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Identification of superior doubled haploid maize (*Zea mays*) inbred lines derived from high oil content subtropical populations

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Abstract

High oil maize (HOM) hybrids, adapted to subtropical regions are needed to meet existing requirements for industrial and livestock purposes. Recently, the doubled haploid (DH) breeding technology is applied to reduce considerably the time to generate homozygous maize lines, however, information of heterotic response, per se productivity, stress, disease reaction, and adaptation to different environments are not known. In this work, field evaluations of per se and testcross high oil content DH lines adapted for subtropical environments were studied to identify superior DH lines with good heterotic response and agronomic characteristics. White and yellow kernel test crosses were formed using the different heterotic population as testers from which DH lines were derived. Test crosses were evaluated at three locations in Mexico. Superior DH lines from each tester presented high GCA effects through test crosses, with a range of grain yield across locations of 12.8 to 10.4 Mg ha^{-1} and 11.1 to 9.1 Mg ha-1 respectively. Per se DH lines were observed and screened for adaptation, productivity, disease reaction and other agronomic traits in five locations/year environments. Per se DH lines screening process across environments was able to obtain information for adaptation, productivity, and healthiness. Also of DH lines with high response of GCA, information regarding heterotic groups, and DH lines oil content determination was used to identify outstanding DH lines for developing HOM high-yielding hybrids adapted for subtropical regions, with fewer costs and time. This research demonstrated that it was feasible to identify subtropical elite DH lines with high oil content.

Introduction

In subtropical regions, an increasing interest exists to develop high yield potential maize hybrids, with value-added properties, such as high oil content (HOC). Because energetic oil value is higher than starch, this type of hybrid represents a potential use for the feed industry to increase caloric intake by poultry, swine, dairy cattle and sheep, and to obtain many other products through diverse industrial processes. Commercial yellow maize contains around 4% oil, whereas improved high oil maize (HOM) genotypes more than 6% oil (Lambert 2000). Around 85% of the total oil is located in the germ, 10% in the aleurone layer, and only 5% in the starchy endosperm (Chaudhary *et al.*, 2014). The direct consumption of HOM varieties could exert positive nutritional effects in humans (Zhang *et al.*, 2015), but also benefits animal performance because it increases feed efficiency, animal growth, and productivity mainly due to their higher metabolizable energy (Weiss and Wyatt 2000). Several studies presented by (Lambert 2000) underline the importance of maize with HOC for animal feed: advantages to laying hens and broiler chicks, formulating swine rations, HOC in maize silage improves dry matter absorption and milk production in dairy cows, and lambs gain weight and require less feed with a high oil and high protein rations.

However, several authors have reported a negative correlation between oil content and grain yield (Alexander and Seif, 1963; Misevic and Alexander, 1989; Dudley *et al.*, 1974, 1977; Rosulj *et al.*, 2002; Pamin *et al.*, 1986). The use of heterosis could be a strategy to compensate for this negative correlation. Lambert (2000), presented a comprehensive revision of efforts realized since the 1940s in temperate regions by different public and private maize breeding programs in order to develop competitive HOC hybrids with no yield reduction.

Conventionally maize inbred lines are developed by six to eight generations of self-pollination, with the aim of fixing desirable traits and obtain maximum segregation to separate groups of genetically different individuals (Hallauer et al., 2010)

According to Bauman (1981), Hallauer *et al.*, (1988) and Hallauer, (1990), pedigree selection used to be the conventional breeding methodology to develop inbred lines. This process of line development used to involve breeder's visual selection, among and within the ear to row progenies for several generations of self-pollination considering agronomic and morphological characteristics; and, ear, stalk and foliar disease reaction. The advanced lines developed by this method used to be healthy and productive. The term 'haploid' is referred to the chromosome number of reproductive cells, such as sperm cells or ovules, containing 50% of the chromosomes of an individual developed. With fecundation, genetic information from both parents are combined, and the offspring obtain a series of chromosome pairs from the genetic divergence of each parent. A doubled haploid (DH) is a genotype formed when haploid (n) cells successfully undergo either spontaneous or artificially induced chromosome doubling (Prasanna *et al.*, 2012). In a broad context, haploid is defined as all the individuals that have similar chromosome contribution of the normal gametes of the species, which is one set of chromosomes (n). Whereas, a DH is an individual where chromosomal composition is equal to the gamete, but duplicated, with two sets of identical chromosomes (2n) resulting in 100% homozygosis, (Prasanna et al., 2012).

