Stability analyses of sunflower (*Helianthus annuus* L.) hybrids for oleic acid and yield traits under multi location trials in Pakistan

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Highlights

- *An important aim for sunflower breeding is to release hybrids with stable high oleic acid, seed yield and oil content.*
- *Heritability of oleic acid content was medium across testing sites.*
- *Oleic acid content and seed yield increased at high temperature and low humidity particularly in the spring season.*
- *Relation of oleic acid content and combining ability suggests selection effectiveness to breed high oleic acid inbreds.*
- *H1, H4 , H5 and H10 were the most promising hybrids due to their high oleic acid content across sites in the spring.*

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Key words: combining ability; degree days; kernel to seed percentage; seed yield.

Contributions: MHS, worked as a PhD scholar under the project, carried out agronomic evaluations; SN, conducted DNA profiling of hybrids; AN, co-principal investigator of project. SR, principal investigator of the project who conceived the research; RO, made major improvement in the manuscript and technical aspects of research.

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Abstract

The development of a hybrid with high oleic acid is an important breeding goal for sunflower. High oleic acid sunflower has better cooking quality due to low oxidation and rancidity. Hence, inbred lines differing for oleic acid content were selected, alongside the development of hybrids where one or both parents exhibited high oleic acid content in edible oil, and then evaluated at various sites (*i.e.* with comparatively low temperature during sunflower reproductive phase at Sargodha and Faisalabad; while high temperature and low humidity at Bhawalpur and Multan) in Pakistan during spring season. Moreover, autumn season was relatively cool and high humid for sites (Faisalabad and Sargodha, Pakistan). DNA profiling of hybrids differing for oleic acid content using N1-3F/N2-1R confirmed the presence of a high oleic acid allele in the hybrids. Oleic acid content and seed yield components were increased at high temperature and low humidity to a greater extent in spring than in autumn season. Among the hybrids, one (H5) had stable high oleic acid content during the spring season with higher seed yield and kernel to seed percentage than the check cultivars (Hysun-33 and FH-331). Analysis of the combining ability of two locations revealed a relationship between mean oleic acid contents and combining ability, thereby suggesting the effectiveness of selection in developing high oleic acid inbred lines. Newly developed inbred C.112.P was a positive combiner for oleic acid at all sites except Sargodha, while restorer populations such as RH.344, RH.345 and RH.347 were positive male combiners.

Introduction

Sunflower seed is a rich source of edible oil (30-50%), protein (20-30%), tocopehrols (vitamin E), and fatty acids. Its oil contains up to 90% polyunsaturated fatty acid (60% linoleic acid) (Rauf, 2019). Improvement and modification of sunflower oil and fatty acid is an important breeding goal (Alberio *et al*., 2017). Regular sunflower oil (with high linoleic acid) is more suitable as a salad dresser. This is partly due to presence of poly-unsaturated fatty acid in sunflower oil. Sunflower oil rich in linoleic acid is ideal for consumption but such oil shows poor oxidative stability as a cooking or deep-frying oil (Chernova *et al*., 2021). Oil rich in polyunsaturated fatty acids becomes rancid quickly due to its susceptibility to oxidation (Romano *et al*., 2021). On the other hand, oils enriched in saturated fatty acids or mono-unsaturated fatty acid (oleic acid) show more stability during cooking and have greater shelf life than polyunsaturated fatty acids (Romano *et al*., 2021). However, saturated fatty acid increases blood cholesterol level and other health hazards (Rauf *et al.*, 2020; Astrup *et al*., 2021). Fatty acid profiles showed that mid- to high oleic hybrids had oleic acid ranging from 44 to 85%, 9 to 46% linoleic acid, 4 to 6% palmitic acid, and 2 to 6% stearic acid (de Carvalho *et al.,* 2019). Sunflower accessions have been characterized into three types with respect to oleic acid content such as standard sunflower types (<50%), mid oleic acid (50-70%) and high oleic acid content (>80%) (Rauf, 2019; Manalili *et al*., 2021). High oleic acid content was controlled by a single dominant gene mutation. This dominant mutation was induced in breeding program through use of a chemical mutagen ethyl methane sulfonate (Dimitrijević *et al.*, 2017; Rauf *et al.*, 2017; Rauf, 2019).

The Pervenent genotype (obtained through a mutation breeding program) was selected in breeding programs as a sunflower cultivar characterized by high oleic acid. Oleic acid content in Pervenent was highly unstable and ranged between 15-51% and 87-91% across a range of environments, depending upon temperature during the reproductive phase (Alberio *et al.*, 2017). NuSun has been released for general cultivation in the USA as mid-oleic acid commercial cultivar of sunflower (Gupta, 2014).

Temperature, particularly minimum night temperature, and intercepted solar radiation have positive effects on oleic acid content in sunflower (Echarte *et al.*, 2010). Sowing also affects the oleic acid content of sunflower due to the influence of temperature, humidity and rainfall (Akkaya *et al.*, 2019). Early sowing decreased oleic acid and palmitic acids and increased linoleic and stearic acids under irrigation, and irrigation decreased oleic acid contents in a sunflower study by Flagella *et al.*, (2002). In Pakistan, regular sunflower hybrid crops can be grown in different seasons (spring and autumn). Spring season crops are generally sown in February while autumn season cultivation was recommended in August in Pakistan (Qadir *et al.*, 2006). Both sowing periods differ in intercepted radiation, humidity and rain fall and thus provide different environmental condition for growth and reproduction associated with oleic acid synthesis (Qadir *et al.*, 2006). Stability analyses have been carried out to select hybrids with high oleic acid content across environments by Van Der Merwe *et al.* (2013), who evaluated 16 sunflower hybrids and found that one displayed high oleic levels (80%) across all environments. Unstable hybrids may be sensitive to temperature, rainfall and humidity, and will not benefit farmers in some years. In addition to stability across environments, the stability of performance of a parent with multiple other lines to develop good hybrids is essential. Combining ability analyses of 15 hybrids generated from breeding lines differing for oleic acid content revealed dominance or partial dominance in the F_1 for some of the parents (Joksimović *et al.*, 2006). Thus, inbred lines differed in their ability to produce high oleic acid hybrids depending on the other parent; however, some produced high oleic acid hybrids with multiple inbred lines, and were positive general combiners (Joksimović *et al.*, 2006). Oleic acid traits measured in these crosses showed a preponderance of additive gene action, while dominance and epistatic effects were non-significant (Joksimović *et al.*, 2006).

On the basis of this background, the aim of our research was to select inbred lines with high oleic acid for further crossing to develop hybrids, and to evaluate these hybrids in various locations during spring [4 locations: Sargodha, Faisalabad (with comparatively lower temperature than Multan and Bahawalpur across 2 years) and autumn seasons (2 locations with low reproductive phase temperature and higher humidity)] in Pakistan.

Materials and Methods

Development of breeding lines

Pervenent high oleic acid sunflower (89% oleic acid) was introduced from the United States Department of Agriculture (USDA), and superior plants with high fertility were selected and backcrossed to cytoplasmic male sterile lines (CMS-89) to develop two inbred lines C.112.P and C.116.P. High oleic acid restorer populations (RH.345, RH.347 and RH.345) were introduced from the USDA and selected (during year 2018-20) and maintained for purity for several generations. These restorers originally had more than 80% oleic acid content. Low oleic acid inbred lines C.250, C.249, RSIN.82 and R.365 were also included in the crossing. Seeds of the inbred lines were multiplied and produced hybrids as indicated in Table 1. Those hybrids having sufficient seeds were

Table 1. Code and salient feature of hybrids developed and evaluated in the study.

