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ORIGINAL ARTICLE

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Identification of simple-sequence-repeat markers linked to Fusarium wilt (*Fusarium oxysporum* f.sp. *carthami*) resistance and marker-assisted selection for wilt resistance in safflower (*Carthamus tinctorius* L.) interspecific offsprings

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Abstract

Safflower (*Carthamus tinctorius* L.) is a multipurpose oilseed crop. Fusarium wilt (*Fusarium oxysporum* f.sp. *carthami*) is the major damaging disease in safflower. In the present investigation, Fusarium wilt resistance was introgressed from two wild species, *Carthamus oxyacantha* and *Carthamus palaestinus*, into susceptible cultivated species through interspecific hybridization. Inheritance of wilt resistance indicated single dominant gene control. Eight simple-sequence-repeat (SSR) markers each in ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) were found to be linked to wilt resistance. Marker-assisted selection for wilt resistance was performed using these markers in F_3–F_7 generations of both crosses. Six wilt resistant interspecific lines evaluated for 2 years under nondisease conditions have recorded 9%–29% higher seed yield than the high yielding cultivar, 'A1'. The wilt resistant lines would serve as new sources of resistance to wilt in safflower. The SSR markers linked to wilt resistance would be useful for precise selection of wilt resistance at seedling stage in large segregating populations without attempting screening in artificially inoculated conditions and pyramiding of wilt resistant genes from wild into a common background.

KEYWORDS
interspecific, MAS, safflower, SSR, wild species, wilt resistance

1 | INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a multipurpose oilseed crop with world adoptability. It is relatively tolerant to abiotic stresses such as temperatures, drought and salinity (Abel, 1975; Bahrami, Arzani, & Karimi, 2014; Yeilaghi, Arzani, & Ghaderian, 2015) which facilitate its expansion in areas around the world, particularly with ecological sustainability and climate change as the looming issues. On the other hand, safflower is susceptible to various biotic stresses such as Alternaria, Fusarium wilt, rust (*Puccinia carthami)* and safflower fly (Karami, Sabzalian, Rahimmalek, Saeidi, & Ghasemi, 2017). Kazakhstan and India are the largest safflower-growing countries followed by Mexico, USA, Argentina, China, Russian Federation, Turkey, Iran, Kyrgyzstan, Tanzania, Australia and Uzbekistan (http://faostat3.fao.org). Wilt caused by *Fusarium oxysporum* f.sp. *carthami* Klisiewiz and Houston is an important disease that can cause complete yield loss in susceptible safflower varieties when grown in heavily infected fields (Schwartz & Gent, 2005). Fusarium wilt is prevalent in most of the safflower-growing areas especially where safflower is cultivated repeatedly year-after-year. Host-plant resistance is a practical and effective means to combat this disease. Although a few wilt resistant safflower germplasm accessions have been identified but they were not stable across locations. Therefore, there is a need to discover novel sources of Fusarium wilt resistance and introduce them into safflower.

Alien gene transfer has opened new ways and opportunities to transfer desirable alien genes and for creating new variability in many crop species (Arzani & Ashra, 2016; Cai, Chen, Xu, Oliver, & Chen, 2005). Various desirable traits including resistance to diseases have been introgressed from wild species in many crops (Croser, Ahmad, Clarke, & Siddique, 2003; Kumar, Imtiaz, Gupta, & Pratap, 2011). Safflower wild species were not fully investigated and exploited for crop improvement. Numerous studies on crossing among *Carthamus* species and associated cytogenetics have been carried out in the past (Ashri & Efron, 1964; Ashri & Knowles, 1960), but very little attempts have been made to improve cultivated safflower using wild species. A cytoplasmic-genic male sterility (CMS) system in safflower was developed through introgressing sterile cytoplasm from *Carthamus oxyacantha* into cultivated species (Anjani, 2008). Six wild species *viz*., *C. oxyacantha* (2*n* = 24), *Carthamus palaestinus* (2*n* = 24), *Carthamus lanatus* (2*n* = 44), *Carthamus turkesthanicus* (2*n* = 64), *Carthamus glaucus* (2*n* = 20) and *Carthamus creticus* (2*n* = 64), were identified as stable sources of resistance to Fusarium wilt (Pallavi, Prasad, & Anjani, 2007). *Carthamus oxyacantha* and *C. palaestinus* are readily crossable to cultivated safflower and produce fertile F_1 hybrids (Ashri & Knowles, 1960). To increase genetic diversity for Fusarium wilt resistance, introgression of wilt resistance genes from resistant wild species needs to be persued vigorously. With this background, attempts were made in the present investigation to introgress resistance to Fusarium wilt from *C. oxyacantha* and *C. palaestinus* into susceptible cultivated species (*C. tinctorius*), and to identify simple-sequence-repeat (SSR) markers for marker-assisted selection of Fusarium wilt resistance in F_3–F_7 generations of interspecific crosses. The successful exploitation of marker-assisted selection in developing Fusarium wilt resistant interspecific lines with limited linkage drag was discussed in this paper.

