14
	50µ	17	125	8
HYA× CIM2GTAIL-P2	100µ	16	196	4
	50µ	7	43	6
(HYA×B73)×CIM2GTAIL-P2	100µ	57	310	3
	50µ	15	63	1

This situation was also reflected in the HIR values (Figure 3). For all donor materials, HIR values showed statistically significant differences among the samples pollinated by 50- and100µ pollens. In the B73 and HYA, haploid seed formation was significantly higher in samples pollinated with 50µ pollen samples compared to100µ samples.

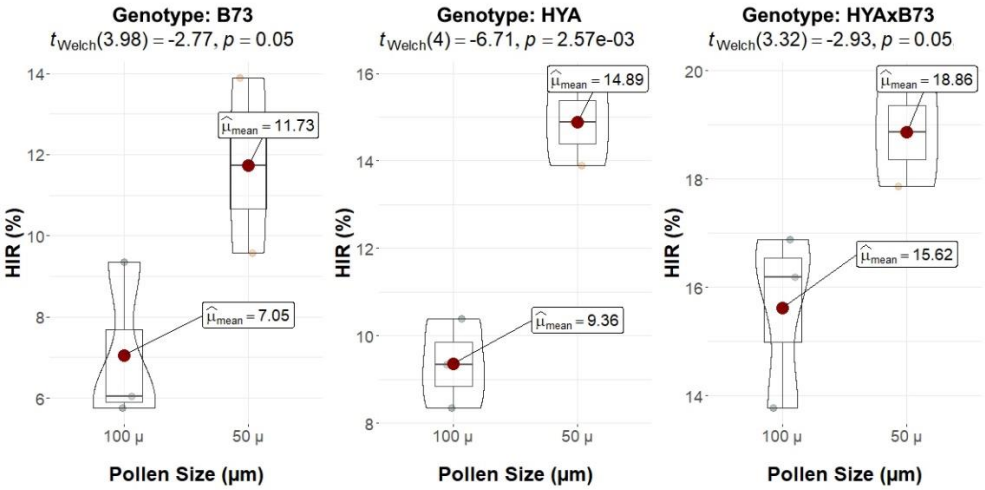

Figure 3. Haploid Induction Rate (HIR) values of different maize pollen size for the tested donor materials

There was approximately 5.1% difference for the HIR value obtained from 50-µ pollen grains compared to the values obtained from 100µ pollen grains' samples in HYA (t_{Welch}=6.71, p≤0.05). Similarly, significant differences were determined as 4.68% in the B73 (t_{Welch}=2.77, p≤0.05). A statistically significant difference (t_{Welch}=2.93, p≤0.05) for HIR value (3.36%) was also noted in the HYA×B73 hybrid. Swapna and Sarkar (2011) suggested several possibilities on the origin of haploid embryos in 'Stock 6' inducer lines. One reason haploid seed formation is related with the meiotic/mitotic division at the time of pollen grain formation resulting in a single diploid sperm nucleus. Diploid sperm nuclei can fuse with polar nuclei resulting

in tetraploid endosperm and haploid embryo after fertilization. Swapna and Sarkar (2011) also found that the ratio of binucleate pollen grains varied across the high haploid inducer line pollen grains, low haploid pollen grains and inbred pollen grains. Li et al. (2017) argued that the haploid induction rate may be related to the pollen division stages. These researchers found that the rate of aneuploidy was higher in the trinuclear stages of pollen than at the tetrad stage. The size of pollen is closely related to plant developmental stage and the pollen life cycle had mainly six different stages which started from pollen mother cell to tricellular structure. Pollen cells vary in size from the pollen grains mother cell to the mature trinuclear stage in maize (Begcy et al., 2017; Zhou et al., 2017). Filtration of collected pollen from the inducer line could influence the separation by developmental stages of pollen grains. The large pollen could be accumulated on the 100µ filter and small ones pass through 50µ filter. Small pollen grains from inducer lines seem to have a potential to form haploid seeds in maize. Although there is no exact evidence on this subject, the results of current study indicated that there is an increasing potential for the formation of haploid seeds when using small sized pollen samples during induction of crosses. In the SDS-PAGE analyses of seed samples, it was determined that there were some differences in terms of storage protein fractions in haploid and diploid seed samples (Table 3).

