

3 **Characterization of high oleic gene pool and validation of the identified genomic**  
4 **regions controlling oleic acid content in Sunflower (*Helianthus annus L.*)**

5  
6 **Abstract:**

7 The increase of oleic acid content has become one of the major goals of plant  
8 breeders to improve sunflower oil quality, as high content of oleic acid increases the  
9 oil's stability to oxidative degradation at high temperatures and as well has been  
10 suggested to reduce cholesterol in blood plasma thereby reduces the risk of coronary  
11 heart disease. In this study 120 inbred lines of high oleic gene pool were characterized  
12 for yield, its attributing traits, oleic content and then validated with two known  
13 microsatellite molecular markers linked to oleic acid content. High phenotypic and  
14 genotypic coefficients of variation as well high heritability and high genetic advance as  
15 percent of mean was recorded for oleic acid content. This indicated the presence of the  
16 additive type of gene action controlling the trait. Further, the two molecular markers  
17 under the study exhibited differentiating bands between all the high and low oleic  
18 inbred lines. Hence the validated markers from this study, linked to the high oleic acid  
19 trait could be further used in marker-assisted selection and would greatly contribute to  
20 develop stable high oleic acid breeding lines.

21 Key words: Oleic acid content, *Ol* gene, Inbred lines, microsatellite molecular markers  
22 and Sunflower

23  
24 **Introduction:**

25 Oleic acid, a monounsaturated omega-9 fatty acid (18:1 cis-9) is found in many  
26 foods, but mainly in olive oil. Even if other mono-unsaturated fatty acids are present in  
27 olive and seed oil, oleic acid is receiving great attention worldwide for its beneficial  
28 health properties (Romano *et al.*, 2021). The FDA has determined the existence of  
29 realistic evidence to support a health claim associated to the oleic acid consumption and  
30 to the reduction of coronary heart disease risk (US FDA, 2018). For this reason, in the

31 recent decades, plants with higher oleic acid content (up to 70% and more) have been  
32 selected, which opened up a new frontier to the possible uses of these crops taking  
33 advance of possible beneficial health effects and triggering at the same time the market  
34 interest for its wider use (Ramadan *et al*, 2013). Similarly, in case of sunflower oil with  
35 a high oleic acid content in the range between 70 to 90 *per cent* is called as “high oleic  
36 content” sunflower oil and presents a fatty acid composition similar to that of olive oil.

37         There were many attempts to carry out the genetic research with this trait in  
38 sunflower crop. The most of them were dedicated to a high oleic mutation of variety  
39 ‘Pervenets’, obtained by Soldatov, 1976 and this was exclusively used as a donor of the  
40 high oleic trait in sunflower breeding programmes worldwide. Several genetic  
41 approaches have been developed to study the high oleic mutation and different  
42 conclusions are reported on the number of genes that control the trait and on their  
43 dominance. Research on genetic control of a high oleic mutation led to the hypotheses  
44 of one major gene *Ol* and gene-modifier *ml* (Miller *et al.*, 1987).

45         Nevertheless, the genetic control of high oleic acid content is still not well  
46 understood, as high oleic acid content was initially identified as monogenic trait  
47 produced by dominant alleles *Ol*, but afterwards several modifying genes were  
48 identified that affect the *Ol* gene and produce reversal of the expected dominance. This  
49 has complicated the practical management of the trait in breeding programmes.  
50 (Dimitrijevic *et al.*, 2017). The oleic acid content, to a certain extent, be considered a  
51 semi-qualitative trait since OAC is dependent not only on the environment, but also on  
52 the genetic background of the receiver line. (Fuller *et al.*, 1967), (Ferfuia *et al.*, 2015)  
53 and (Regitano Neto *et al.*, 2016). A partial duplication of the Fatty Acid Desaturase 2-1  
54 (FAD2-1) allele caused by chemical mutation leads to an increase in OAC by silencing  
55 the FAD2-1 gene encoding FAD 2 (Lacombe *et al.*, 2004). This enzyme catalyzes the  
56 synthesis of linoleic acid from oleic acid and by silencing its activity oleic acid is  
57 accumulated. Different markers were employed in mapping and detecting the *Ol*  
58 mutation in sunflower, however Premnath *et al.* (2016) identified two QTL’s,  
59 HO\_Fsp\_b and ORS762 explaining about 60% of the phenotypic variation in OAC.