2 | MATERIAL AND METHODS

2.1 | Plant material

Fusarium wilt susceptible safflower (*C. tinctorius*) variety, "Nira" was crossed as a female parent to wilt resistant wild species, *C. oxyacantha* (IP-16) and *C. palaestinus* (PI-235663-2) in 2007 and the F_1s of ("Nira" × *C. oxyacantha*) and ("Nira" × *C. palaestinus*) crosses were advanced to F_8 generation. The F_1s were backcrossed to "Nira." The flower buds of female parent were hand-emasculated prior to anthesis, and the stigmas were brushed with pollen collected from male parent. The flower buds of both parents were covered with butter paper bags right from bud stage to harvesting; in addition, the entire experimental plot was covered with nylon net cages to avoid pollen contamination through honeybee. Morphological traits of each interspecific progeny were recorded in every generation.

2.2 | Disease screening

Screening of experimental material against Fusarium wilt was taken up in a typical wilt sick plot at the Indian Council of Agricultural Research-Indian Institute of Oilseeds Research (ICAR-IIOR), Hyderabad, India. The soil in wilt sick plot is characterized as red sandy loam. The wilt sick plot was artificially infested with *Fusarium oxysporum* f.sp. *carthami*. The inoculum load maintained in this plot was approximately 2–3 × 10^3 cfu. Inoculum growing on sorghum seeds was incorporated to wilt sick plot prior to sowing as well intermittently up to flowering period to maintain required load and uniform spread of the pathogen in the wilt sick plot. The susceptible parent, Nira, was grown after every five rows of experimental material to check the uniform spreading of wilt pathogen across the wilt sick plot. The parents and F_1–F_7 generations of the crosses, ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*), were grown in wilt sick plot and were phenotyped for their reaction against Fusarium wilt. Wilt data were recorded at 15 days of interval from germination to 120 days after planting. Wilt incidence (%) was recorded using the formula, (Number of wilted plants/total number of plants) × 100. The degree of susceptibility and resistance to disease of each interspecific derivative in F_6 and F_7 was determined using 1–9 rating scale given by Mayee and Datar (1986). However, the plants with any intensity of wilt symptom were designated as susceptible and those free from wilt symptoms were designated as resistant in F_2–F_5 generations.

The F_1 generations of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) crosses were planted in 2008. The BC_1F_1 generations of ('Nira' × *C. oxyacantha*) 'Nira', ('Nira' × *C. palaestinus*) 'Nira', and F_2 generations of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) were planted in 2009. In F_2 generation of both crosses, Fusarium wilt resistant and susceptible plants were identified by phenotyping in wilt sick plot as well by genotyping using SSR markers linked to wilt resistance. The resistant plants in both crosses were advanced in progeny-rows from F_3 to F_7 generation in consecutive years. The experimental materials were planted in augmented block design with spacing of 30 cm between rows and 20 cm between plants; each row was of 5 m length. Wilt sick plot was irrigated regularly to maintain the inoculum load.