Code	Parentage	Salient feature
FH.331	Cultivar check	Local hybrid, dwarf and early maturing hybrid, low oleic acid hybrid
Hysun-33	Commercial check	High yielding, tall and late maturing hybrid, low oleic acid hybrid
H1	C.112.P × RH.344	Both high oleic acid parents
H2	C.112.P × RSIN.82	High × low oleic acid parent
H3	C.112.P × RH.365	High × low oleic acid parent
H4	C.250 × RH.345	Low × high oleic acid parent
H5	C.112.P × RH.347	Both high oleic acid parents
H6	C116.P × RH.365	High × low oleic acid parent
H7	C.249 × RH.345	Low × high oleic acid parent
H8	C.116.P × RH.344	Both high oleic acid parents
H9	C.250 × RH344	Low × high oleic acid parent
H10	C.249 × RH.447	Low × high oleic acid parent

evaluated in multisite trials (4 locations: Sargodha, Faisalabad, Bahawalpur and Multan) over spring and autumn seasons. Hybrid seed was produced by growing cytoplasmic male sterile lines (CMS) and restorer (R) lines (2:1). Synchronized plants of CMS and R combinations were bagged together before opening of ray florets to avoid insect pollinators and CMS lines were manually pollinated from R lines to improve seed set. CMS lines were also maintained from their B lines while restorer and maintainer lines were maintained through sib mating by bagging two plants of same inbred lines. Mature hybrid seed was manually threshed, dried and put in paper bags and stored at room temperature.

Evaluation of hybrids

The F_1 hybrids were planted in two locations on 15th August 2020 for evaluation during the autumn season; and at four locations on 16th February 2020 for evaluation during the spring. Details of all locations and meteorological features are given in Table 2 and Supplementary Figure S1. Daily mean minimum and maximum temperature is shown in Figures 1 and 2. Mean monthly temperature (°C) fluctuation during growth cycles of sunflower crop is shown in Tables 3 and 4. Temperature during the spring season was low during the vegetative phase and high during the reproductive phase (Figure 2; Table 3). In contrast, the autumn season had relatively higher temperatures during vegetative phase (Figure 1; Table 4). Among the experimental sites, Multan and Bahawalpur had very high temperature stress during the reproductive and grain filling stages (April-May) when compared with Sargodha and Faisalabad (Table 3). Sargodha had temperature stress free growth season during spring season (Table 3).

The soil of the Faisalabad site was a sandy clay loam type with a pH of 7.4±0.27, EC equal to 2.19±0.1 deci Siemen meter^{-1} (dS m^{-1}) and a water holding capacity 18.5% by weight determined through the gravimetric method (Reynolds, 1970). Soil at the Faisalabad site contained Potassium (K) levels of 161.3±4.00 mg kg^{-1}, and phosphorous (P^-) of 8.31±0.58 mg kg^{-1}. The Sargodha site has a sandy loam type of soil with a pH of 7.6±0.11, EC equal to 1.67±0.08 dS m^{-1}, organic matter 0.82±0.11, and 17.5% water holding capacity by weight, while K^+ contents were 172.3±3.17 mg kg^{-1}, and P^- 8.39±0.42 mg kg^{-1}. Multan has a loam soil type with a pH of 8.86±0.19, EC equal to 1.85±1.08 dS m^{-1}, organic matter being 0.42% ±0.05, K^+ contents were 177.5±5.00 mg kg^{-1}, and P^- was 7.47±0.72 mg kg^{-1}, and 18.3% water holding capacity by weight. Bahawalpur has a sandy soil with a pH of 7.63±0.09, EC equal to 2.15±0.22 dS m^{-1}, organic matter 0.64% ±0.04, K^+ of 124.5±7.13 mg kg^{-1}, P^- 11.37±1.01 mg kg^{-1}, and 15.2% water holding capacity. Each hybrid was sown in three rows of 6 m following a randomized complete block design with three replications. Each row was about 40 m long, plants to plant distance was maintained at 22 cm while the distance between rows was 75 cm. Soils were fertilized with inorganic fertilizer, 60 Kg ha^{-1} phospho-

Table 2. Characteristics of the experimental sites used for field trials across seasons in Pakistan.

Trait	Spring				Autumn	
	Sargodha	Faisalabad	Bahawalpur	Multan	Sargodha	Faisalabad
Location	32.07°N, 72.69°E	31.45°N, 73.14°E	29.35°N, 71.69°E	30.16°N, 71.52°E	32.07°N, 72.69°E	31.45°N, 73.13°E
Elevation (m)	190	186	214	122	190	186
Vegetative days	721	880.5	1406.5	1240	861.5	931.75
Reproductive days	835	969.5	1336.5	1570	830	678
Rainfall (mm)	181.5	170.0	71.6	120.6	43.1	56
Preceding crop	*Brassica juncea*	*Brassica juncea*	*Brassica juncea*	*Brassica juncea*	*Triticum aestivum*	*Triticum aestivum*
Supplemental irrigation	1.0 (76 mm)	1.0 (76 mm)	4.0 (304 mm)	4.0 (304 mm)	1.0 (76mm)	1.0 (76mm)
Relative humidity	52	62	29	39	68	73

Table 3. Monthly temperature (°C) fluctuation among the experiment sites during spring season.

Month	Multan		Bahawalpur		Faisalabad		Sargodha	
	Max	Min	Max	Min	Max	Min	Max	Min
Feb (sowing)	26.20±4.60	15.60±2.87	29.80±3.71	15.93±2.05	24.56±2.34	12.56±2.80	22.81±3.23	10.06±1.53
March (vegetative)	33.63±4.45	22.10±3.80	32.47±4.46	18.70±3.67	25.22±4.25	14.41±1.19	17.55±4.05	12.44±2.09
April (anthesis)	41.10±2.90	28.76 ±2.71	40.34±2.63	25.85±2.49	33.17±3.13	20.57±2.40	29.67±3.50	17.93±2.33
May (grain filling)	44.96±3.69	34.25 ±2.41	43.12±3.30	28.65±3.30	38.22±3.87	24.39±2.46	28.47±3.27	21.17±2.72

Table 4. Monthly temperature (°C) fluctuation among the experiment sites during autumn season.

Month	Sargodha		Faisalabad	
	Max	Min	Max	Min
August (sowing)	34.81±1.47	26.06±1.76	39.41±1.25	28.88±1.41
September (vegetative)	29.85±3.20	22.78±2.89	34.22±2.45	23.23±3.40
October (anthesis)	29.57±1.50	16.57±2.33	33.22±3.14	15.78±2.51
November (grain filling)	14.70±2.53	12.04±1.77	22.83±2.48	10.17±2.38

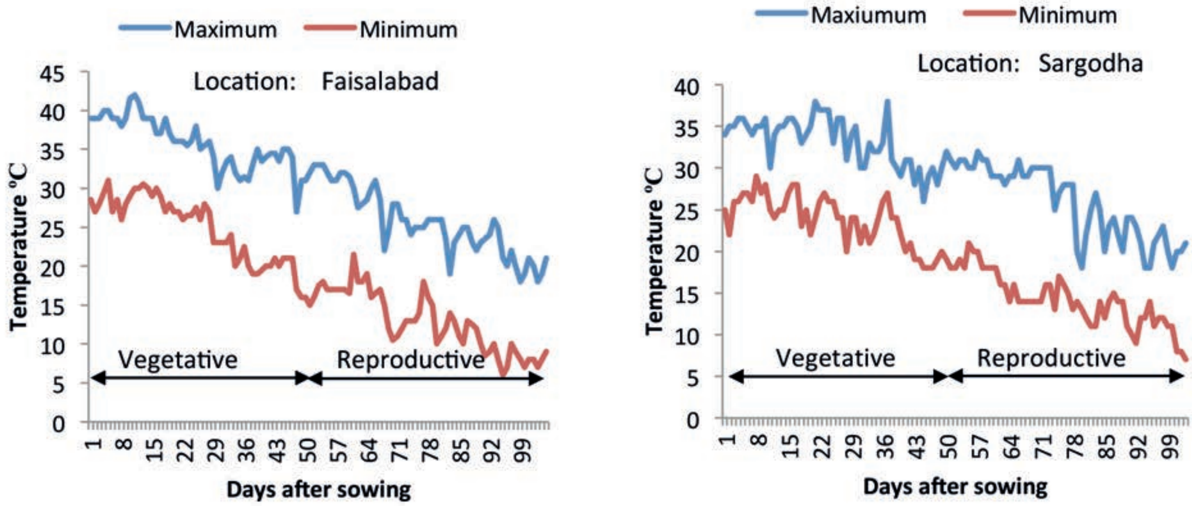

Figure 1. Daily mean minimum and maximum temperature during autumn season.