According to Zhang et al., (2015), in conventional maize breeding, obtaining 99% homozygous inbred lines is time-consuming (6-8 generations; 3-4 years using off-seasons). However, using DH breeding methodology it takes only one year to obtain 100% homozygous inbred lines, which significantly shortens the breeding process and dramatically improves the breeding efficiency. The advantages of using DH lines in maize breeding programs compared with the traditional self-pollination process includes the following: i) increased selection efficiency, by increasing genetic variance among lines, for perse and testcross performance; ii) complete homozygosity; iii) reduced breeding cycle length, (short "time to market"); iv) perfect fulfillment of distinctness, uniformity and stability criteria, for variety protection; v) reduced expenses for self-pollination and maintenance breeding; vi) simplified logistics; and vii) increased efficiency in marker-assisted selection (Gallais and Bordes, 2007; Geiger and Gordillo, 2009; Prasanna *et al.*, 2012; Zhang *et al.*, 2015).

Even though DH lines are 100% homozygous, they might present several drawbacks because heterotic response, perse productivity, adaptation to different environments, agronomic performance, diseases, biotic and abiotic stresses reactions are unknown. Another concern commented by Prigge *et al*. (2012) for the efficient production of DH lines is the low number of seeds often harvested after chromosome doubling.

Currently, DH approaches have been successful employed to accumulate favorable alleles related to agronomic performance (Geiger *et al.*, 2013), drought stress (Beyene *et al.*, 2013; Odiyo *et al.*, 2014), and for maintaining and recovering genetic diversity in maize landraces (Wilde *et al.*, 2010; Strigens *et al.*, 2013). Success in DH production and development of high yielding DH hybrids derived from tropically adapted populations have been achieved (Beyene *et al.*, 2011; Battistelli *et al.*, 2013), but until now this technology has not been reported to accelerate the breeding of new DH lines for hybrids adapted to subtropical environments. Furthermore, to our knowledge, no DH technology has been applied to assist conventional breeding programs to accelerate the production of HOM hybrids for commercial purposes.

For the production of competitive commercial hybrids, most breeding programs should emphasize the identification of genetic potential of parental lines with good combining ability, productivity, adaptation, and per se performance, to facilitate seed production (Hallauer et al., 2010). Although it is possible to obtain 100% homozygous DH lines through DH breeding, the major problem is the evaluation, selection, and identification of elite parental DH lines to be used in hybrid seed production. Per se lines evaluation in different environments enables the selection of adaptation, productivity agronomic performance, disease reaction, and specific traits.

Geiger et al., (2013) commented that per se selection in DH lines may be practiced for four purposes: i) to rid deleterious recessive alleles of the gene pool of breeding materials, ii) to improve the usefulness of DH lines in hybrid production, iii) to enhance the performance of open-pollinated diploid populations and iv) to develop high-yielding hybrids.

Jeffers and Mahuku (2012) commented on the evaluation value of new DH lines under stress screening nurseries and multilocation trials for use in commercial hybrids.

Preliminary testing of new inbred lines by crossbred combinations allowed for the identification of lines with general combining ability (Jenkins and Brunson, 1932). The term "general combining ability" (GCA) is used to designate the average performance of a line in hybrid combinations (Sprague and Tatum, 1942). The use of test crosses makes it possible to discard a portion of inbred lines that are inadequate in hybrid performance (Hallauer *et al*., 1988). Longin *et al*. (2007) commented that to estimate the GCA of new lines, they needed to evaluate the combination with one or several testers; SCA acts as a masking effect in determining GCA. SCA´s influence can be reduced by using genetically broad testers, or it can be increased with a higher number of line testers.

In other testcross studies, Prigge et al. (2012) evaluated test crosses with DH lines derived from tropical open-pollinated populations and single crosses, using a tropical single cross tester. Although they were not able to classify a heterotic pattern response of their DH lines, they found that DH technology had the potential to generate useful inbreds derived from tropical and subtropical open-pollinated populations to gain access to novel genetic variation. Geiger et al. (2013) evaluated different heterotic group testers in DH lines derived from single crosses; they found strong correlations among different traits including grain yield, which indicated that GCA of the genotypic variance was much more critical than the SCA component.

Gallais and Borders (2007) compared the use of DH lines, with the conventional self-pollination method, found that when a line is used as a tester, the advantage was to produce DH lines that could be used directly as parents of hybrids. Theoretically, it was the best method in genetics for variety development, even with medium heritability, it was and less expensive compared with the conventional method.

The objectives of this research were (1) identify superior DH lines, with significant effects of GCA, by using different heterotic broad populations as testers, and (2) identify HOC parental elite DH lines to develop high yield potential subtropical hybrids by per se performance field evaluation. This information could help to accelerate the development of high-yielding and agronomically competitive subtropical HOC hybrids, using DH technology.

Materials and Methods

Germplasm population

The germplasm used in this research was derived from four subtropical populations: Bajio White Population (BWP), Northwest White Population (NWP), Bajio Yellow Population (BYP), and Northwest Yellow Population (NYP). The four populations, two white and two yellow kernels, were developed by a recurrent selection scheme to increase oil content. The four subtropical populations belonged to different heterotic groups, involved improved germplasm as well as several landraces. A full description of the genetic background and adaptation of each population are in Preciado-Ortíz et al. (2013) and Ortega-Corona et al. (2015).