In addition, for further confirmation of resistant reaction, parents of both the crosses and the homozygous resistant F_6 and F_7 plants identified using SSR makers were planted in wilt sick pots in glasshouse. Inoculum load of approximately 3–4 × 10^3 cfu of *Fusarium oxysporum* f.sp. *carthami* was incorporated in pots prior to sowing. The soil and sand used for filling the earthen pots were sterilized prior to inoculation. 'Nira', *C. oxyacantha*, *C. palaestinus*, F_6 and F_7 generations of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) were planted in two pots each; each pot had 25 plants. Observations on disease incidence in wilt sick pots were recorded at every 5 days of interval up to 45 days after planting. In pots also, the plants with any intensity of wilt symptom were designated as susceptible and the one without symptoms was designated as resistant.

2.3 | Yield evaluation of interspecific lines

Twenty-two interspecific lines derived from ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) crosses were evaluated along with wilt

susceptible high yielding check variety, 'A1' belonging to cultivated species and the parents, 'Nira', *C. oxyacantha* and *C. palaestinus* in a nondisease plot at research farm of ICAR-IIOR during 2014–2015 and 2015–2016 in RBD with three replications. The plot size was 11.25 sq.m/entry/replication and the soil in the experimental plot was Vertisol. The spacing followed was 45 cm between rows and 20 cm between plants. Recommended doses of fertilizers (40:40:20 of N, P_2O_5, K_2O kg/ha) were applied, and plant protection measures were taken up as and when required. Irrigation was given with sprinklers immediately after sowing in 2nd week of October in both years. In 2014–2015, the total rainfall during crop period (October–February) was 107.2 mm; maximum and minimum temperatures were 20.8–34.2 and 5.2–23.8°C, respectively, and relative humidity was 78%–94%. The year 2015–2016 was a drought year in India which received 12.2 mm rainfall during safflower crop period and had 26.8–34.2 and 9.6–19.7°C maximum and minimum temperatures, respectively, and 77%–92% relative humidity. The data on seed yield (kg/ha), oil yield (kg/ha), 100-seed weight (g) and oil content (%) were recorded, and the 2-year mean values of the traits were considered for selecting promising interspecific lines for high seed and oil yields. Of 22 interspecific lines evaluated, the data of six high yielding interspecific lines were reported.

2.4 | Marker analysis

Genomic DNA was extracted from fresh leaves according to the protocol of Doyle and Doyle (1987) with minor modifications. The fresh leaves were collected from 15 to 20-days-old plants growing in wilt sick plot. The quantity and quality of isolated DNA was determined using 3% Agarose gel electrophoresis.

Total 142 previously published SSR primer-pairs (Hamdan, Garcia-Moreno, Redondo-Nevado, Velasco, & Perez-Vich, 2011; Mayerhofer, Archibald, Bowles, & Good, 2010) were used in this study. The SSR markers polymorphic to parents, 'Nira', *C. oxyacantha* and *C. palaestiuns* were used in bulked-segregant analysis (Michelmore, Paran, & Kesseli, 1991) employed in F_2 mapping populations of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) to identify markers linked to Fusarium wilt resistance. The resistant and susceptible DNA bulks in a cross were made separately by mixing equal quantity of DNA from 30 resistant and 30 susceptible F_2 plants. The SSR markers flanked to wilt resistance were validated by genotyping the individual plants in F_2–F_7 generations for wilt resistance and susceptibility.

Amplification was carried out in 15 μl of reaction mixture containing 1.5 μl *Taq* buffer, 0.75 μl dNTP mix, 8.5 μl distilled water, 0.75 μl primer (both forward and reverse), 2.5 μl template, and 0.25 μl *Taq* DNA polymerase. PCR was performed in a thermal cycler (Eppendorff, Germany) and the PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing as per primer's melting temperature (Tm) for 30 s at 53-57°C, and extension at 72°C for 1 min, final extension at 72°C for 7 min. All PCR products were size-separated in 3% (w/v) Agarose gel (Bangalore Genei, India), stained with ethidium bromide and visualized with UV transilluminator. In all cases, λ100 bp (Merck make) ladder was used as molecular size marker.