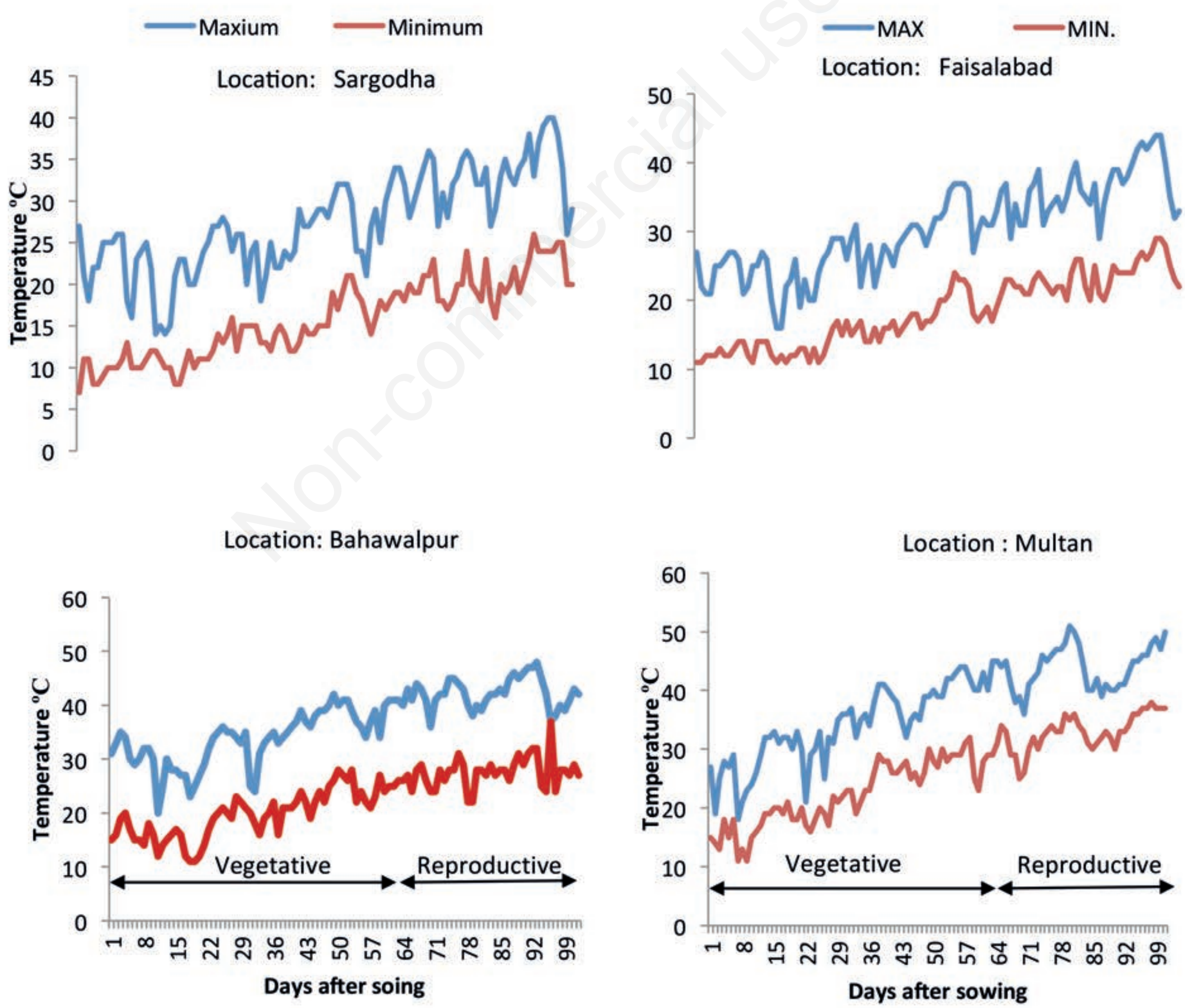

Figure 2. Daily mean minimum and maximum daily temperature during spring season.

rus [6 bags (each 50 Kg) of diammonium phosphate, Engro, Pakistan], and 40 kg ha^{-1} of Nitrogen [2 bag (each 50 kg) of urea, Engro, Pakistan] during field preparation. The pre-emergence herbicide S-metolachlor (dual gold, Syngenta, Pakistan) was sprayed at all sites to control the growth of weeds. Pest scouting was done throughout, and recommended pesticide was sprayed (20 mL dissolved in 20 L water) when armyworm (Coragen, FMC, Pakistan) populations reached above threshold levels during the spring season. No specific infestation was seen at either site during the autumn season.

Hybrid seed production for on farm yield trials

Inbred lines were planted during the 2021 autumn season (15th August 2021) for mass production of hybrid seed. Inbred lines (C-112.P, C-116P, C.250, RSIN.82, RH.344, RH.347, RH.345) were sown as (2:1) of female and male parents to yield several cross combinations. Cross combinations were obtained by bagging each of the floral head as bud was of sufficient size before opening. All unbagged buds were removed before their opening. Each male line was sown in the middle of two CMS lines. Pollen was transferred manually from the bagged male floral heads by shaking male head over CMS heads. The procedure was continued until all the stigma withered and seed started to develop within floral head. Mature floral heads were harvested and dried under the shade. Seeds were manually threshed, cleaned and dried, and put in a Kraft paper bag and stored at room temperature.

On farm yield trials

Spring season sunflower yield trials were performed at four locations (Shahkot, Sargodha, Multan and Bahawalpur) with seeds sown on 12th February 2021. About 100 g seed of each hybrid (H1, H2, H4, H5, H8, H9 and Hysun.33) were given to farmers for plantation in the field on ridges with plant-to-plant distance of 22 cm. Each hybrid was sown in 12 rows of 67 m, which were 75 cm apart.

Measurement of seed yield and components

Five heads from consecutive plants within each of 3 rows (I m^{-2}) were harvested and threshed manually. Seed from all hybrids from each replication within each location were dried to a constant moisture (8%) under natural sunlight. Seed yield (g m^{-2}) was determined on a digital balance. Seed lots of 100 g were obtained after de-hulling using a rotary machine. The mass of dehulled seed was determined to calculate kernel to achene ratio as follows:

$$\textit{Kernel to achene ratio} = \frac{\textit{mass of kernel after dehulling}\ (g)}{\text{mass of 100 g of achene}} \times 100$$

Kernel oil content after de-hulling was determined on a soxhlet apparatus (64826, Sigma, USA). About 10 g of kernels were crushed and put in thimble to extract oil through petroleum ether. Kernel oil contents were determined by following equation:

$$\textit{Kernel oil contents}\ (\%) = [\textit{KM before extraction}\ (g) - \textit{KM after extraction}\ (g)] \times 100\ \textit{KM}\ \text{before extraction}$$

where KM is kernel mass (g)

Fatty acid profile

A manual hand oil extractor (hand extractor, locally assembled, Pakistan) was used to obtain a small quantity of oil (1.5 g) without applying heat. The oil was put in Eppendorf tubes to profile the fatty acids. Small amounts of seed (10 g) were put in the extractor and pressed to obtain about 1.5 g of oil. A 50 μL sample of this oil was methylated using 4 mL KOH for one hour at room temperature. Methylated fatty acids were extracted with hexane. Fatty acid profiles of all edible oils were analyzed using gas chromatography (M-3900, Varian, USA). Analysis was done using the fused capillary column, flame ionizing detector and nitrogen gas carrier at 3.5 mL min^{-1}. Injector and detector temperature were set at 260°C, while column oven temperature was set at 222°C. Methylated esterified fatty acids were injected manually while fatty acids were identified through peak retention time when compared with a known standard (Sigma Aldrich, USA, purity ≥98%).