60         With this background, the objective of this study was to characterize the 120  
61 inbred lines of high oleic gene pool for yield, its attributing traits, oleic acid content and  
62 as well to validate the reliability of two gene-based microsatellite molecular markers

63 for utilization in marker assisted breeding programme to improve oil quality of  
64 sunflower.

## 65 **Material and methods:**

### 66 **Phenotypic characterization of high oleic gene pool:**

67 The plant material of this study constituted of 120 inbred lines of R x R  
68 (Restorer x Restorer) high oleic gene pool developed at AICRP Sunflower, ZARS,  
69 GKVK, Bangalore. The characterization of these 120 inbred lines was carried out for  
70 yield, its attributing traits and oleic acid content in alpha lattice design in 12 blocks  
71 with 10 inbred lines each in a block with two replications during summer 2021.

### 72 **Validation of microsatellite markers linked to oleic acid content:**

73 From the study conducted by Premnath et al. (2016), the *Ol* gene was mapped to  
74 linkage group (LG) 14 and tightly linked to the markers HO\_Fsp\_b and ORS 762.  
75 These two reported linked markers from this study were used to ascertain oleic acid  
76 content and demonstrate the utility of gene-based microsatellite molecular markers for  
77 accurate identification of high oleic lines. Hence the reliability of these identified  
78 markers was carried with 17 selected high oleic inbred lines (>70 %) and 13 low oleic  
79 inbred lines (<40 %) to find out whether the identified markers could discriminate the  
80 low and high oleic acid content of the inbred lines under study. The details of the inbred  
81 lines used for validation, along with respective oleic acid content is presented in Table  
82 1 while the details of the microsatellite molecular markers are presented in Table 2.

### 83 **Estimation of fatty acid composition**

84 The validation was carried out using 30 samples with known oleic acid content  
85 as estimated using standard gas chromatography (GC) available at Indian Institute of  
86 Oilseed Research, Hyderabad.

### 87 **Genomic DNA isolation**

88 DNA was extracted from 15–20-day-old fresh fully expanded leaves of the 30  
89 inbred lines using the modified cetyl trimethyl ammonium bromide (CTAB) extraction  
90 method as described in Doyle (1991). The DNA quality and quantity were checked on  
91 0.8 % agarose gel and DNA concentration was normalized to 10 ng/L.

92 **Results and discussion:**

93 The knowledge of genetic variability helps the breeder to improve the suitable  
94 breeding strategy, therefore it is necessary to know genetic variability, heritability and  
95 genetic advance as *per cent* in the available genetic material. Genetic variability  
96 together with heritability estimates would give a better idea on the amount of genetic  
97 gain expected out of selection (Burton and De Vane 1953) and (Panse and Sukhatme,  
98 1957). Through this study an attempt was made to assess the mean performance and  
99 extent of variability in the 120 inbred lines of high oleic gene pool, for nine quantitative  
100 traits. The estimates of range, mean, phenotypic coefficients of variation (PCV),  
101 genotypic coefficient of variation (GCV), heritability ( $h^2$  broad sense) and genetic  
102 advance as *per cent* of mean (GAM) are presented in Table 3.

103 The analysis of variance revealed significant differences among the entries for  
104 all the characters studied indicating the existence of a high degree of variability in the  
105 material. Genetic parameter studies showed that the magnitude of difference between  
106 PCV and GCV was relatively low for all the traits and the magnitude of PCV was little  
107 bit higher than GCV for all the traits, which revealed less influence of environment on  
108 the expression of these traits.

109 The oleic acid content recorded a wide range of variation from 36 *per cent* (L-  
110 12-1) to 86.23 *per cent* (K-10) with a mean value of 56.20 *per cent*. The estimates of  
111 PCV (21.89) and GCV (21.76) were high, indicating wider variability. High heritability  
112 (98.85) accompanied by high genetic advance as *per cent* of mean (44.58) was  
113 observed for this trait indicating the role of additive gene action and also that the  
114 inheritance of this trait was less influenced by environmental effects and therefore  
115 selection of the inbred lines under the study, for oleic acid content would be effective.  
116 These findings were in line with the results obtained by Neelima *et al.* (2016), Hassan  
117 *et al.* (2012) Baraiya and Patel (2018) and Nasreen *et al.* (2011).