Table 3. Protein band profiles of haploid (H) and diploid (D) maize seeds of donor materials

	HYA				B73				HYA×B73			
	50 µ		100 µ		50 µ		100 µ		50 µ		100 µ	
Molecular	H	D	H	D	H	D	H	D	H	D	H	D
23	+	+	+	-	+	+	-	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+	+	+
30	+	-	+	-	+	+	+	+	-	+	+	+
34	+	+	-	-	-	+	+	+	+	+	-	+
39	+	+	+	-	+	+	-	+	-	+	+	-
43	+	-	-	-	+	-	-	-	-	-	-	-
45	+	-	-	+	+	+	-	+	-	+	+	+
54	+	+	+	+	+	-	+	+	+	+	-	+
60	+	-	+	+	+	+	-	-	-	+	-	-
69	+	+	+	+	+	+	+	+	+	+	+	+
80	-	-	-	-	-	-	-	+	-	-	-	-
87	-	-	-	+	-	-	-	-	+	+	-	-
90	+	+	+	-	+	+	+	+	-	-	+	+
94	-	-	-	-	+	+	+	+	+	+	+	+
98	+	+	+	+	-	-	-	-	-	-	-	-
100	-	-	+	-	-	-	-	-	-	-	-	+
103	+	-	-	-	-	+	-	+	+	-	-	-
108	-	-	-	+	+	+	+	+	+	+	+	+
113	+	-	-	-	+	-	-	+	-	-	-	-
117	-	-	-	+	-	-	-	-	-	-	-	-

A total of 20 bands were detected and it was observed that all of them were polymorphic except two bands. The estimated molecular weights of the bands obtained from the seed analyses varied between 23-117 kDa. Three bands (30 kDa, 103 kDa and 113 kDa) in HYA, two bands (43 kDa and 60 kDa) in B73, and one band (103 kDa) in the HYA×B73 were detectable in the haploid samples but not in diploid seed samples. Therefore, the result indicates that these bands can be distinctive in detecting the haploid seed samples. Protein band analyses in maize have been widely used for genotypic classification (Ünlü et al., 2018; Tuna et al., 2019). Some studies

dealing with the differences of proteins isolated from haploid and diploid maize kernels have also been reported. Birchler and Newton (1981) observed comparable band intensities on 1D SDS-PAGE protein gels when comparing maize genotypes with different ploidy levels and their results showed that diploids have double the amount of protein per cell as their haploid counterparts. Our results also agree with this finding.

4. Conclusion

The results of this study revealed that haploid seed formation can be increased if pollen samples collected from the inducer lines are graded into different sizes and small diameter pollen are used for induction of hybridization using *in vivo* doubled haploid technique in maize. The proposed method can be tested and used for maize inducer lines with low haploid induction capacity such as Stock-6. It has been found that spectral and molecular analyses can be used to characterize the pollen samples with different sizes of inducer maize lines. The numbers of bands were relatively lower in 50µ pollen samples than obtained in 100µ pollen samples. Average spectra indicated that small sized pollen samples had different spectral fingerprints compared to big-sized samples.

Testing this method for different inducer lines and donor materials may allow more comprehensive results. It is established that testing various sizes of pollen could give more detailed results with possibility to develop new approaches based on the spectral measurement methods to screen potential pollen samples that may form haploid seeds. It is also shown that single pollen measurements can be possible using spectroscopy instruments. It is concluded that a system based on Raman measurements allow separation of pollens increasing potential haploid seed formation making important contributions in scientific and applied studies.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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References

Begcy, K., Dresselhaus, T., 2017. Tracking maize pollen development by the Leaf Collar Method. *Plant Reproduction*, 30(4): 171–178.