2.5 | Data analysis

The data obtained from F_2 generation of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) and the BC_1F_1 generations, ('Nira' × *C. oxyacantha*) 'Nira' and ('Nira' × *C. palaestinus*) 'Nira' were used for chi-square (χ^2) test, and the analysis of variance in yield evaluation trial was computed using INDOSTAT statistical software (Indostat services, Hyderabad, India, www.indostat.org.

3 | RESULTS

Wilt resistant reaction of wild species parents, *C. oxyacantha* and *C. palaestinus* and highly susceptible reaction of cultivated species parent, 'Nira' were confirmed in wilt sick plot and wilt sick pots in glasshouse (Supporting information Figure S1). Wilt incidence in resistant species was zero per cent, whereas it was 95%–100% in 'Nira'. The F_1 of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) were highly resistant to wilt with zero per cent wilt incidence. Morphologically F_1 of each cross was intermediate to its parents (Supporting information Figure S2). Furthermore, the true-hybrid nature of F_1s of both crosses was confirmed by SSR markers (Figures 1a, 2a). The F_2 population of ('Nira' × *C. oxyacantha*) consisted of 687 plants, of which, 525 were classified as resistant and 162 as susceptible. The resistant and susceptible segregation was in accordance with 3:1 ratio (χ^2: 0.73; *p*: 0.39). The backcross population of ('Nira' × *C. oxyacantha*) 'Nira' had 123 resistant and 127 susceptible plants confirming 1:1 segregation ratio (χ^2: 0.064; *p*: 0.80). The F_2 generation of ('Nira' × *C. palaestinus*) has segregated into 312 resistant and 120 susceptible plants confirming 3:1 segregation ratio (χ^2: 1.77; *p*: 0.18). ('Nira' × *C. palaestinus*) 'Nira' backcross showed 1:1 segregation of resistant and susceptible plants (115:112; χ^2: 0.28; *p*: 0.59). Segregation pattern in F_2 and backcross generation has confirmed a single dominant gene control of Fusarium wilt resistance introgressed from *C. oxyacantha* and *C. palaestinus*.

Using bulked-segregant analysis (BSA), the SSR markers associated with Fusarium wilt resistance were identified in F_2 mapping populations of ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) crosses (Figures 1b, 2b). Eight SSR markers each in ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*) were found to be associated with Fusarium wilt resistance. The DNA sequence of these markers is given in Table 1. These markers were validated by phenotyping of F_2 plants in wilt sick plot for reaction against wilt. The markers were further validated by both genotyping and phenotyping for wilt reaction in F_3–F_7 generations in both crosses.

3.1 | Marker-assisted selection for wilt resistance

Based on genotyping, the resistant F_2 plants in both crosses were classified into heterozygous and homozygous resistant groups. The