Testcross Generation and Evaluation

Simultaneously, as DH lines seeds were increased by self-pollination, test crosses were integrated by the use of DH lines as male parents from each population, and the opposite heterotic populations were used as female testers, (BWP was crossed by NWP DH lines, BYP were crossed by NYP DH lines, and vice versa). Populations were used as testers in order to identify opposite heterotic group inbred lines with good GCA, also because HOC elite subtropical DH lines were not yet available to be used as testers.

Test crosses were evaluated at three locations in Mexico (Table 1): two experiments were conducted during 2013 summer growing season at Celaya, Gto. and Morelia, Mich. and the other was conducted during the 2013-

Table 1 - Agro-climatic description of the locations where maize DH testcross hybrids and per se DH lines were evaluated and screened, from 2012 to 2014.

Site	Mega Environment	Latitude	Longitude	Elevation (masl)	Mean Rainfall (mm)	Mean Temperature (°C)	Growing season
Celaya, Guanajuato	Subtropical	20°34′ N	100°50′ W	1,765	630	18.4	Summer
Morelia, Michoacán	Subtropical	19°42′ N	101°11′ W	1,941	760	17.6	Summer
Obregon, Sonora	Subtropical	27°22′ N	109°55′ W	40	377	25	Fall-Winter
Iguala, Guerrero	Tropical	18°21 N′	98°30′ W	732	977	26	Fall-Winter

2014 Fall-Winter growing season at Obregon, Son. For the evaluation of white, kernel test crosses a 10 x 10 lattice design was used with two replications. For yellow kernel test crosses a 9 x 8 lattice design was used with two replications. The following agronomic traits were analyzed: grain yield (GY), days to male flowering (pollen shed) (MF), days to female flowering (silking) (FF), plant height (PH), ear height (EH), stalk and root lodging (TL), and ear rot (ER).

DH lines perse evaluation

The evaluation of DH lines based on per se performance a screening was conducted in six environments, with the aim to score for productivity, adaptation, disease reactions, agronomic characteristics, and other traits. At Celaya location in 2012 summer season, the evaluation began with the seeds obtained after chromosome duplication. The following seasons, an ear to row observation were conducted. At Iguala, Gro. during 2012-2013 and 2013-2014 fall-winter growing seasons; at Celaya, in 2013 and 2014 summer seasons; and at Obregon, during 2013-2014 fall-winter season. Table 1 summarizes the agro-climatic information of the locations used in this study. Subtropical location ranged with elevation between 40 to 1941 masl, mean rainfall of 377 to 760 mm, and mean temperature between 17.6 to 25°C.

Oil content determination

To derive DH lines, from each of the four subtropical populations, 200 HOC seeds selected by NIR spectroscopy were used as donors. DH lines derived from the four populations and a sample of the top-yielding test crosses of selfed plants from Celaya trials were also analyzed by conventional methods. Briefly, oil content was determined using 50 gr of kernels. Samples were prepared from three separate replicates that were dried and ground to a fine powder using a mixer ball mill (MM 400; Retsch/Verder Scientific, Col. Germany) and stored at –20°C for analysis. Oil determinations were performed with the Goldfish methodology described by the AOAC (1990).

Statistical Analysis

Data were subjected to analysis of variance using the statistical software Statistics v.8 (Analytical Software, Tallahassee, FL) and differences among means for testers and genotypes were compared by the LSD test at $p < 0.05$.

Results and discussion

White Kernel Testcross evaluation

Analysis of variance for GY and other agronomic traits for each environment and the combined analysis across locations are presented in Table 2. For genotypes, highly significant differences ($P \leq 0.01$) were observed for all traits at Celaya 2013 except for ER that was significant at $P \leq 0.05$. At Morelia 2013, all traits presented highly significant differences ($P \leq 0.01$), with the exception of ER that was not significant. In the case of Obregon 2013-2014, testcross performance presented highly significant differences ($P \leq 0.0$)1 for GY, MF, PH, EH, and ER; FF and TL were not significant. The lower part of Table 2 contains the combined analysis across environments, wherein the main components of genotypes and locations all evaluated traits presented highly significant differences at $P \leq 0.01$. For Genotype x Location interaction, all traits were not significant, except ER which presented highly significant differences ($P \leq 0.01$).

Concerning the coefficients of variation, (Table 2), GY, FF, MF, PH, and EH, had acceptable values. For TL and ER, CV values were higher, and the reason for that is because these traits are expressed in percentage.

Table 3 presents the average values across environments for MF, FF, PH, EH, TL, and ER. GY is listed for each environment and is also presented as an average across environments, for the purpose of illustrating the GY response through environmental variation.