Bell, K.L., De Vere, N., Keller, A., Richardson, R.T., Gous, A., Burgess, K.S., Brosi, B.J., 2016. Pollen DNA barcoding: current applications and future prospects. *Genome*, 59(9): 629-40.

Birchler, J.A., Newton, K.J., 1981. Modulation of protein levels in chromosomal dosage series of maize: the biochemical basis of aneuploid syndromes. *Genetics*, 99: 247–266.

Bujang, J.S., Zakaria, M.H., Ramaiya, S.D., 2021. Chemical constituents and phytochemical properties of floral maize pollen. *PLoS One*, 16(2): e0247327.

Chaikam, V., Molenaar, W., Melchinger, A.E., Boddupalli, P.M., 2019. Doubled haploid technology for line development in maize: technical advances and prospects. *Theoretical and Applied Genetics*, 132: 3227-3243.

Chaikam, V., Prasanna, B.M., Mahuku, G., 2012. Doubled haploid technology in maize breeding: Theory and practice. In: B.M. Prasanna, V. Chaikam, G. Mahuku (Eds), *Maternal Haploid Detection Using Anthocyanin Markers*, Mexico, pp. 21-24.

Chase, S.S., 1969. Monoploids and monoploid-derivatives of maize (*Zea mays*L.). *The Botanical Review*, 35: 117–167.

Chidzanga, C., Muzawazi, F., Midzi, J., Hove, T., 2017. Production and use of haploids and doubled haploid in maize breeding: A review. *African Journal of Plant Breeding*, 4: 201-213.

Fonseca, A.E., Westgate, M.E., Doyle, R.T., 2002. Application of fluorescence microscopy and image analysis for quantifying dynamics of maize pollen shed. *Crop Science*, 42(6): 2201-6.

Geiger, H.H., 2009. Doubled haploids. In: J.L. Bennetzen, S. Hake (Eds.), *Maize handbook -volume II: genetics and genomics*. Springer Science and Business Media, New York, pp. 641-657.

Gilles, L.M., Martinant, J.P., Rogowsky, P.M., Widiez, T., 2017. Haploid induction in plants. *Current Biology*, 27: 1095-1097.

Iqbal, J., Shinwari, Z.K., Rabbani, M.A., Khan, S.A., 2014. Genetic variability assessment of maize (*Zea mays* L.) germplasm based on total seed storage proteins banding pattern using SDS-PAGE. *European Academic Research*, 2(2): 2144–60.

Jacquier, N.M., Gilles, L.M., Martinant, J.P., Rogowsky, P.M., Widiez, T., 2021. Doubled haploid technology. In: J.M. Segui-Simarro (Ed), *Maize in Plant a Haploid Inducer Lines: a Cornerstone for Doubled Haploid Technology*, Mexico, pp. 25 – 48.

Joujeh, R., Zaid, S., Mona, S., 2019. Pollen morphology of some selected species of the genus *Centaurea* L. (Asteraceae) from Syria. *South African Journal of Botany*, 125: 196-201.

Kendel, A., Zimmermann, B., 2020. Chemical analysis of pollen by FT-Raman and FTIR spectroscopies. *Frontiers in Plant Science*, 11: 352.

Li, X., Meng, D., Chen, S., Luo, H., Zhang, Q., Jin, W., Yan, J., 2017. Single nucleus sequencing reveals spermatid chromosome fragmentation as a possible cause of maize haploid induction. Nature Communications, 8(1): 1-9.

Liu, Q., Yang, J., Wang, X., Zhao, Y., 2023. Studies on pollen morphology, pollen vitality and preservation methods of gleditsia sinensis Lam. (*Fabaceae*). *Forests*, 14(2): 243.

Liu, Z., Wang, Y., Ren, J., Mei, M., Frei, U.K., Trampe, B., Lübberstedt, T., 2016. Maize doubled haploids. *Plant Breeding Reviews*, 40: 123-66.

