Exploring Sunflower Genetic Potential: Insights from Combining Ability Analysis through Lines × Tester Analysis and DNA-based Hybrid Authentication.

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Abstract

The study used the RCBD approach with three replications, L×T design was used where restorer and CMS lines were crossed and sample data from five plants with three replications were collected. Seeds of the resulting hybrids were saved for the following growing season. Statistical analysis was used to examine the differences between parents and crosses, and heterosis and combining ability were used to assess the best-performing combination. Maximum mean performance was shown by lines T3 for plant height, T4 for head diameter, T-3 and T-1 for leaf number, and T1 for 100 achene weights. Line T-3 had maximum GCA performance for all the characters and was the best to study further. For leaf area T-3×B-1, for number of whorls of achene per head T1×B1 cross showed significant SCA effects. T-3×B-3 showed maximum positive SCA value for No. of leaves/plants in testers. T-4×B-4 showed a non-significant effect for intermodal length. For achene yield, almost all combinations exhibited a significant SCA. Hybrid with RAPD L4 primer authentication was done with one line and one tester. The bands of the parents were seen in hybrid.

Keywords: **L×T** (Line × Tester), **CMS** (Cytoplasmic Male Sterile), **SCA** (Specific Combining Ability), **GCA** (General Combining Ability), **RAPD** (Random Amplified Polymorphic DNA)

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Introduction:

Agriculture defines the economy of Pakistan as it is considered the backbone of the country. Pakistan spends a huge amount on importing things from different countries among which edible oil is significant. Due to the population explosion, the demand for edible oil is increasing day by day. To meet the required demand for oil we import it as we are unable to produce oil per demand. Basically, there are two types of crops growing in Pakistan; conventional and non-conventional crop. Sunflower is one of the non-conventional crops which can be used to fulfil the demand for oil (Khalifa et al., 2000).

The oilseed crop sunflower belongs to the family Asteraceae, its scientific name is *Helianthus Annuus L.* It can be grown all over Pakistan as an oilseed crop as well as for ornamental purposes. There are almost 65 species of sunflower which have been discovered (Andrew et al., 2013). More studies reveal that fertilizers play an important role in the enhancement of the yield of sunflowers. Hybrids of the sunflower require high amount of Nitrogen than OPV's (Ozer et al., 2004). Sunflower is the potential crop which can be used to fulfill the requirement of the country regarding oil production. It is reported that the total cultivated area of Pakistan was 140000 hectares in 2015-2016 (Anonymous, 2015-16). Sunflower seed is known as achene. Achene has 40%-50% fat with a protein range from 20-30% (Arshad et al., 2013), along with 90% oleic acid and 10% Linoleic acid. Sunflower oil is used for cooking purposes as well as in salad preparations. Sunflower oil has high smoke point with excellent oxidative properties and it does not contain any Linolenic acid (Ismail et al., 2014).

The main aim of this study is to identify diverse parents and use them as Lines and Testers to identify high yielding crosses. In this experiment, different crosses were made to improve the yield. Crosses with high yield potential can be obtained through Combining abilities (Machkowa et al., 2011). The line*Tester method used by Kemp thorn (1957) was the effective method to perform the combining ability analysis. GCA and SCA estimation is used for the selection of better parents for high-yielding hybrid development (Sprague Tatum and., 1942). A breeder can get a good combiner from genetically diverse parental lines. Estimation of combining abilities is the significant technique for developing sunflower hybrid for more yield having high oil contents (Fehr, 1987).

Objectives:

1. To evaluate different Sunflower lines with different genetic characteristics.
2. For the authentication of good hybrids by using RAPD Markers.

Materials and Method:

Experiment 1:

The research was conducted at Raja Wala University of Agriculture, Faisalabad. The parent material consisted of four lines and testers respectively that were grown in three replications using RCBD design in the fall season of 2020. To develop hybrids CMS lines and testers were crossed in spring following agronomic practices. The plant × plant (25cm) and row × row distance (75cm) was maintained. For selfing purpose flowers were covered with butter paper bag while to develop hybrids emasculation was done. Hand

emasculation was carried out through forceps and gametocide spray for chemical. Crosses were made during the initial season. The following season, three blocks of hybrid seeds were planted, each block containing 16 hybrids and 8 parents. Data was collected at maturity stage of crop for selected traits: Plant height (cm), Internodal distance (cm), Days to 50% flowering, Days to 100% flowering, Head diameter (cm), Number of whorls per head, 100-achene weight/head (g).