118 Wider variability was observed in case of plant height, head diameter, seed  
119 yield plant<sup>-1</sup> which indicated its amenability towards directional selection. stem girth  
120 and 100 seed weight recorded moderate variability whereas days to 50 *per cent*  
121 flowering, volume weight and oil content showed low variability. Plant height, head  
122 diameter, stem girth, seed yield plant<sup>-1</sup>, 100 seed weight and oleic acid content showed  
123 high heritability coupled with high genetic advance of mean, suggesting the role of

124 additive gene action in the inheritance of these traits and hence, improvement for these  
125 characters could be achieved by mass selection and progeny selection methods. The  
126 high heritability observed for most of the traits, which might not be realistic as the  
127 experiment was conducted in one location with two replications. More realistic  
128 estimates could be obtained by testing genotypes in a multi-environment trial.  
129 However, variability together with heritability and genetic advance gave some  
130 indication of the nature of gene action governing the traits.

131 Further from the same gene pool, 17 high oleic inbred lines (>70 %) and 13 low  
132 oleic inbred lines (<40 %) were selected and screened with previously reported  
133 microsatellite molecular markers to find out whether the identified markers could  
134 discriminate the low and high oleic acid content among these inbred lines. The markers  
135 under the study were basically derived from the study conducted by Premnath et al.  
136 (2016). They had developed an F<sub>2</sub> mapping population of sunflower and phenotyped for  
137 oleic acid content. The *Ol* gene was mapped to linkage group (LG) 14 and tightly  
138 linked to the marker HO\_Fsp\_b. In addition, two more quantitative trait loci (QTLs) for  
139 oleic acid content were identified in LG8 and LG9. Further the study was conducted  
140 with 13 genotypes differing in oil quality as well as quantity over three seasons to  
141 assess the reliability of the identified QTLs over seasons. It resulted in the identification  
142 of two potential QTLs for oleic acid content with the markers ORS 762 and HO\_Fsp\_b.  
143 These markers explained more than 57.6–66.6 *per cent* of phenotypic variation in their  
144 study. With this background to further validate the genomic regions controlling the  
145 oleic acid content the current study was envisaged utilizing the high oleic restorer gene  
146 pool.

147 The results from the current study revealed that both the markers exhibited  
148 differentiating bands between high and low oleic lines. The high oleic containing  
149 individual lines showed a specific band at about 890 bp length which was absent in low  
150 oleic lines (Fig. 1) for the primer HO\_Fsp\_b. Similarly, the high oleic containing  
151 individual lines showed a specific band at about 750 bp length which was absent in low  
152 oleic lines (Fig. 2) for the primer ORS 762. It was evident from the result that both the  
153 markers exhibited the expected amplicon size as per the study conducted by Premnath  
154 et al. (2016) and successfully differentiated all the high oleic and low oleic lines

155 **Conclusion:**

156 The oleic acid content is highly influenced by environmental factors such as the  
157 temperature and the amount of moisture in the soil. In addition, high oleic genes show  
158 unstable expression for oleic acid content in different genetic backgrounds and  
159 therefore phenotypic selection for the high oleic acid trait could be difficult across  
160 different environments and seasons. The standard method to determine oleic acid  
161 content is Gas Chromatography (GC) which produces accurate result but is expensive,  
162 time consuming and involves hazardous chemicals. The DNA markers are not  
163 influenced by the environment and therefore selection based on molecular markers  
164 linked to the high oleic acid trait will allow further advance in breeding for this  
165 character. Hence the validated molecular markers from this study, linked to the high  
166 oleic acid trait could be further used in marker-assisted selection and would greatly  
167 contribute to develop stable high oleic acid breeding lines.