Each of the testers separated the superior white kernel testcrosses. The upper part of Table 3 lists the top 10 yielding test crosses using population BWP as female tester, and the lower part of the Table depicts the top 10 yielding test crosses using NWP as tester. To be able to compare test crosses within and between groups, the bottom of the Table lists the mean of the combined experiments for all traits, as well as the values of the mean comparison test ($P \leq 0.05$) to determine statistical significance within traits. The top-yielding test crosses across environments was formed by BWP as female tester by NWP DH 9. While the average GY across locations was 12.82 Mg ha^{-1}; this testcross presented the highest GY at Celaya and Morelia. The other traits of this testcross were statistically similar to the experiment mean. Concerning the testcross formed with NWP as female tester, by BWP DH 180 presented the highest GY across environments, and that GY was maintained at outstanding performance for each location.

The bottom of Table 3 also lists per se average performance of the populations NWP and BWP by agronomic traits and GY at each location and

Table 2 -Analysis of variance of grain yield and other traits, by location and across locations of test crosses from BWP and NWP DH lines evaluated at Celaya, Gto, Morelia, Mich., during summer 2013 season and at Obregon, Son., México during 2013-2014 fall-winter season

White Kernel Testcrosses								
CELAYA, GTO. 2013								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	9	3030327.70	14.42	16.32	0.05	0.03	2.68	0.65
Genotype	**99**	**4564271****	**10.24****	**12.11****	**0.06****	**0.04****	**2.07****	**0.56***
Error	81	2419413.60	1.75	3.08	0.03	0.03	1.05	0.37
CV (%)		15.87	1.80	2.32	6.49	10.62	74.66	21.65
MORELIA, MICH. 2013								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	9	15166656.3	13.68	14.78	786.73	382.44	59.94	1.08
Genotype	**99**	**5942052.3****	**15.98****	**17.05****	**522.98****	**373.1****	**4.98****	**0.66NS**
Error	81	2320481	2.79	3.54	215.08	196.65	2.74	0.59
CV (%)		16.33	2.13	2.33	5.34	9.92	53.74	27.06
OBREGÓN, SON. 2013-2014								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	8	2194513	108.36	111.48	285.31	129.06	3.97	4.47
Genotype	**89**	**6501993.8****	**12.17****	**11.96NS**	**259.76****	**201.72****	**1.65NS**	**3.71****
Error	73	5418875.5	6.83	10.05	105.49	104.77	1.22	1.20
CV (%)		17.87	3.09	3.60	4.43	7.92	85.31	30.60
COMBINED								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Location	2	107913153.1**	5458.95**	6924.03**	108310.14**	19971.65**	185.04**	34.53**
Genotipe	**89**	**8051175.2****	**29.38****	**30.77****	**884.23****	**608.72****	**5.51****	**2.34****
Genotype*Loc	**178**	**3852869NS**	**8.71NS**	**9.37NS**	**271.34NS**	**198.46NS**	**3.08NS**	**1.45****
Error	180	3783019	8.48	10.05	473.72	303.25	2.78	0.72

across locations, allowing for comparisons with GY performance of test crosses providing an estimate of the heterosis manifested by DH lines. The use of BWP as tester enhanced yields compared with NWP group.

Based on this information, it is possible to assume that DH lines which participate in the top-yielding test crosses had superior GCA: NWP DH 9, NWP DH 10, NWP DH 57, NWP DH 85, and BWP DH 180, BWP DH 198, BWP DH 193, and BWP DH 197. The top-yielding test crosses were the ones that had GY above the mean of the experiment and above the GY of the parental populations per se. Also, analyses for oil contents in the top-yielding test crosses are shown in the last column of Table 3. In this group of the test crosses which presented the highest oil contents were also the top-yielding genotypes.

Yellow Kernel Testcross evaluation

Evaluations of yellow kernel test crosses were also conducted at three locations, and the analysis of variance of each location and across locations are presented in Table 4. At Celaya 2013, Genotypes presented highly significant differences ($P \leq 0.01$) for GY, FF, MF, PH, and EH whereas TL and ER were not significant. At Morelia 2013, all traits presented highly significant differences ($P \leq 0.01$) except for ER that was significant at $P \leq 0.05$. For Obregon, highly significant differences ($P \leq 0.01$) were observed for GY, FF, MF, PH, EH, and ER whereas TL did not present significant differences. Concerning the combined analysis of variance across locations, for the main components genotype and location, all traits presented highly significant differences ($P \leq 0.01$). For genotype x location interaction, TL and ER presented highly significant differences at $P \leq 0.01$ whereas GY presented significant differences at $P \leq 0.05$. Furthermore, the interactions were not significant in terms of MF, FF, PH, and EH. In addition to TL and ER, the rest of the coefficients of variation had acceptable values.