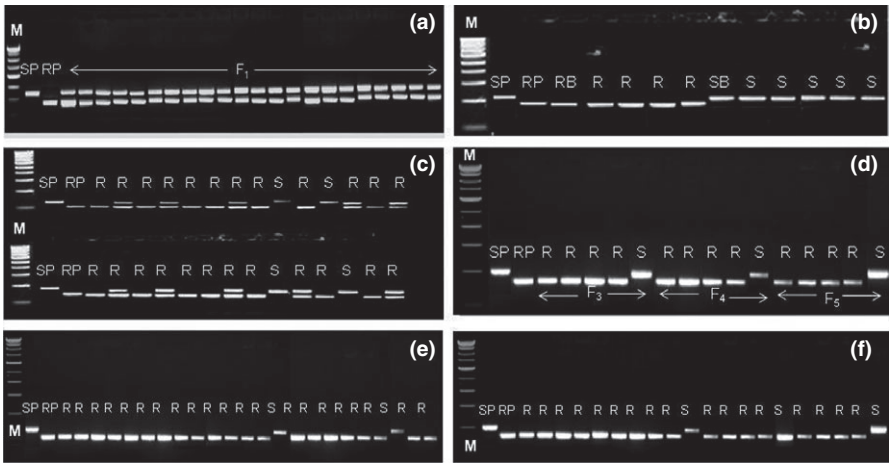

FIGURE 1 Confirmation of true-hybrid nature of F_1 of ('Nira' × *Carthamus oxyacantha*), bulked-segregant analysis (BSA) and marker-assisted selection (MAS) for wilt resistance in different filial generations of ('Nira' × *C. oxyacantha*). (a) SSR marker, CAT-70 confirming true-hybrid nature of F_1, (b) BSA revealing cosegregation of SSR marker, CAT-70 with wilt resistant F_2 individuals, (c) MAS for homozygous and heterozygous resistant F_2 individuals, and (d,e,f) MAS for wilt resistant individuals in F_3–F_5, F_6 and F_7 generations, respectively. M, molecular weight marker; R, resistant individual; RB, resistant bulk; RP, resistant parent; S, susceptible individual; SB, susceptible bulk; SP, susceptible parent (λ 100 bp ladder)

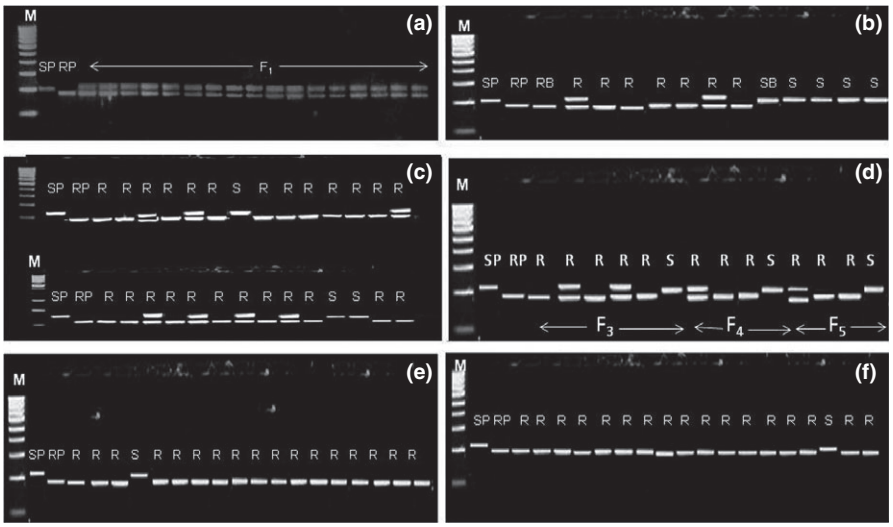

FIGURE 2 Confirmation of true-hybrid nature of F_1 of ('Nira' × *Carthamus palaestinus*), bulked-segregant analysis (BSA) and marker-assisted selection (MAS) for wilt resistance in different filial generations of ('Nira' × *C. palaestinus*). (a) SSR marker, ct-47 confirming true-hybrid nature of F_1, (b) BSA revealing cosegregation of SSR marker, ct-47 with wilt resistant F_2 individuals, (c) MAS for homozygous and heterozygous resistant F_2 individuals, (d,e,f) MAS for wilt resistant individuals in F_3–F_5, F_6 and F_7 generations, respectively. M, molecular weight marker; R, resistant individual; RB, resistant bulk; RP, resistant parent; S, susceptible individual; SB, susceptible bulk; SP, susceptible parent (λ 100 bp ladder)

heterozygous resistant plants showed double bands while the homozygous resistant plants had a single band in the Agarose gel (Figures 1c, 2c). Using the SSR markers linked to Fusarium wilt resistance, the wilt resistant and susceptible plants could be differentiated at seedling stage in F_3–F_7 generations of both crosses (Figures 1d,e,f & 2d,e,f). The resistant progenies selected in each generation with assistance of markers were simultaneously confirmed by phenotyping in wilt sick plots for resistance reaction (Supporting information Figure S3). The homozygous resistant plants produced only resistant progenies in all filial generations while the heterozygote plants produced both resistant and susceptible progenies. The resistant plants in every generation were simultaneously selected for agro-morphological traits close to *C. tinctorius*. Thus gradually, the undesirable traits introgressed from wild species were eliminated and finally all the resistant interspecific F_6 and F_7 plants resembled mostly *C. tinctorius* with very minute morphological resemblance to wild species parent (Supporting information Figure S4). Morphological description of