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232 **Table 1. List of selected high and low oleic lines for validation of molecular**  
233 **markers for oleic acid**

Sl. No	Code	Inbred Lines	Oleic acid content (%)
1	HO-1	K-10	87.50
2	HO-2	F-20	84.40
3	HO-3	L-3-1	83.20
4	HO-4	K-11	81.60
5	HO-5	G-5	81.05

234	6	HO-6	G-17-1	80.26
	7	HO-7	L-17	78.85
235	8	HO-8	B-29-2	78.28
236	9	HO-9	G-12	76.75
	10	HO-10	D-33-2	76.40
237	11	HO-11	N-16	75.85
238	12	HO-12	D-11	72.36
239	13	HO-13	C-30	74.10
	14	HO-14	L-1-1	70.78
240	15	HO-15	A-16	70.75
241	16	HO-16	M-25	70.25
242	17	HO-17	M-19-1	70.50
	18	LO-1	A-6	40.00
243	19	LO-2	B-22	39.50
244	20	LO-3	C-31	39.00
245	21	LO-4	F-6-2	37.50
	22	LO-5	G-13-2	38.00
246	23	LO-6	K-6	38.50
247	24	LO-7	G-36-1	37.50
248	25	LO-8	I-18	39.50
	26	LO-9	L-23	39.00
249	27	LO-10	M-21-1	40.00
250	28	LO-11	N-17	39.00
251	29	LO-12	RHA-95-C-1	36.00
	30	LO-13	RHA-6D-1	38.00

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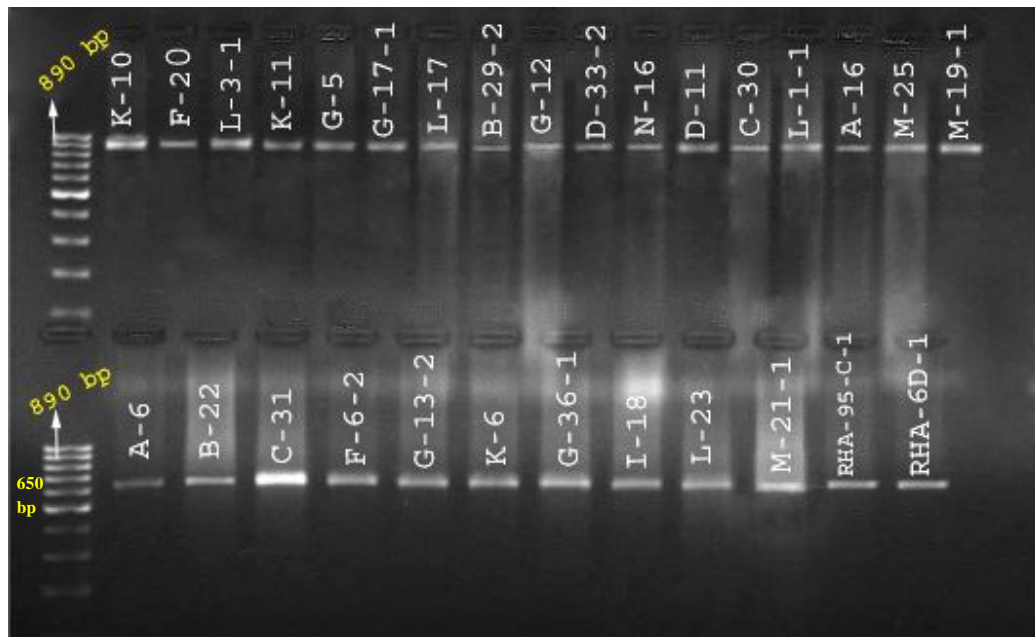
\*LO- Low Oleic Lines      HO- High Oleic Lines

**Table 2. The list of microsatellite molecular markers, primer sequence and expected PCR amplicon**

Primer Name	Primer Sequence	Gene	Expected Amplicon Size
ORS 762	Forward- 5'-TGCACATGAGGGTATTCTTGTC-3' Reverse- 5'- TCGAGGAGAGTGTGACGTTG-3'	<i>Ol</i>	750 bp
Ho_Fsp_b	Forward- 5'- GCACCATGAGGGCTGTTATTGT-3' Reverse- 5'- TGCATGGAAGTGGAGTCTAT-3'	<i>Ol</i>	890 bp