Table 5 contains the mean values of the superior yellow kernel test crosses across locations for MF, FF, PH, EH, TL, and ER. For the purpose of illustrating environmental variation, both GY for each environment and the GY averaged across environments are shown. The upper part of Table 5 contains the top 10 yielding test crosses formed by BYP tester population and NYP DH lines. The top-yielding testcross from this group was formed by BYP as female tester, crossed by NYP DH 133, which presented an average GY across locations of 9.98 Mg ha-1. This cross was slightly later in flowering and slightly shorter compared with the mean of the experiments. The lower group of Table 5 lists the top 10 yielding test crosses, integrated with NYP as the female tester by BYP DH lines. The highest yellow kernel testcross, was NYP x BYP DH 218, which when averaged across environments, presented a GY of 11.14 Mg ha^{-1}. Also listed is the GY at each location, where Morelia and Obregon were the highest followed by Celaya. This finding illustrates the adaptation of

Table 3 -Grain yield and agronomical traits of the superior white kernel testcrosses across locations grouped by the female testers BWP and NWP.

Genotype	Pedigree	COMBINED 2013-2014						COMBINED		CELAYA	MORELIA	OBREGÓN
		MF	FF	PH	EH	TL	ER				GY	Oil content
		(days)	(cm)	(%)	(Mg ha-1)							%
Test crosses of NWP DH lines using BWP as FF tester												
8	BWP X NWP DH9	77	81	266	148	1.98	2.02	12.82	13.63	14.92	9.91	8.0
9	BWP X NWP DH10	79	84	274	154	1.43	2.85	11.66	13.43	10.62	10.95	7.6
46	BWP X NWP DH57	82	82	288	163	3.08	2.42	11.33	12.16	10.30	11.53	7.2
62	BWP X NWP DH85	80	83	280	154	2.03	3.17	11.14	12.22	12.55	8.66	7.1
73	BWP X NWP DH124	80	85	272	148	1.02	2.67	11.14	11.04	11.49	10.89	7.5
35	BWP X NWP DH39	82	83	281	146	1.12	2.87	10.94	9.95	14.02	8.86	6.9
43	BWP X NWP DH53	80	82	263	152	1.93	2.88	10.74	10.98	12.10	9.13	7.3
23	BWP X NWP DH24	79	83	271	154	2.47	2.28	10.67	11.33	10.50	10.19	6.5
45	BWP X NWP DH56	81	81	257	135	3.53	2.45	10.28	11.70	8.83	10.31	6.5
38	BWP X NWP DH42	79	81	253	131	1.80	2.87	10.19	12.17	9.99	8.40	6.6
Test crosses of BWP DH lines using NWP as FF tester												
77	NWP X BWP DH180	82	86	280	164	2.25	2.48	11.02	12.06	10.48	10.53	8.2
87	NWP X BWP DH198	84	83	272	147	1.58	2.58	10.74	12.80	9.43	9.97	6.0
84	NWP X BWP DH193	81	80	261	147	2.23	2.20	10.44	10.21	9.73	11.38	7.5
86	NWP X BWP DH197	78	83	261	135	0.70	3.32	10.18	11.09	10.18	9.26	7.7
75	NWP X BWP DH178	80	84	248	141	1.53	4.15	9.92	12.52	10.37	6.88	7.7
78	NWP X BWP DH181	82	83	265	162	0.70	2.92	9.91	9.35	9.89	10.49	5.8
80	NWP X BWP DH185	81	81	280	137	2.85	3.43	9.77	9.40	8.97	10.93	5.7
82	NWP X BWP DH187	78	81	269	143	1.88	3.28	9.47	11.96	9.89	6.56	6.0
83	NWP X BWP DH191	78	79	260	148	1.22	3.35	9.01	9.69	10.28	7.07	6.6
76	NWP X BWP DH179	77	79	253	114	0.70	2.98	9.00	11.90	7.92	7.19	7.3
Checks												
90	NWP	80	82	260	139	0.90	3.98	8.14	8.68	7.76	7.99	
89	BWP	78	81	259	139	2.53	4.28	6.41	7.01	6.63	5.57	
C.V. (%)	3.70	3.90	8.38	12.42	87.69	27.52	21.27	15.87	16.34	17.88		
MEAN	79	81	259	140	1.82	3.05	9.07	9.69	9.19	8.09		
LSD (P ≤ 0.05)	3.31	3.61	24.79	19.83	1.9	0.96	2.216	3.095	3.031	2.952		

GY= Grain Yield (Mg ha^{-1}); MF=Male Flowering (days); FF= Female Flowering (days); PH= Plant Height (cm); EH= Ear Height (cm); TL=Total Lodging (Stalk+root) (%); ER=Ear Rot (%).
Oil content %= Oil content percentage determined with samples obtained from two selfed pollinated plants per replication in the yield trial conducted at Celaya.

testcross across environments.

Table 5 also lists the average GY per se of the BYP, which was only out-yielded by four test crosses. For NYP, because data from Obregon was not available, the average of this population per se was calculated only from two locations. In general the groups of superior test crosses suggest that NYP was a better tester than BYP.

DH lines, which formed the top-yielding test crosses, can be assumed to have a good GCA. Thus, the information presented in Table 5 shows that the BYP DH 218, BYP DH 226, BYP DH 235, BYP DH 214, BYP DH 236, NYP DH 133, NYP DH 139, NYP DH 157, and NYP DH 147, had superior effects of GCA.