Experiment 2:

1. DNA isolation was done in statistical analysis for which disease free two samples of parents and one from hybrid were collected. Samples were kept at -30˚C in refrigerator for 2 hours. 2ml of CTAB (Acetyl trimethyl ammonium bromide) was used (500 µl once) to make volume up to 2ml for grinding purposes.
2. Placed it in water bath after grinding. The temperature of the water bath was kept 65˚ for 30 mints and inverted the sample 3 times.
3. Centrifuged the sample at 1365 rpm for 15 mints.
4. The pallet was discarded and supernatant was placed into another 2ml E-tube and centrifuged again.
5. Supernatant was placed into a second 2 ml E-tube after the pallet was discarded.
6. Required 900 ml of supernatant was used. Equal volumes of PCI (Phenol Chloroform Isoamyl alcohol) were introduced to the Eppendorf tube and centrifuged. Removed the green upper layer. After that 1/10 sodium acetate was added. Isopropanol was then added after chilling. The entire volume was kept at 0° C for 40 minutes.
7. The supernatant was discarded and pallet was centrifuged at 12500 rpm for 5 minutes after being cleaned with 500 l of ethanol. Supernatant was once more discarded. The pallet was placed for two hours in the incubator. The DNA sample was kept for 30 minutes before being run on a gel to establish its identity.

Gel Electrophoresis:

80ml of buffer 1X was used and 0.8g of agarose was added. The mixture was microwaved for 40 seconds. After that, 0.3 l of ethidium bromide was added after heating the mixture to 65 °C. The slurry was poured into a casting tray, and combs were then pressed into the gel to create wells. The gel at room temperature solidified after 25 to 30 mints. The tray was moved into the buffer tank after the comb was taken out. DNA samples were put onto a gel. The findings indicated that RNA and DNA were both present.

RNAse Treatment:

Samples were treated with 3µl RNAses for 30 mints at 37C˚.

Quantification:

Nano drops Spectrophotometer was used to check the quality of the DNA. A good quality DNA was present in all samples.

PCR:

Below mentioned ingredients were added in PCR tubes and switched on the PCR cycler.

Chemical name	Quantity
D_3H_2O	9.5 µl
Taq Polymerase	9.5 µl
Primer	3 µl
DNA	5 µl
Total	25 µl

Results and Discussion:

Proportional contribution of lines, testers and line x tester interaction to the total variance

In case of Plant height, Head diameter, Number of Whorls of per head, Achene weight per head, 100 seed Weight, Leaf area (cm^3), Number of leaves per plant, Internodal length (cm^2), these traits varied equally in all directions. The results revealed that these parameters were significant for all lines, testers and interactions as were reported by Qamar *et al.* (2015), Rameeh and Andarkhor (2017).

The cross combination had a major contribution for plant height (94.97%) followed by line (4.37%) and tester (0.66%). For head diameter, lines have a very important role (10.49%) followed by line tester (7.06%) and tester (82.45%). For number of leaves per plant for tester 19.06% contribution followed by line (45.08%) and line × tester (35.86%). For Internodal length, the line× tester had highest contribution (41.92%) followed by line (23.47%) and testers (34.61%).

In the variance of leaf area, line played important role (49.13%) followed by line × tester (43.88%) and testers (6.99%). Number of whorls of achene per head variance was more contributed by line (83.79%) followed by line tester (12.75%) and testers (3.47%). For 100 Seed weight, the line× tester interaction contributed (2.83%) followed by lines (91.29%) and tester interaction (5.87%). For achene weight per head the line x tester was more important in the value of variance (2.90%) followed by line (91.69%) and the testers had very low contribution (5.41%) to the variance.