DNA profiling

DNA of sunflower hybrid seed obtained after crossing among various parental lines were amplified with primer N1-3F/N2-1R. This primer has been extensively utilized for identification of high oleic acid sunflower germplasm (Bervillé *et al.*, 2009; Dimitrijević *et al.*, 2017; Bilgen *et al.*, 2018). DNA profiling was carried out by the Agricultural Biotechnology Research Institute, Pakistan. Sunflower seed samples of various hybrids were first frozen in liquid nitrogen and ground into a fine powder. About 200 mg of the samples were used to isolate the total genomic DNA using cetyltrimethylammonium bromide methodologies as described by Rogers and Bendich (1985) with some modifications. Briefly, about 100 ng of isolated DNA was used in polymerase chain reaction (PCR) for validation of the presence of the allele causing high oleic acid using specific primers *i.e.* N1-3F/N2-1R given by Bilgen *et al.,* 2018. The PCR profile was comprised of 35 cycles of 95°C for 30 seconds, 56°C for 45 seconds and 72°C for 1 minute with a final step of 72°C for 7 minutes. The amplified PCR product was separated on 1.5% agarose gel and visualized under UV gel documentation system.

Statistical and biometrical analyses

All data were analyzed using the computer-based R software (Ferreira *et al.*, 2014) considering a randomized complete block design with 3 replications at 4 locations during the spring season, while 3 replications in each of the 2 locations were used for the autumn season. R functions GGEbiplotGUI were used for GGE biplot analysis, which was originally outlined by Yan and Kang (2002). Stability parameters such as ecovalence (W_i^2; Wricke, 1962), regression coefficient (b_i; Finlay and Wilkinson, 1963), deviation from regression (s^2d_i; Eberhart and Russel, 1966), stability variance ($\sigma^2_{i,}$; Shukla, 1972), coefficient of variation (CV, Francis and Kannenberg, 1978); mean and genotype × environment variance component, ($\theta_{(i)}$ and θ_i: Plaisted and Peterson, 1959), and ranking (KR; Kang, 1988) were estimated using STABILITYSOFT (Pour-Aboughadareh *et al.*, 2019). The estimated eco-valence (wi^2) is the contribution of each genotype to its interaction with the environment. A low value indicates trait stability. The regression coefficient (b_i) also allows identification of stable genotypes. Combining ability effects were estimated on an Excel worksheet following Kempthorne (1957). Mean values of traits were compared using least significant differences at a probability threshold below or equal to 0.05.

Results

PCR identified the high oleic allele of 870 bp in medium and high oleic acid sunflower hybrids (2, 3, 4 and 6; lane

2=C.112.P×R.SIN.82 (H2); 3=C.112.P×RH.344; 4=C.249×RH.344; 6=C.250×RH. 344); while the high oleic acid band was absent in other hybrids (lane 1=Hysun.33, 5=C.224×R.SIN.82; 7=FH.33) (Figure 3).

There were significant (P≤0.05) differences among the hybrids, locations and the interaction of hybrids × locations according to the analysis of variance (Table 5). The significant (P≤0.05) hybrids × location interaction indicated that hybrids changed their relative performance across locations. Hence, means were compared with reference to their specific location. Oleic acid had a medium heritability estimate, while kernel oil contents percentage (KOC%) and seed yield (SY) had low heritability estimates across four locations during the spring season (Table 5).

Among inbred lines B.250, B.249 and R.SIN.82 had the highest de-hulling percentage (KTS%). while RH.344 had the highest KOC%. Oleic acid content of parental lines during the spring season are given in Table 6. Parental lines B.116.P and B.112.P, which were selected from the Pervenent population, had the highest oleic acid content as female lines. Introduced restorer populations produced 70 to 75% oleic acid averaged across the four locations (Table 6).

The hybrids had the highest SY (g m^{-2}) at Multan. Among the hybrids, H5 had the highest seed yield at Multan and Faisalabad. Lowest SY values were noted at Sargodha during the spring season (Figure 4A), where hybrid H1 had the highest SY. Hybrid H3 had the highest SY at Bahawalpur (Figure 4A). With respect to location responses for KOC% and kernel to seed percentage (KTS%), mean values of these traits were highest at Sargodha, where on average the lowest SY was observed during the spring season. Hybrids FH.331, H3, H5 and H10 had the highest KTS% at Sargodha, while H4 had the highest KTS% at Multan. H1 had the highest KTS% at Bahawalpur, and FH.331 at Faisalabad during spring season (Figure 4B). Hybrid FH.331 (check cultivar) and hybrids H1, H2, H3 and H7 had the highest KOC% at Sargodha (Figure 4A).

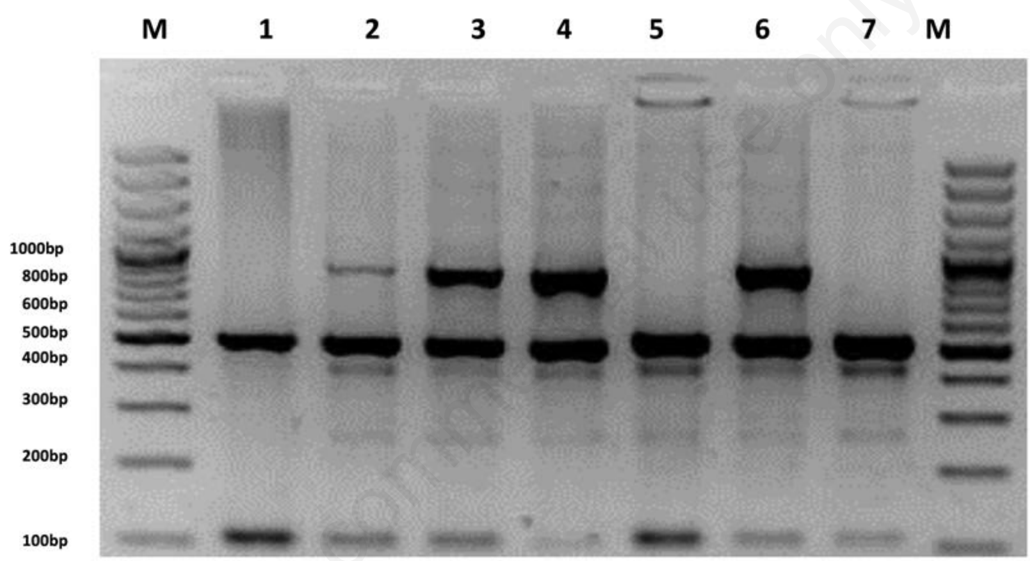

Figure 3. Amplification 870 bp fragment in lanes 2, 3, 4 and 6 that are specific for high oleic (HO), thus confirming the successful cross combinations for HO in sunflower. Lane 1=Hysun.33, 2=C.112.P×R.SIN.82 (H2); 3=C.112.P×RH.344; 4=C.249×RH.344; 5=C.224×R.SIN.82; 6=C.250×RH. 344; 7=FH.331.

Table 5. Analyses of variance for seed yield and quality traits during spring season in sunflower and estimated variances (σ²).