TABLE 1 Forward and reverse sequences of polymorphic SSR primers flanked to Fusarium wilt resistance in ('Nira' × *C. oxyacantha*) and ('Nira' × *C. palaestinus*)

Primer name	5′ Forward sequence 3′	5′ Reverse sequence 3′	Tm (°C)
'Nira' × C. oxyacantha			
CAT 7	AACCCGATGTCTAATTGGGTTG	ATGTATTGCTTCCGGGTGTTAC	55
CAT 31	CTCATCAAACGTATCACTGGAAC	GAACTTCTCTTAGACGCCAACTG	55
CAT 43	AGCTTGGTCTAGATGAACAC	GCAGTAGTAACCGATATGCTA	53
CAT 46	CAAATAGGTGCTAGAAAACAC	ACTCAATCCTCATAGCAATTG	53
CAT 48	GAAATCCGATGGTAGCCGGA	CTTCAACCTTCATCCCTCCC	53
CAT 96	CATGCAATCATCAAGGGGTG	GTGCTCAAGTGTGTTTAATCA	54
CAT 52	GAAACCCTAGATTCATTCA	CGCATGATTACAGCTTGAG	55
CAT70	TACCCTCAATTATGATGCATGAA	CATAATTCATTCACCTCTCACC	55
'Nira' × C. palaestinus			
CAT 92	CAAATAGGTGCTAGAAAACAC	ACTCAATCCTCATAGCAATTG	57
ct32	CCACCGTAACCGAAGATGTG	TCTAAAGGTAACCTTCGTAGTGA	55
ct34	GAGAACCTTCGCGTGAAATC	TGGAAGAAGAAGGGGTGATG	54
ct44	ACACTGGGGTCCATACTTGC	CGACGGTTAAATATGATGGGA	55
ct47	GGGCTTGCTTCATTCAGGT	TGGTGGATTGAAATTGGGTT	53
ct137	GAGCTCTTCACGCACCTCAC	TAGAAATCGAACACATGGCG	55
ct138	TGAAATGGTTTCTGGGTGAA	GAAAGCCATTGGTGGAAGTGT	55
ct169	TCACACACACAACACACACCT	CTTCAACGACGAGAACGTCA	55

Tm, annealing temperature.

parents and interspecific offsprings was given in Supporting information Table S1.

3.2 | Yield performance of wilt resistant interspecific lines

ANOVA of pooled analysis of experimental material for seed yield, oil content and 100-seed weight was given in Supporting information Table S2. Of the 22 wilt resistant interspecific lines evaluated, six have recorded higher seed yield than the high yielding commercial variety, 'A1' (Table 2). Of these, four lines *viz*., ISF-22-15, ISF-18-15, ISF-31-15 and ISF-13-15, were derived from ("Nira" × *C. oxyacantha*) and the remaining two namely, ISF-19-15 and ISF-21-15, were from ('Nira' × *C. palaestinus*). These lines exhibited zero per cent wilt incidence in wilt sick pots in glasshouse when screened prior to yield evaluation. Significant seed yield increase of 9%–29% over check variety, 'A1' was observed among these interspecific lines. The highest seed yield was recorded in ISF-19-15 (1,777 kg/ha) followed by ISF-31-15 (1,726 kg/ha). All the interspecific lines yielded much higher than their parents. The wild species, *C. oxyacantha* and *C. palaestinus*, recorded much lower seed yields as compared to cultivated species. Oil content in interspecific lines was significantly higher than both parents and 'A1'. The interspecific lines gave 18%–61% significantly higher oil yield (396–538 kg/ha) than 'A1' (334 kg/ha). *Carthamus oxyacantha* and *C. palaestinus* were late in flowering and maturity as compared to interspecific derivatives. Phenologically and morphologically the interspecific lines were more close to *C. tinctorius* (Supporting information Table S1).