Table 1.2. Proportional contribution of lines, testers and L× T

Annexure A

Annexure B

GENERAL COMBINING ABILITY EFFECTS:

The tester B-1 exhibited the maximum negative GCA effects for plant height. Because hybrids were low in stature, it was preferred for GCA to have its greatest detrimental effects. Line T-1 and tester B-1 had significant and positive mean effects for head diameter, and these results were positive for both testers and lines. More yield will be produced by lines with higher heads than by smaller ones. More whorls can accommodate a larger head size. For the number of whorls per head, Line T-1 showed significant and positive effects. For 100 seeds' weight, the line B-4 had significant effects on overall combining ability. For achene yield/head, lines B-4 and B-3 had positive and significant additive effects. T-1 showed extremely significant positive impacts among lines for the number of leaves/plants, whereas T-2 showed a highly significant negative GCA. For the number of leaves per plant, B-3 had a large negative GCA. According to the results, all parents displayed non-significant GCA effects for internodal length, with lines T-1 and T-4 showing positive GCA and line T-2 exhibiting negative GCA. Testers B-1 and B-4 showed negative GCA for internodal length, while B-2 and B-4 showed positive GCA. While testers B-3 and B-4 reported highly significant negative GCA values for leaf area, T-3 and T-4 revealed significantly positive GCA effects. The remaining parents' results were not statistically significant.

Table 1.3 General Combining ability effects of lines and testers for yield related traits

Annexure C

SPECIFIC COMBINING ABILITY EFFECTS:

The specific combining ability for plant height was negative and significantly affected by the crosses T-2× B-4 and T-3× B-3. The SCA effects were significant in six crosses for head diameter, as shown in Table 1.4. The combination T-4×B-3 yielded estimates of significant and positive SCA for head diameter. Combinations T-1×B-1, T-1×B-2, T-3×B-4 and T-4×B-3 had strong and positive SCA effects for the number of whorls/head. Crosses for 100 achene weight had significant and positive effects on the ability to combine certain traits. Every combination showed a considerable and favorable estimation of their ability to combine for 100-achene weight but cross T-1×B1 showed the highest value. Almost all combinations had positive and significant specific effects for achene yield. Among all crosses T-3 × B-3 showed maximum positive SCA value for number of leaves per plant. For internodal length, the T-4 × B-4 revealed unfavorable SCA effects. So, for internodal length, this cross is the best one. All other crosses, besides one, had non-significant internodal length values. The leaf area of T-3× B-1 showed the highest SCA impacts. For T-1× B-3, negative SCA effects were found. For SCA impacts on leaf area, several crosses were not statistically significant.

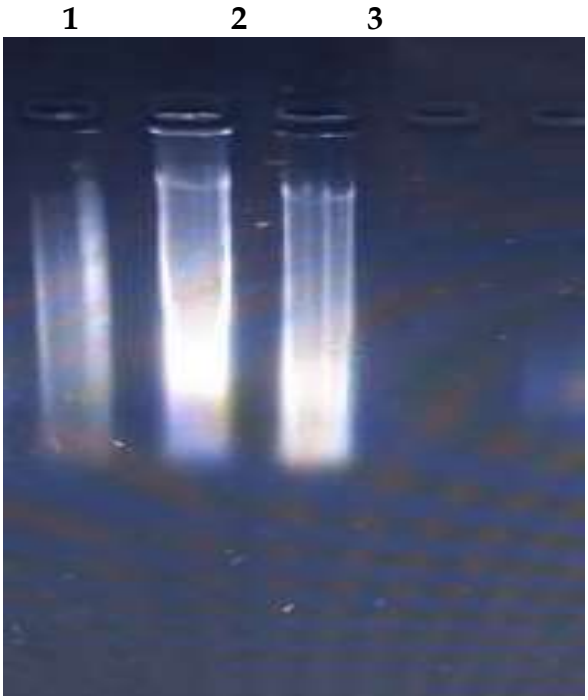
Table 1.4 Specific combining ability effects of crosses for yield related traits

Annexure D

SOV= Source of variation, P.H= Plant Height, H.D= Head Diameter, No.L= Number of Leaves, I.N.L= Internodal Length, No.of Wh. = Number of Whorls, 100 seed W= 100 Seed Weight, Ach W/H= Achene Weight per Head

DNA isolation

In the below diagram bands showed that good quality DNA was present in sample 1,2 and 3 that can be used further PCR process.