Sources of variation	Degrees of freedom	Mean squares			
		Oil contents (g)	Oleic acid (%)	Kernel to seed (%)	Seed yield (g m^{-2})
Blocks	2	1.30^{NS}	70.05NS	3.60^{NS}	377.55NS
Hybrids (H)	11	35.38**	1256.37**	51.64**	16088.46**
Locations (L)	3	183.26**	1607.93**	456.04**	438595.16**
H × L	33	22.97**	203.18**	52.01**	5863.14**
Residual	94	3.16	35.20	9.29	1254.55
Total	143	13.96	201.38	31.70	12621.85
σ^{2}genotype		1.03	87.77		852.11
σ^{2}phenotype		10.80	176.03		3642.86
Heritability (h^2)		0.10	0.49		0.23

**,^{NS}indicate significant at P≤0.01 and non-significant at P>0.05, respectively.

H1 had the highest KOC% at Faisalabad, Bahawalpur and Multan (Figure 5A). There were significant differences (P≤0.05) among the hybrids for oleic acid (%) at all locations and seasons. Both commercial hybrids had lower oleic acid percentage at all locations (Figure 5B). The highest oleic acid was obtained at Multan and Bahawalpur while hybrids showed the lowest values of oleic acid% at Sargodha and Faisalabad during the spring season. H1 and H5 had the highest oleic acid percentage (≥80%) at Bahawalpur, while hybrid H9, H2 and H5 (≥80%) had the highest oleic acid (≥70%) at Multan. H1 had the highest oleic acid percentage at (≥70%) at Faisalabad while H7 and H8 showed the highest oleic acid percentage (≥60%) at Sargodha (Figure 5B). Multan and

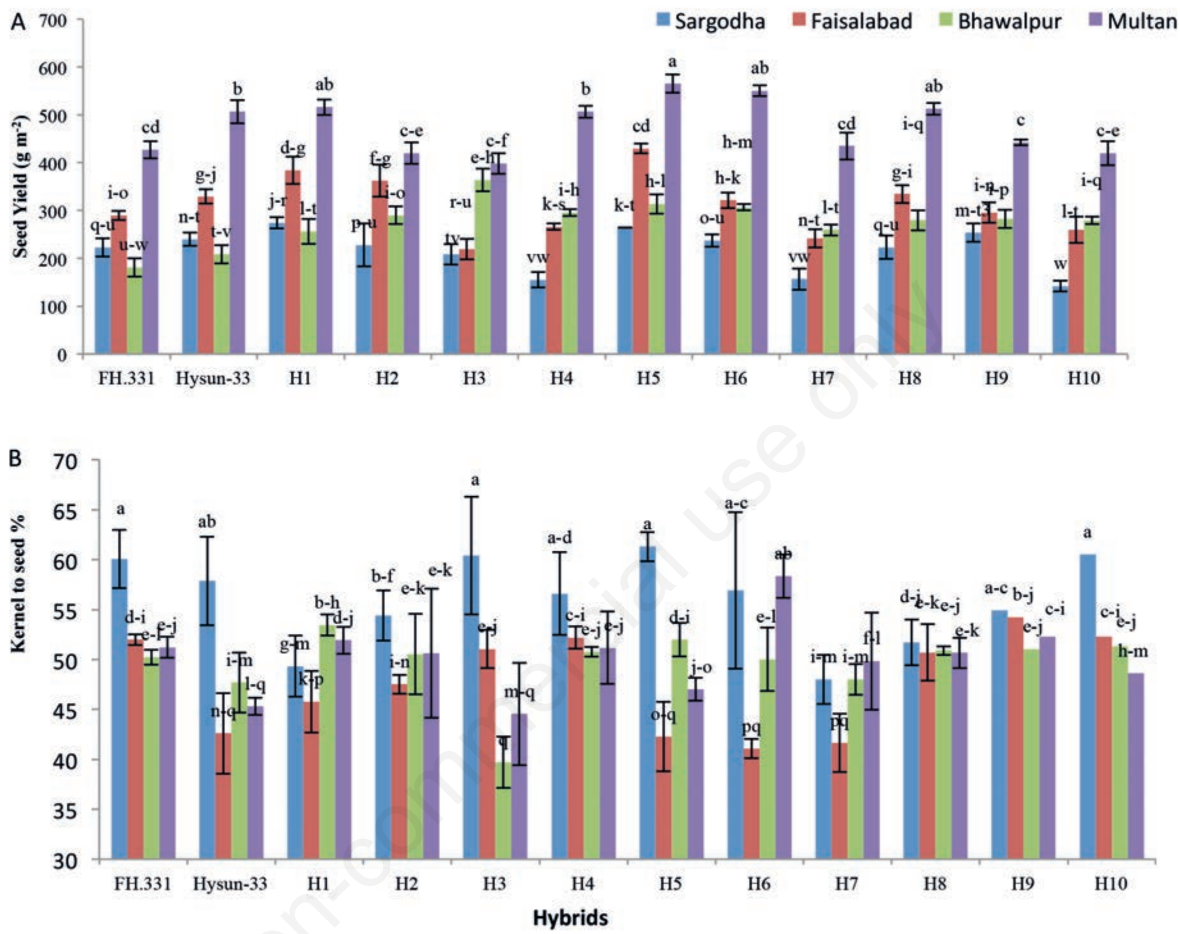

Figure 4. Characteristics of newly developed hybrids along with cultivar checks in the spring season. A) Seed yield (g m-2); B) de-hulling percentage (kernel to seed percentage).

Table 6. Average seed quality traits of various maintainer and restorer lines used for the development of hybrids to be grown in the spring season.

Parents	Kernel to seed (%)	Kernel oil content (%)	Oleic acid (%)
B.208	56.00±3.18	43.10±1.74	35.55±1.47
B.116.P	36.40±2.27	40.12±2.21	71.24±1.52
R.SIN.82	62.00±2.38	45.34±1.92	35.55±1.67
RH.344	56.40±4.15	52.22±1.34	70.00±2.12
B.250	68.40±1.67	44.11±2.12	52.31±2.33
RH.345	51.00±2.93	42.15±1.69	69.27±1.22
RH.365	55.60±3.71	44.10±3.54	40.93±1.39
B.112.P	48.00±3.57	43.19±3.19	78.38±4.12
B.249	63.20±4.19	45.33±2.54	44.09±2.19
RH.347	61.20±3.38	50.13±1.69	75.23±2.16
RH.447	53.24±4.19	47.21±1.86	74.29±3.19

Bahawalpur had favorable environmental conditions for SY and oleic% during the spring season. Sargodha had the highest oleic acid percentage in comparison to Faisalabad during the autumn season. Hybrid H10 had the highest oleic acid percentage (≥60%) at Sargodha and Faisalabad followed by H3 at both locations (Figure 6A). FH.331 and H10 showed similar SY values across both locations during the autumn season. Hybrid H7 had the highest SY during the autumn season followed by H2 and H3. H10 had the highest SY at Faisalabad during the autumn season (Figure 6B). Biplot analysis of oleic acid% showed that H4, H5 and H10 had relatively stable oil content across locations during the spring season (Figure 7). FH.331 had stable but low oleic acid %. H1 had the highest oleic acid at Multan and Bahawalpur during the spring season. Although Hysun.33 was characterized as being a low oleic acid hybrid, but it produced comparatively higher oleic acid at Multan than in other locations (Figure 7).

Stability parameters estimated for oleic acid percentage and seed yield are given in Tables 7 and 8, respectively. Hybrid H5 had the highest oleic acid contents across locations (Table 7), followed by H7 and H8 during spring season. As per wi^2, H10 (mid-oleic acid%) had the lowest wi^2 value followed by H1 (high oleic acid hybrid). H7 and H10 had the smallest Shukla's σ^2_i stability values, thus being stable. Genotypes with b_i about 1 and non-significant S^2_{di} were regarded as stable. Hybrids H10, H4 and H5 had regression coefficient about 1 with lowest non-significant S^2_{di}, and high SY. The hybrids H5, H4, H7 and H10 were the most stable and with the highest oleic acid content.

Hybrid H5, H1 and H6 had the highest SY (g m^{-2}) across locations (Table 8). H8 had the lowest eco-valence and Shukla's σ^2_i. H7, H1 and H10 had b_i about 1. H8 had a non-significant S^2_{di} that confirms its stability for SY. Hybrid H8 also had the lowest Kang's ranking (KR), which indicates that this hybrid as having the highest stability along with high SY when grown in the spring season.