For the yellow kernel, the last column of Table 5, shows the oil content values of some of the top test crosses. The higher testcross of the upper group of BYP tester contained 8.2% oil. In the NYP tester group, the testcross NYP x BYP DH 236 contained the highest oil content of the group (8.1%).

DH lines per se evaluation

Due to a large amount of information generated in this study, Table 6 provides a summary of the effects of selection and screening DH lines with the aim of identifying those lines with better adaptation across environments. Table 6 shows the number of DH lines derived from the four populations and the number of DH lines planted and harvested at each screening environment. The entire DH lines obtained after doubling from each population were planted at Celaya, during 2012 summer season. In almost all cases, just a few seeds were obtained and planted. From BWP, for example, only 37 DH lines were obtained, which were planted, and only 26 DH lines produced acceptable

Table 4 -Analysis of variance of grain yield and other traits, by location and across locations of test crosses from BYP and NYP DH lines evaluated at Celaya, Gto., Morelia, Mich., during 2013 summer season and 2013-2014 fall-winter season at Obregon, Son., México.

Yellow Kernel Testcrosses								
CELAYA, GTO. 2013								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	7	1941484.6	17.25	16.35	362.77	386.49	0.87	0.51
Genotype	63	4344221.8**	9.69**	11.16**	609.99**	596.82**	0.51NS	0.73NS
Error	49	1586049.3	1.76	2.39	199.23	206.25	0.59	0.51
CV (%)		13.98	1.90	2.15	5.42	10.05	72.18	23.58
MORELIA, MICH. 2013								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	7	3094577.8	13.47	20.83	551.75	376.67	18.25	1.53
Genotype	63	4664954.2**	10.33**	13.98**	354.71**	258.32**	5.79**	0.73*
Error	49	1696974	1.94	2.49	103.35	134.51	1.71	0.44
CV (%)		14.15	1.87	2.06	3.71	8.12	48.89	23.89
OBREGÓN, SON. 2013-2014								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Block	6	1840589.7	48.70	25.57	434.57	440.55	5.08	13.97
Genotype	55	3854770.4**	36.73**	31.17**	233.97**	191.28**	2.14NS	8.78**
Error	43	1035095.1	11.79	10.06	117.36	96.77	1.62	2.65
CV (%)		15.14	4.32	3.83	5.00	7.80	92.65	29.75
COMBINED								
SV	**DF**	**GY**	**MF**	**FF**	**PH**	**EH**	**TL**	**ER**
Location	2	214109711.5**	2599.66**	3443.53**	100122.32**	10350.59**	98.8**	241.33**
Genotyipe	55	6718007.5**	47.39**	47.36**	906.97**	743.12**	4.42**	4.31**
Genotype*Loc	110	2427821.1*	6.96NS	6.35NS	181.72NS	207.18NS	2.91*	3.44**
Error	112	1655848	7.27	6.41	160.75	158.40	2.05	1.20
CV (%)		15.48	3.62	3.29	5.07	9.17	82.10	29.02

SV= source of variation; DF= Degrees of freedom; GY= Grain Yield (Mg ha-1); MF=Male Flowering (days); FF= Female Flowering (days); PH= Plant Height (cm); EH= Ear Height (cm); TL=Total Lodging (Stalk+root) (%); ER=Ear Rot (%).

**= highly significant at P ≤ 0.01; *= significant at P ≤ 0.05; NS = Not significant.

ears at harvest.

At the evaluated environments, DH lines faced different types of selection pressure. At all of the environments, selection for adaptation was practiced, and disease reaction was observed at each environment. For example at Celaya, stalk Fusarium resistance is an essential issue with applying selection. For offseasons, at Iguala, the presence of different fungi that cause ER is highly essential, as is the presence of insect damages in all stages. Additionally, the presence of high temperatures at flowering and the grain filling period contribute to the selection pressure. For Obregon, foliar diseases and high temperatures are also important and an excellent opportunity to select and discard DH lines, with the objective to identify productive, healthy and more stable DH lines.

Looking at planted and harvested values presented in Table 6 allow for analysis of the adaptation of the DH lines. In some cases where DH lines were not harvested, the remaining seeds were used to plant the following season. The per se evaluation across environments created a record of agronomical information for the future cross formation and potential use of commercial seed production.