Results after DNA isolation

PCR results:

Six polymorphic primers were used to amplify the genomic DNA of sunflower. But the best amplification was observed by L4. Line T-1 indicated as P1 and tester B-1 was expressed as P2 and hybrid as H1. In the figure 4.1 results indicated hybrid showed similar bands were appeared like parents. The reproducibility of results among laboratories was affected by two factors. First, different laboratories amplified different size ranges of DNA fragments, and, consequently, small and large polymorphic fragments were not always reproduced stated by (Sardar et al., 2022).

Table 4.12 Primers used for amplification

Sr. No	RAPD primers
1	RAPD L1
2	RAPD L2
3	RAPD L3
4	RAPD L4
5	RAPD L5
6	RAPD L6

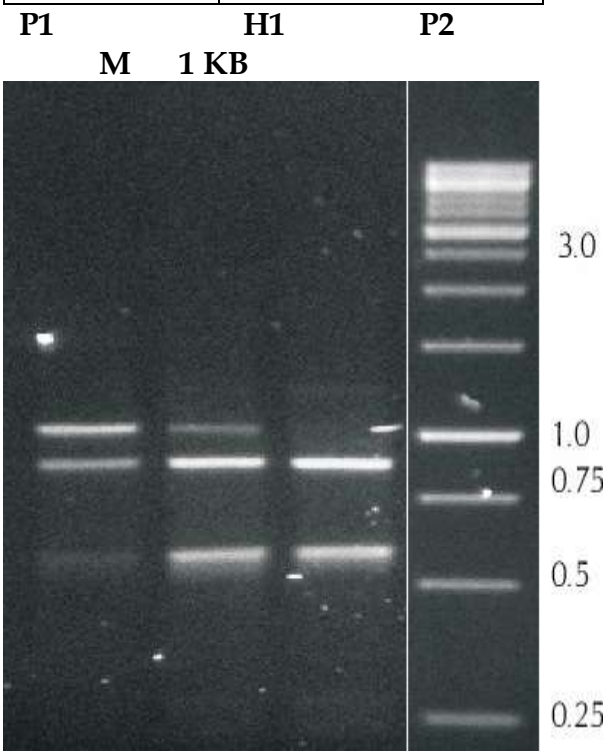


Fig.4.1 Agarose gel of amplified bands using Primer RAPD L4 on 3 sunflower genotypes.

Conclusion:

The findings of research has shown that the cross combinations are significant to proceed further for hybrid development. The DNA studies have indicated that there is a huge research gap in sunflower germplasm that must be explored to overcome needs of the country. This research gap is the main reason why most of sunflower varieties don't have lodging and disease resistance. During investigation, the crosses T1×B1 and T-3×B-3 showed variation from other crosses and showed maximum diversity. These two crosses can be used in future for further breeding as they have diversity.

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Annexure A

Proportional contribution of lines, testers and line x tester interaction to the total variance

Table 1.2. Proportional contribution of lines, testers and L× T

Traits	Lines	Testers	Lines× Testers
P.H	94.97	4.37	0.66
H.D	10.49	7.06	82.45
No. L	45.08	19.06	35.86
I.N.L	23.47	34.61	41.92
L.A	49.13	6.99	43.88
No. of whorls	83.79	3.47	12.75
Achene W/ head	91.69	5.41	2.90
100 seed W	91.29	5.87	2.83

Table 1.1: Analysis of variance for yield related traits in sunflower

Mean Squares

SOV	DF	P.H	H.D	No. L	I.N.L	L.A	No.of Wh.	100 seed W	Ach W / H
Replication	2	31.7**	0.02	13.5	0.08	0.36	0.08	0.61**	1.00*
Treatment	23	657.7**	5.2**	42.54**	2.7*	32.6**	207.4**	12.91**	16.6**
Parents (c)	7	131.4**	0.4	19.3*	1.5	2.87**	371.6**	8.29**	7.6**
Crosses(c)	15	305.1**	7.8**	35.25**	3.6**	32.4**	140.0**	2.71**	4.31**
P vs C	1	9631.7**	0.7	314.1**	2.07	245.7	69.0**	198.34**	265**
Lines	3	1449.0**	0.28	39.5**	1.3	4.7**	0.83**	0.8**	1.4**
Testers	3	66.6**	0.69*	3.0**	0.12	1.1	5.9**	5.9**	5.9**
L×T	9	3.36**	0.05	7.9	5.9**	2.6**	37.7**	37.7	31.7