Mitiku, T., 2022. Review on haploid and double haploid Maize (*Zea mays*) breeding technology. *International Journal of Agricultural Science and Food Technology*, 8(1): 52-58.

Mondol, A.S., Patel, M.D., Rüger, J., Stiebing, C., Kleiber, A., Henkel, T., Popp, J., Schie, I.W., 2019. Application of high-throughput screening Raman spectroscopy (HTS-RS) for label-free identification and molecular characterization of pollen. *Sensors*, 19(20): 4428.

Patil, I., 2021. Visualizations with statistical details: The 'ggstatsplot' approach. *Journal of Open Source Software*, 6(61): 3167.

Pereira, S.G., Guedes, A., Abreu, I., Ribeiro, H., 2021. Testing the Raman parameters of pollen spectra in automatic identification. *Aerobiologia,* 37: 15-28.

Pospiech, M., Javůrková, Z., Hrabec, P., Štarha, P., Ljasovská, S., Bednář, J., Tremlová, B., 2021. Identification of pollen taxa by different microscopy techniques. *PloS One*, 16(9): e0256808.

Qiu, F, Liang, Y, Li, Y, Liu, Y, Wang, L, Zheng, Y., 2014. Morphological, cellular and molecular evidences of chromosome random elimination in vivo upon haploid induction in maize. *Current Plant Biology*, 1: 83-90.

Radev, Z., 2018. Variety in protein content of pollen from 50 plants from Bulgaria. *Bee World*, 95(3): 81-83.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7): 671-675.

Soares, T.L., Jesus, O.N., Souza, E.H., Rossi, M.L., Oliveira, E.J., 2017. Comparative pollen morphological analysis in the subgenera Passiflora and Decaloba. *Anais da Academia Brasileira de Ciências,* 90: 2381-2396.

Swapna, M., Sarkar, K.R., 2011. Anomalous fertilization in haploid inducer lines in maize (*Zea mays* L). *Maydica*, 56(3).

Tuna, G.S., Uyanik, B., Özdemir, E.E., Dalgiç, G., Mengi, Y., Korkut K.Z., 2019. Electrophoretic characterization of inbred maize lines. *International Advanced Researches and Engineering Journal*, 3(2): 86-92.

Uliana Trentin, H., Frei, U.K., Lübberstedt, T., 2020. Breeding maize maternal haploid inducers. *Plants (Basel)*, 9(5): 614.

Ünlü, E., Mutlu, E., Polat, M., Çeri, S., Kahrıman, F., 2018. Diversity among Turkish maize landraces based on protein band analyses and kernel biochemical properties. *Journal of Crop Improvement*, 32(2): 175-187.

Vanous, K., Vanous, A., Frei, U.K. Lübberstedt, T., 2017. Generation of maize (*Zea mays*) doubled haploids via traditional methods. *Current Protocols in Plant Biology*, 2(2): 147-157.

Wang, Y., Lv, Y., Liu, H., Wei, Y., Zhang, J., An, D., Wu, J., 2018. Identification of maize haploid kernels based on hyperspectral imaging technology. *Computers and Electronics in Agriculture*, 153: 188-195.

Williams, P.J., Kucheryavskiy, S., 2016. Classification of maize kernels using NIR hyperspectral imaging. *Food Chemistry*, 209: 131-138.

Wizenberg, S.B., Weis, A.E., Campbell, L.G., 2020. Comparing methods for controlled capture and quantification of pollen in Cannabis sativa. *Applications in Plant Sciences*, 8(9): e11389.

Yu, J., Roy, S.K., Kamal, A.H.M., Cho, K., Kwon, S-J., Cho, S-W., So, Y-S., Holland, Jb., Woo, Sh., 2014. Protein profiling reveals novel proteins in pollen and pistil of W22 (ga1; Ga1) in Maize. *Proteomes*, 2: 258-271.

Zhou, L.Z., Juranić, M., Dresselhaus, T., 2017. Germline development and fertilization mechanisms in maize. *Molecular Plant*, 10(3): 389-401.

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