Table 6 also depicts the ranges of kernel oil content in different types of genetic materials used in this research. The 200 seeds used as donors to induce haploids from each of the four subtropical populations had a range of 6.5% to 7.4% oil. Not all DH lines presented HOC. From 17 BWP DH lines analyzed, nine presented percentage values below 6% and eight DH lines were HOC with values above 6%. Therefore, the range of oil contents of BWP DH lines varied from 3.6 to 8.0%. For NWP, 117 DH lines were analyzed, 58 presented percentages values below 6% and 59 DH lines were HOC, with values above 6%. Thus, the range of oil contents of NWP DH lines varied from 3.8% to 9.3%. For BYP, 43 DH lines were analyzed, 25 DH lines were HOC with values

Table 5 -Grain yield and agronomical traits of the superior Yellow kernel test crosses across locations grouped by the female testers

Genotype	Pedigree	COMBINED 2013-2014						COMBINED	CELAYA	MORELIA	OBREGÓN	
		MF	FF	PH	EH	TL	ER	GY				Oil content
		(days)	(cm)	(%)		(%)			(Mg ha^{-1})			%
Test crosses of NYP DH lines using BYP as tester												
1	BYP X NYP DH133	78	81	242	142	1.73	3.22	9.98	10.94	11.46	7.56	8.2
5	BYP X NYP DH139	75	78	258	149	1.55	2.92	9.52	10.23	10.33	8.00	6.1
16	BYP X NYP DH157	74	77	252	136	2.02	2.77	9.14	10.43	8.51	8.49	4.7
12	BYP X NYP DH147	75	78	263	141	1.02	3.43	8.98	8.40	11.24	7.30	7.6
20	BYP X NYP DH169	75	77	248	138	1.02	3.87	8.43	9.35	8.63	7.32	5.6
7	BYP X NYP DH141	75	77	245	134	1.23	3.93	8.35	9.21	8.90	6.95	6.8
2	BYP X NYP DH136	74	77	243	129	1.48	4.05	8.35	8.40	11.75	4.90	8.0
15	BYP X NYP DH155A	73	75	245	133	1.00	5.20	8.30	9.80	10.29	4.83	4.7
4	BYP X NYP DH138	73	75	263	143	1.43	2.88	8.25	8.33	10.04	6.38	7.0
21	BYP X NYP DH171	72	75	251	136	1.43	2.90	8.21	9.61	7.68	7.34	4.8
Test crosses of BYP DH lines using NYP as tester												
35	NYP X BYP DH218	77	80	267	151	0.90	3.37	11.14	10.92	12.43	10.08	7.6
41	NYP X BYP DH226	76	79	278	153	3.87	2.95	10.71	11.86	10.53	9.73	7.6
46	NYP X BYP DH235	76	80	272	170	2.63	2.58	10.06	10.99	11.56	7.62	7.2
33	NYP X BYP DH214	79	81	249	133	3.27	3.35	9.51	12.11	8.77	7.65	7.2
47	NYP X BYP DH236	76	78	246	126	0.90	2.97	9.47	10.27	10.39	7.75	8.1
40	NYP X BYP DH225	78	81	257	146	2.45	3.40	9.41	9.85	9.63	8.77	5.5
48	NYP X BYP DH238	77	80	271	160	1.10	3.10	9.28	10.05	9.98	7.81	5.9
30	NYP X BYP DH211	76	78	266	161	3.80	2.15	9.24	10.42	7.99	9.32	6.5
39	NYP X BYP DH223	75	77	228	127	2.80	2.28	9.24	10.70	10.45	6.59	6.8
51	NYP X BYP DH246	74	76	257	145	1.02	2.07	9.17	8.81	10.50	8.19	6.2
Checks												
90	NYP§	73	75	255	170	2.0	3.75	8.92	8.45	9.4	----	
89	BYP	77	79	248	131	1.83	2.47	8.45	8.05	10.32	6.99	
C.V. (%)	3.62	3.29	5.07	9.17	82.10	29.02	15.48	13.98	14.15	15.14		
MEAN	74	77	250	137	1.65	3.39	8.28	8.98	9.13	6.57		
LSD (P ≤ 0.05)	3.08	2.89	14.5	14.39	1.63	1.25	1.472	2.531	2.618	2.051		

§Information of NYP is coming from the average of Celaya and Morelia because data from Obregon do not exist.

GY= Grain Yield (Mg ha-1); MF=Male Flowering (days); FF= Female Flowering (days); PH= Plant Height (cm); EH= Ear Height (cm); TL=Total Lodging (Stalk+root) (%); ER=Ear Rot (%).

Oil content %= Oil content percentage determined with samples obtained from two selfed pollinated plants per replication in the yield trial conducted at Celaya.