Combining ability effects showed significant (P≤0.05) relationships with mean oleic acid contents of inbred lines at Faisalabad and Sargodha. The variability among inbred lines was

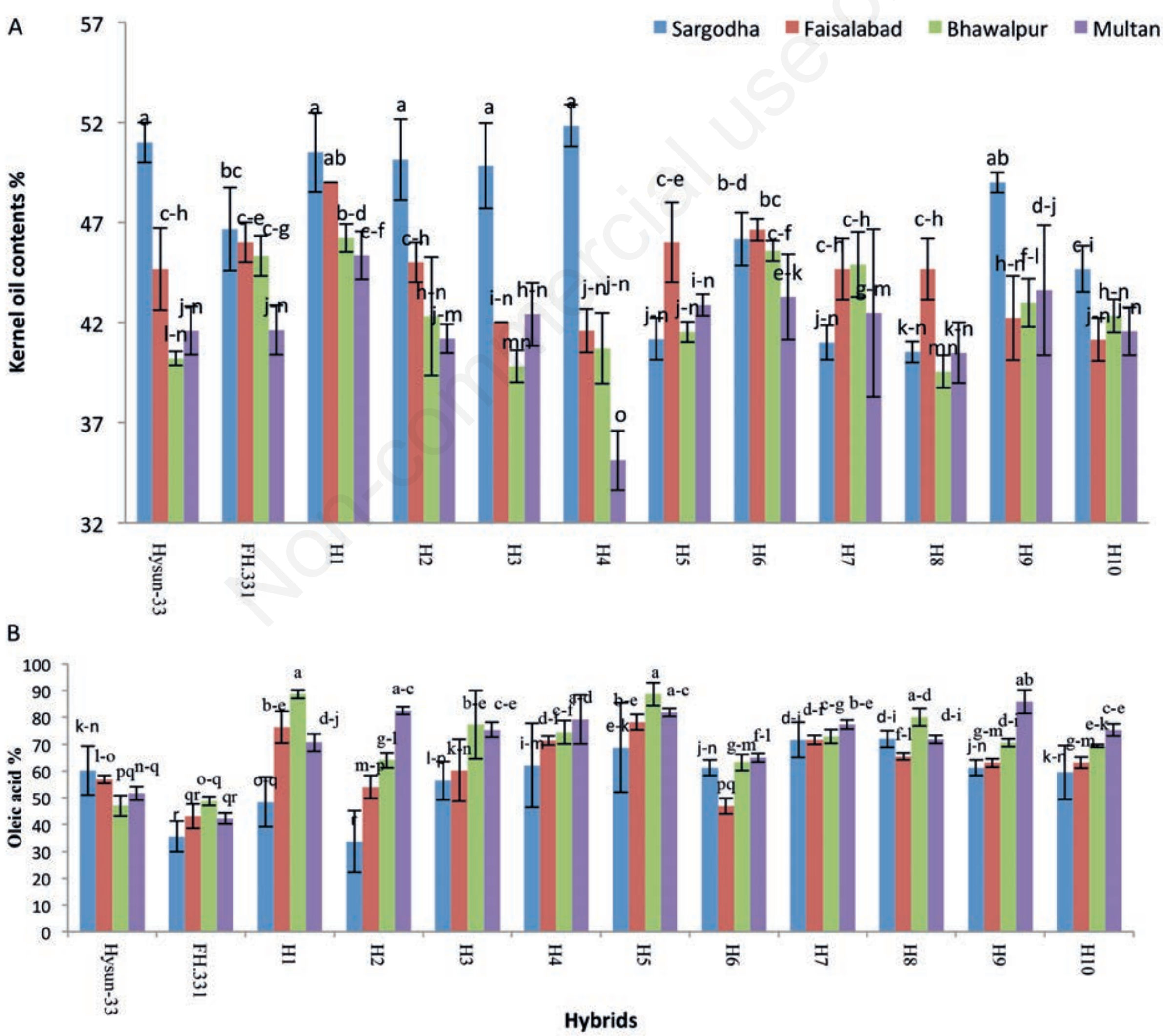

Figure 5. Characteristics of newly developed hybrids along with cultivar checks in the spring season. Bars showing similar alphabets are statistically insignificant (P≥0.05) estimated by least significant difference test. A) Kernel oil content (%); B) oleic acid percentage.

masked by the environment at both Sargodha and Multan, where relationship between combining ability effects and mean oleic acid contents were not high (Figure 8). Among inbred lines, RH>347, RH.344 and B.112.P had higher combining ability values for oleic acid content across locations except Sargodha, where RH.347 and RH.345 were positive combiners. B.112.P had high mean oleic acid content but with negative combining ability effects (Figure 8).

Analysis of variance showed significant variation due to hybrids and hybrids × locations. Hybrid H2 (oleic acid contents 75.01%) had the highest seed yield averaged over all locations followed by H1 (oleic acid content 81.38%) and standard check (oleic acid contents 35.06%) (Figure 9). Hybrids had the highest seed yield at the Multan where Hysun.33 (oleic acid contents 35.06%) and H2 (oleic acid contents 80.5%) showed the highest seed yield potential (Figure 9). H8 (oleic acid content 76.03%) and H1 (oleic acid content 82.6%) had the highest yield at Bahawalpur (Figure 9).

Discussion

Our research was initiated to develop high oleic acid hybrids with better SY. Breeding lines with high oleic acid and high fertility were derived from Pervenent. These newly developed breeding lines were further used as female lines in the breeding program. They were mated with low oleic or high oleic acid restorer populations. DNA profiling of the hybrids provided a low cost and reliable method of identification of high oleic acid hybrids. Field screening of the high oleic acid sunflower is complicated due to instability of the gene at high temperature as identified in our research. The high oleic acid allele was detected in sunflower hybrids that were successful in breeding programs to develop high oleic acid breeding lines. The developed hybrids were evaluated at four locations, which differed for the total degree days received during their reproductive phase.

Generally, those hybrids whose parents were selected on the

Table 7. Oleic acid contents % and stability parameters ^{Z} estimated from four locations in Pakistan during spring season.

Hybrid	Y	W_i^2	σ^2_i	s^2d_i	b_i	CV_i	$\theta_{(i)}$	θ_i	KR
Hysun-33	53.96	446.94	172.00	2.18	-0.79	10.70	58.25	118.51	21
FH.331	42.45	55.25	15.33	5.20	0.63	12.81	72.49	47.30	17
H1	71.01	484.03	186.84	54.77	1.87	23.71	56.90	125.25	16
H2	58.55	607.65	236.29	19.78	2.87	34.77	52.40	147.73	22
H3	67.32	54.86	15.17	2.25	1.54	15.67	72.50	47.22	11
H4	71.76	16.15	-0.31	2.30	1.02	10.03	73.91	40.19	6
H5	79.38	41.59	9.86	5.68	1.12	10.46	72.99	44.81	4
H6	59.06	186.90	67.99	23.02	0.56	13.94	67.70	71.23	18
H7	73.33	74.16	22.89	1.34	0.30	3.68	71.80	50.73	8
H8	72.32	125.15	43.29	11.86	0.44	8.33	69.95	60.00	10
H9	70.14	130.99	45.62	15.37	1.42	15.98	69.74	61.07	14
H10	67.10	11.29	-2.26	1.59	1.03	10.67	74.09	39.30	9

Y, mean seed yield (g m^{-2}) across all locations; Wi2, Wricke's (1962) ecovalence; σ^2i, Shukla's (1972) stability variance; s^2d_i, Finlay and Wilkinson's (1963) deviation from regression; b_i, Eberhart and Russell's (1966) regression coefficient; CV, Francis and Kannenberg's (1978) coefficient of variation; $\theta_{(i)}$ and θ_i, Plaisted and Peterson's (1959) mean and genotype × environment variance component; KR, Kang's (1988) ranking.