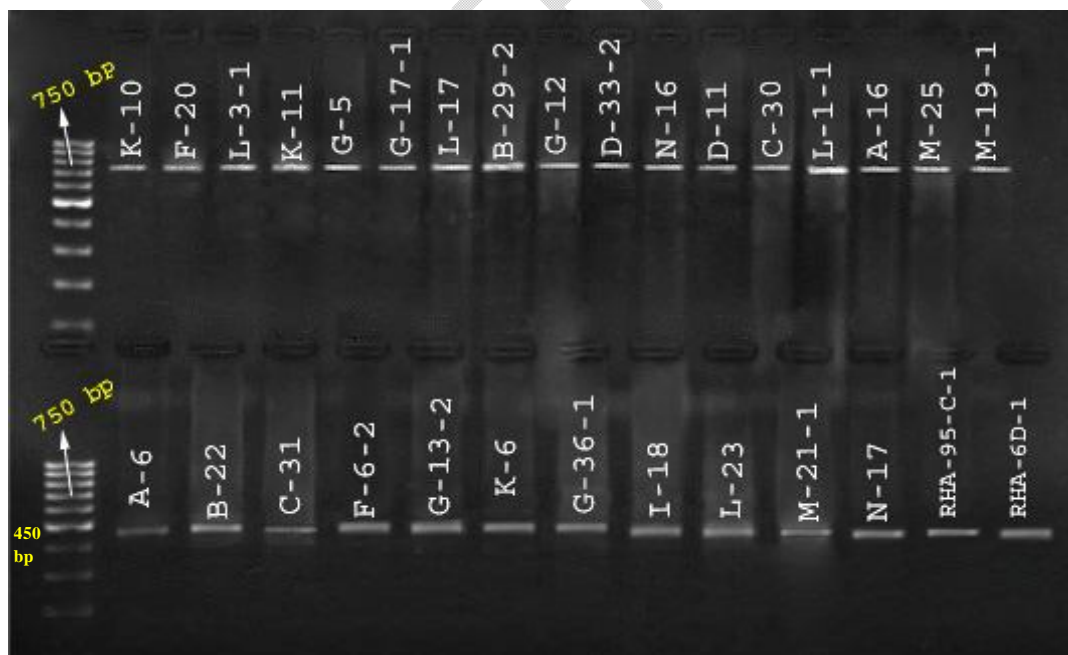
**Table 3. Mean performance and genetic variability of 120 sunflower inbred lines for yield, its attributing traits and oleic acid content**

Character	Mean	Standard Error of Mean	Range		Coefficient of variation (%)		Heritability (%)	Genetic Advance as Mean (%)
			Low	High	Genotypic Coefficient of Variation	Phenotypic Coefficient of Variation		
<b>Days to 50 % flowering</b>	57.40	0.251	52.00	68.00	5.39	5.42	98.70	11.03
<b>Plant height (cm)</b>	117.88	0.812	92.50	144.50	11.38	11.43	99.27	23.37
<b>Head diameter (cm)</b>	10.95	0.577	5.00	15.60	21.66	22.02	96.76	43.90
<b>Stem girth (cm)</b>	1.78	0.048	1.30	2.37	11.01	11.66	89.07	21.41
<b>Seed yield plant<sup>-1</sup> (g)</b>	11.89	0.308	6.00	19.10	20.45	22.04	86.07	39.10
<b>Volume weight (g/100ml)</b>	37.78	0.517	31.00	44.30	7.68	7.92	94.02	15.34
<b>100 seed weight (g)</b>	3.83	0.155	2.19	5.20	16.54	17.51	89.27	32.20
<b>Oil content (%)</b>	34.19	0.451	28.31	40.48	6.47	6.73	92.39	12.82
<b>Oleic acid content (%)</b>	58.33	0.968	36.00	86.23	21.76	21.89	98.85	44.58



High oleic lines with presence of band at 890 bp

Fig 1. PCR amplification of High oleic and Low oleic lines for HO\_Fsp\_b marker



High oleic lines with presence of band at 750 bp

Low oleic lines with absence of band at 750 bp

Fig 2. PCR amplification of High oleic and Low oleic lines for ORS 762 marker