Error	46	1.6	0.17	7.6	31.19	0.39	0.09	0.09	0.06

Annexure B

GENERAL COMBINING ABILITY EFFECTS:

Table 1.3 General Combining ability effects of lines and testers for yield related traits

Lines	**P.H**	**H.D**	**No. L**	**I.N.L**	**L.A**	**No. of whorls**	**100 seed W**	**Ach W/H**
T-1	-14.0**	0.50**	2.82**	0.64 ns	-2.85**	8.87**	-1.21	-1.56**
T-2	-2.99**	-0.05ns	-3.41**	-2.40ns	-1.45**	-0.73ns	-0.42**	-0.51**
T-3	5.94**	0.35**	0.09 ns	0.72 ns	2.60**	0.08 ns	0.54**	0.83**
T-4	11.05**	-0.81**	0.50 ns	1.04 ns	1.71**	-8.21**	1.08**	1.25**
Testers	**P.H**	**H.D**	**No. L**	**I.N.L**	**L.A**	**No. of whorls**	**100 seed W**	**Ach W/H**
B-1	-2.88 **	0.69**	0.45 ns	-1.81ns	-1.14**	1.30 **	-0.28**	-0.4**
B-2	0.35 ns	-0.42**	1.39 ns	2,18 ns	-0.2ns	-1.09*	0.03 ns	0.05 ns
B-3	2.85 **	-0.20 *	-2.43 *	-1.47ns	0.26 ns	-1.36**	0.33 **	0.36 **
B-4	-0.32 ns	-0.06ns	0.59 ns	1.10ns	1.17 **	1.15 *	-0.08**	-0.02ns

Annexure C

Table 1.4 Specific combining ability effects of crosses for yield related traits

Crosses	**P.H**	**H.D**	**No. L**	**I.N.L**	**L.A**	**No. of whorls**	**100 seed W**	**Ach W/H**
T-1×B1	-1.03ns	-1.85**	2.72ns	-4.10ns	0.24ns	2.84 **	26.24**	30.19**
T-1×B2	0.01 ns	0.46*	0.45ns	3.20 ns	1.44 **	3.33 **	18.82**	19.81**
T-1×B3	0.34 ns	1.33**	-4.43 *	-0.71ns	-1.10**	-5.26**	29.27**	17.88**
T-1×B4	0.68 ns	0.06 ns	1.26 ns	1.61ns	-0.58ns	-0.91ns	18.82**	19.81**
T-2×B1	1.03 ns	1.96 **	-0.61ns	2.93 ns	-2.06**	1.81 ns	22.01**	19.78**
T-2×B2	0.50 ns	-0.36ns	1.68 ns	-0.70ns	-3.32**	-0.77ns	15.24**	22.35**
T-2×B3	-0.00ns	-3.12**	-0.33ns	-0.11ns	1.67 **	-0.76ns	11.16**	14.79**
T-2×B4	-1.53*	1.51 **	-0.74ns	-2.12ns	3.72 **	-0.28ns	24.03**	23.93**
T-3×B1	0.11 ns	0.33 ns	-3.29ns	-0.75ns	3.95**	-2.60**	12.82**	14.05**
T-3×B2	0.31 ns	0.44*	-0.66ns	-1.65ns	0.76*	-1.94*	16.68**	16.19**
T-3×B3	-1.39 *	-0.82**	3.80*	1.00 ns	-1.98**	2.73 **	21.29**	20.78**
T-3×B4	0.98 ns	0.05 ns	0.15 ns	1.40 ns	-2.73**	1.81 ns	11.34**	12.79**
T-4×B1	-0.11ns	-0.44*	1.17 ns	1.93 ns	-2.12**	-2.04*	12.15**	13.73**
T-4×B2	-0.81ns	-0.54**	-1.47ns	-0.86ns	1.12 **	-0.62ns	15.23**	15.07**
T-4×B3	1.06 ns	2.61 **	0.96 ns	-0.18ns	1.41 **	3.29 **	19.67**	19.52**
T-4×B4	-0.14ns	-1.63**	-0.66ns	-0.89ns	-0.41ns	-0.63ns	11.54**	12.68**

Level of significance at 0.05% = *, level of significance at 0.01% = *