Table 8. Seed yield (Y, g m^{-1}) and stability parameters ^{Z} estimated from four locations in Pakistan during spring season.

Hybrid	Y	W_i^2	σ^2_i	s^2d_i	b_i	CV_i	$\theta_{(i)}$	θ_i	KR
HFH.331	280.17	5587.83	2039.70	744.97	0.90	38.42	1946.58	2090.85	18.00
Hysun-33	321.04	6426.11	2375.01	806.63	1.15	41.66	1916.09	2243.27	16.00
H1	357.63	4941.27	1781.07	703.68	1.02	33.48	1970.09	1973.30	9.00
H2	324.92	5792.15	2121.43	386.23	0.71	25.81	1939.15	2128.00	14.00
H3	297.33	20103.92	7846.14	2104.95	0.62	32.86	1418.72	4730.14	21.00
H4	305.71	6440.79	2380.88	461.98	1.30	48.02	1915.56	2245.94	19.00
H5	393.13	4323.06	1533.79	454.16	1.18	34.07	1992.57	1860.90	7.00
H6	353.92	2489.33	800.30	91.97	1.22	38.42	2059.25	1527.49	6.00
H7	273.17	1608.61	448.01	222.89	1.04	42.70	2091.28	1367.36	14.00
H8	337.29	774.11	114.21	18.15	1.13	37.13	2121.62	1215.63	5.00
H9	318.42	2662.75	869.67	67.03	0.76	26.47	2052.94	1559.02	11.00
H10	275.17	3343.18	1141.84	476.58	0.99	41.37	2028.20	1682.74	16.00

Y, mean seed yield (g m^{-1}) across all locations; Wi2, Wricke's (1962) ecovalence; σ^2i, Shukla's (1972) stability variance; s^2d_i, Finlay and Wilkinson's (1963) deviation from regression; b_i, Eberhart and Russell's (1966) regression coefficient, $\theta_{(i)}$ and θ(i); CV, Francis and Kannenberg's (1978) coefficient of variation; $\theta_{(i)}$ and θ_i, Plaisted and Peterson's (1959) mean and genotype × environment variance component; KR, Kang's (1988) ranking.

basis of high oleic acid production, also produced high oleic acid at warmer and low humidity sites, especially Bahawalpur. This finding agrees with previous research showing that high temperatures stimulate higher production of oleic acid (Echarte *et al.*, 2010, Akkaya *et al.*, 2019). However, there was no clear-cut advantage of using both parents in breeding programs to develop hybrids with stable high oleic acid (Van Der Merwe *et al.*, 2013, Alberio *et al.*, 2017).

Oleic acid, seed yield and oil content traits are known to be affected by environment. Generally high temperature favored the production of oleic acid (Figure 5B). Evaluation showed that hybrids had higher oleic acid and seed yield at locations with high

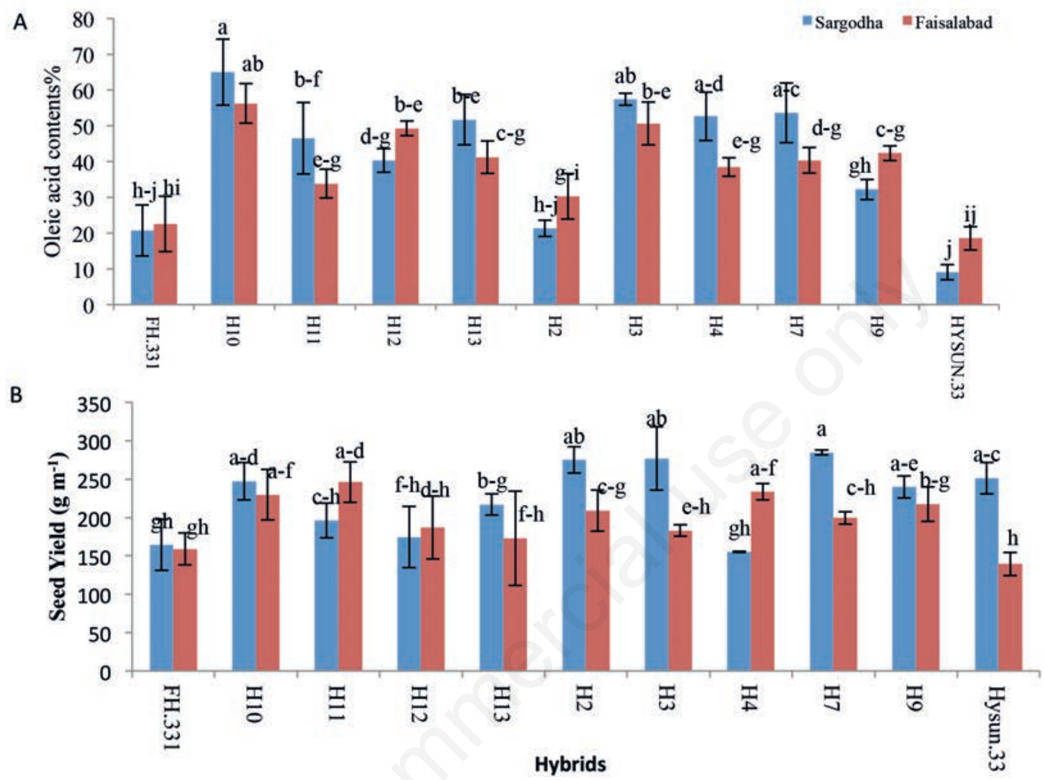

Figure 6. Characteristics of newly developed hybrids along with cultivar checks in the spring season. A) Seed yield (g m^{-2}); B) oleic acid percentage. Bars showing similar alphabets are statistically insignificant (P≥0.05) estimated by least significant difference test.

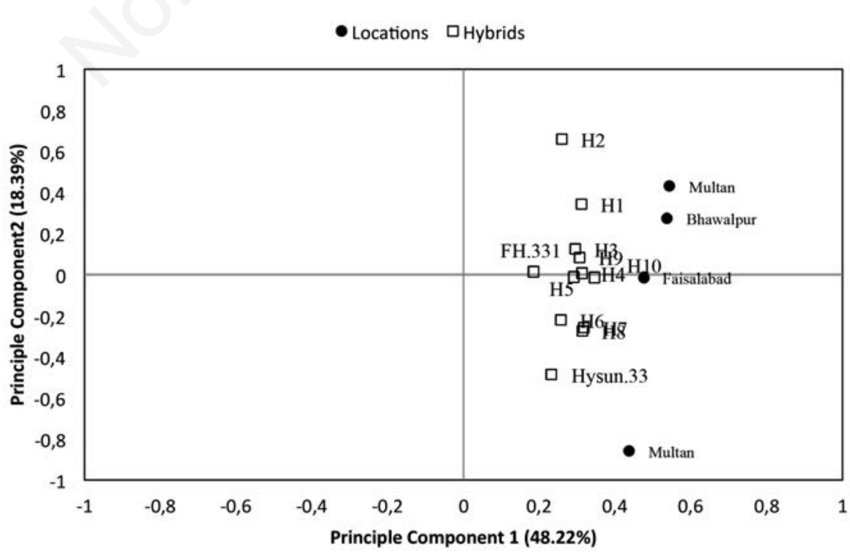

Figure 7. Biplot analysis of hybrids + (hybrid × location) for oleic acid content in newly developed hybrids.

temperature along with supplementary irrigation during the key reproductive phase. While temperature was optimal for growth at Sargodha, the oleic acid and SY (g m^{-2}) were low, possibly due to high humidity and water stress negatively affecting SY due to infestation by fungal diseases (Venkataramanamma *et al*., 2020). Seed oil contents percentages were favored under mild temperature and site such as Sargodha had higher oil contents than other sites.