Table 6 -Ear to row per se evaluation of DH lines derived from HOC populations BWP, NWP, BYP, and NYP. Several planted and harvested DH lines, under different environments in México (above table), Oil content of different type of germplasm described in this research. (lower part)

Environment	BWP		NWP		BYP		NYP		Selection Pressure	Data collected
	Planted	Harvested	Planted	Harvested	Planted	Harvested	Planted	Harvested		
Celaya 2012	37	26	152	132	101	60	58	46	A, D,	P, H, MF, FF, PH, EH, SL, RT
Iguala 2012-2013	16	6	120	57	42	11	28	8	A, D, HT, FI	P, H, MF, FF
Celaya 2013	18	16	127	124	46	45	31	28	A, D	P, H, MF, FF, PH, EH, SL, RT
Iguala 2013-2014	18	6	124	78	46	14	30	16	A, D, HT, FI	P, H, MF, FF
Obregon 2013-2014	9	7	90	74	23	14	16	15	A, D, HT, FI	P, H, MF, FF, PH, EH, SL, RT
Celaya 2014	4	3	81	71	10	5	16	11	A, D	P, H, MF, FF, PH, EH, SL, RT

Oil Content

	BWP	NWP	BYP	NYP
200 Donor Seeds (Range)	6.7-7.4	6.5-7.4	6.7-7.4	6.7-7.4
DH lines (Total)	17	117	43	28
DH Lines (Above 6 %)	9	58	18	8
DH Lines (Below 6 %)	6	59	25	20
DH oil content (Range)	3.6-8.0	3.8-9.3	3.9-7.6	4.2-8.2
Testcrosses (Range)	6.5-8.0	4.8-8.2	4.7-8.2	5.9-8.1

Selection Pressure: A= Adaptation; D= Disease reactions; HT= High Temperatures; FI=Foliar Insects;
Data collected: P= Productivity; H= Healthiness, MF=Male Flowering; FF= Female Flowering; PH= Plant Height; EH= Ear Height; SL= Stalk lodging; RT= Root Lodging

above 6% and 18 presented percentages values below 6%; the range varied from 3.9% to 7.6%. For NYP, 28 DH lines were analyzed, 20 DH lines were HOC, with values above 6% whereas eight contained values below 6% oil. The range of oil contents for these materials varied from 4.2 to 8.2%. The oil content range of test crosses shown in the bottom of Table 6 indicated that the BWP materials went from 6.5 to 8.0% oil whereas the NWP counterparts from 5.7 to 8.2% and for BYP and NYP from 4.7 to 8.2% and 5.5 to 8.1%, respectively.

Our study contributes with useful information for national maize breeding programs and small regional seed companies who might be interested, in using DH breeding methodology, and also the use of test crosses to identify DH lines with GCA considering the heterotic pattern concept. Although DH lines are 100% homozygous, the major problem is the evaluation, selection, and identification of elite parental DH lines to be used in hybrid seed production. Nevertheless, this work supports the idea, of using different heterotic broad populations as testers, which would enable the identification of superior DH lines, with significant effects of GCA (Hallauer and Lopez-Perez, 1979; Geiger et al., 2013). This research also contributes information to the efforts described by Lambert (2000) for developing HOC hybrids, which can be useful for both the animal feed and food industries. The results of this work showed differences in the evaluation environments of the white and yellow kernel test crosses, which help to identify genetic materials that contributed to a better performance across environments, assuming that adaptation is related with good performance across locations. This information is complementary to the per se DH lines evaluation in different environments, to identify DH lines with good per se adaptation and through superior crosses. In subtropical regions, an increasing interest exists to develop high yield potential maize hybrids, with value-added properties, such as HOC. This research used an original strategy to identify superior DH lines since no HOC elite lines or commercial testers exist for subtropical regions, and broad genetic populations had to be used as the source of DH lines; essential and useful information was generated for the integration and consolidation of a HOC hybrid breeding program. Also, according to Prigge et al. (2012) because test crosses were from broad genetic populations, DH lines were expected to have access to novel genetic variation. For the heterotic pattern concept, which had been considered for the integration and development of the genetic populations used for this work, previous studies have been identified that show the white kernel populations manifested a stronger heterotic response compared to yellow kernel populations (Ortega-Corona et al. 2015). The use of BWP, as female tester in white kernel testcrosses presented higher GY than NWP as tester. One interpretation of this is that BWP was a better tester than NWP, but another interpretation might be that NWP DH lines, used in these test crosses manifested significant heterotic effects. A similar trend was observed for yellow kernel test crosses, where NYP used as female tester presented higher GY heterotic combinations than BYP as tester. In general, it was observed that necessary heterotic combinations in white and yellow kernel materials could corroborate the heterotic response between populations after DH line derivation process has been maintained.

Conclusions

Utilizing the advantages of DH technology, this work identified agronomic superior DH lines with GCA and HOC. Test crosses were formed with different heterotic testers and evaluated for different environment properties *per se*. These finer lines can be used as parents for generation of new high yielding HOC subtropical hybrids. The previously identified heterotic response between BWP and NWP populations was maintained in the derived DH lines. The use of double haploid hybrid technology allowed us to reduce costs and time in obtaining hybrids for subtropical environments.

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Conflict of Interest

This manuscript does not have any conflict of interest, since genetic materials and the field evaluation data, belongs to INIFAP. Data information contained in this manuscript had not been published before. The other collaborative authors from the other participant institutions (Tecnológico de Monterrey and CIMMYT) performed the analysis of samples for oil content, and the Instituto Tecnológico de Roque was involved in the educational process which the first author of this manuscript obtained her Master in Science Degree.

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