Development of sunflower hybrids with stable high oleic acid, seed yield and oil content was considered an important breeding objective for potential rapid commercialization (Ghaffari *et al*., 2021). Therefore, this study evaluated sunflower hybrids under diverse environmental conditions and seasons to select hybrids

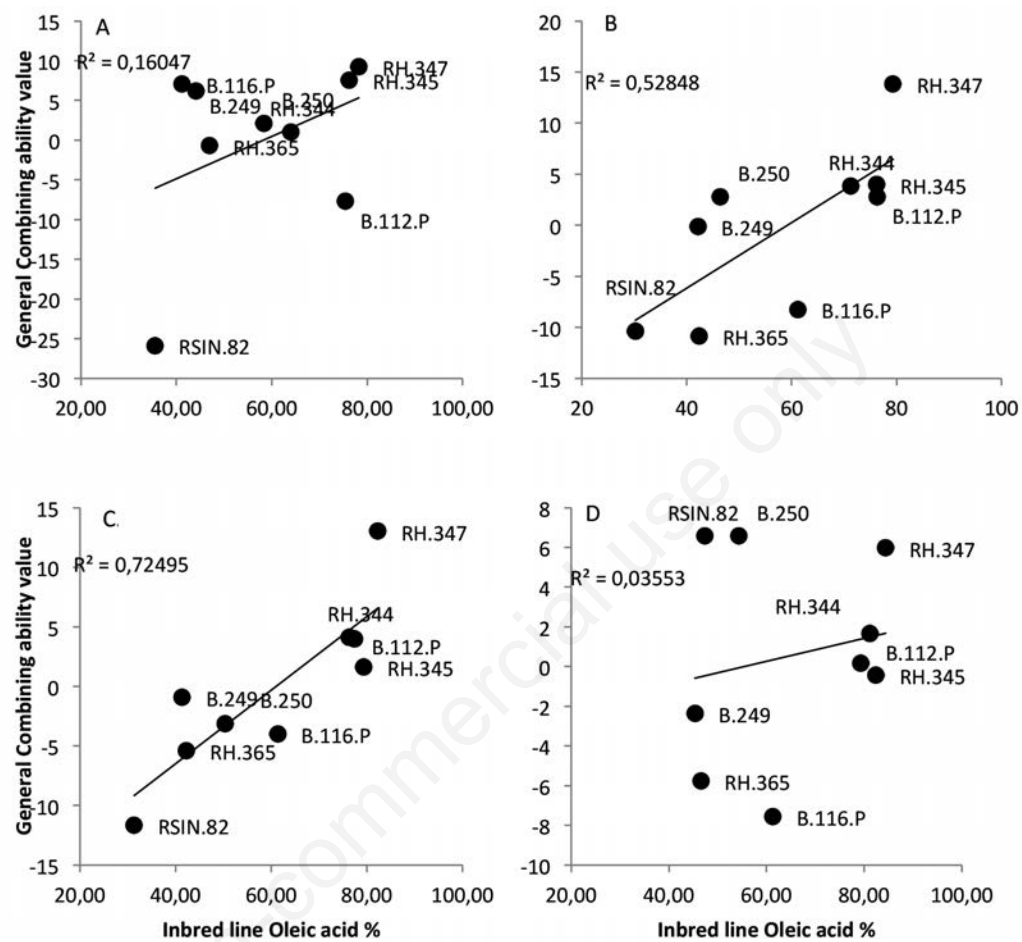

Figure 8. Relationship between combining ability effects and mean oleic acid contents of inbred lines at four locations, namely, Sargodha (A), Faisalabad (B), Bahawalpur (C) and Multan (D) in Pakistan.

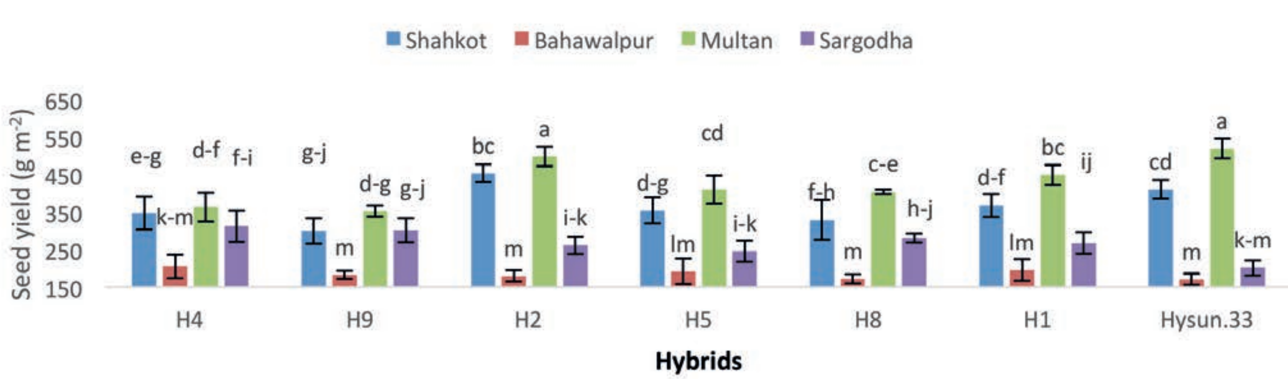

Figure 9. Farm yield trial of high oleic acid sunflower hybrids. Bars showing similar alphabets are statistically insignificant (P≥0.05) estimated by least significant difference test.

with relatively stable oleic acid and SY. Among the evaluated hybrids, H1 (both parents with high oleic acid), H4 (only one parent with high oleic acid), H5 (both parents with high oleic acid) and H10 (one parent with high oleic acid) were the most promising due to their high oleic acid content across different locations during the spring season (Figure 4B). Among these hybrids, H10 was the most promising due to its stable and medium oleic acid content (67%) across locations. This hybrid also showed medium oleic acid content during the autumn season (Figure 5A). Another promising hybrid H5 (oleic acid 79%) was also stable according to the biplot analysis and KR. Previous research demonstrated that high oleic acid content was due to a dominant mutation, which seems contradictory to the response of hybrids in our study, *i.e.*, hybrids with one oleic acid parent had medium oleic acid showing partial dominance (Joksimović *et al.*, 2006). Some high oleic acid hybrids such as H5 also had the highest SY across locations and had higher yield than a low oleic acid standard hybrid. H8 was the most stable hybrid with the lowest environmental variances for SY. This seems contradictory to previous findings which, show that high oleic acid genotypes had low seed yield potential (Zambelli, 2021).

The selection of breeding lines with better combining ability could lead to the development of superior highly heterotic hybrids. Generally, lines with higher combining ability values may be utilized in hybrid breeding programs. Combining ability effects showed a high positive relationship with mean oleic acid content at Faisalabad and Bahawalpur (Figure 8), indicating useful gain from selection of this trait. Furthermore, heritability of oleic acid content was medium across locations. This may be due to the response of the high oleic acid mutant genes to environmental factors such as temperature, water availability and humidity (Echarte *et al.*, 2010; Akkaya *et al.*, 2019). Several genomic regions are known to modify oleic acid content in sunflower (Premnath *et al.*, 2016). Inbred lines RH.344, RH.347. RH.345 and C.112. were positive combiners for high oleic acid content and are regarded as promising lines for the development of high oleic acid hybrids.

Conclusions

This study aimed to develop hybrids with high oleic acid contents and SY. Hybrids were evaluated at multiple locations across two seasons. Among the evaluated hybrids, H1, H4, H5 and H10 were the most promising due to their high oleic acid content across the locations during the spring season. Hybrid H10 was the most promising due to its stable and medium oleic acid content (67%) across the locations and between seasons. Hybrid H5 had high oleic acid (79%) and was stable according to the biplot analysis and Kang's ranking. The H5 hybrid also had the highest SY across locations and exhibited higher yield than a low oleic acid standard hybrid. Breeding lines such as RH.344, RH.347. RH.345 and C.112. were positive combiners for high oleic acid content and are regarded as promising lines for the development of high oleic acid hybrids.

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