4 | DISCUSSION

Fusarium wilt is economically the most important disease of safflower. There is no information on existence of *Fusarium oxysporum* f.sp. *carthami* races in India although three pathogenic races of safflower wilt were identified in the USA (Klisiewicz & Thomas, 1970). Screening of vast safflower germplasm for resistance to Fusarium wilt has identified a few resistant sources but these were not stable and not directly resulted in the development of resistant varieties (Mundel & Huang, 2003). Diverse Fusarium wilt resistance sources are desired for durable resistance. Among the *Carthamus* species, either of *C. oxyacantha* or *C. palaestinus* or both were suggested to be progenitors of cultivated species (Sehgal, Rajpal, & Raina, 2008), and the recent studies on species-genomic relations indicated that *C. palaestinus* was genetically the closest species to the cultivated species (Agrawal, Tsujimoto, Tandon, Rama Rao, & Raina, 2013; Chapman & Burke, 2007; Sasanuma, Sehgal, Sasakuma, & Raina, 2008; Sehgal, Raina, Devarumath, Sasanuma, & Sasakuma, 2009). In the present investigation, Fusarium wilt resistance from *C. oxyacantha* and *C. palaestinus* was successfully introgressed into cultivated species through interspecific hybridization. This was confirmed by SSR markers linked to Fusarium wilt resistance as well by phenotying for resistance in wilt sick plot. With the assistance of SSR markers linked to wilt resistance, resistant progenies could be selected in F_2–F_7 generations and were simultaneously confirmed in wilt sick plot for resistant reaction. As SSR markers are codominant markers, they could distinguish resistant homozygotes from resistant heterozygotes. SSR markers have been applied to assess interspecific and intraspecific polymorphism in cultivated and wild safflower (Barati &

TABLE 2 Mean performance of Fusarium wilt resistant interspecific lines tested in 2 years at Hyderabad, India

Entry	Pedigree	Seed yield (kg/ha)	Oil content (%)	Oil yield (kg/ha)	100-seed weight (g)
ISF-19-15	(‘*Nira*’ × *C. palaestinus*)	1,777	26.03	463	3.93
ISF-21-15	(‘*Nira*’ × *C. palaestinus*)	1,520	26.11	396	4.91
ISF-22-15	(‘*Nira*’ × *C. oxyacantha*)	1,495	29.17	437	4.07
ISF-18-15	(‘*Nira*’ × *C. oxyacantha*)	1,544	29.16	450	4.86
ISF-31-15	(‘*Nira*’ × *C. oxyacantha*)	1,726	31.15	538	4.04
ISF-13-15	(‘*Nira*’ × *C. oxyacantha*)	1,604	29.11	467	4.13
‘A1’ (check)	Cultivated species	1,372	24.35	334	6.16
‘Nira’	-do-	676	26.10	177	4.49
C. oxyacantha	Wild species	109	24.66	27	0.91
C. palaestinus	Wild species	112	24.53	28	2.5
CV (%)		15	0.74	15	1.91
CD (p = 0.05)		102	0.34	30	0.13

Arzani, 2012), but SSR markers cosegregating with Fusarium resistance were not indentified earlier in safflower. RAPD markers associated with Fusarium wilt resistance in safflower were reported (Anjani et al., 2012); however, marker-assisted selection for wilt resistance was not practiced using these markers. Nonetheless, use of molecular markers and wild species in safflower breeding is negligible hitherto.

While selecting the resistant progenies using SSR markers in F_2 and subsequent generations, utmost care was taken to reject those plants possessing undesirable traits from wild species parent and select only those close to *C. tinctorius* parent. Hence, all the resistant interspecific lines developed were more of like *C. tinctorius* in morphological and phenological traits. Rejection of morphological traits close to wild species and other undesirable traits such as late flowering and maturity, small size capsules, low seed number per capitulum, low seed weight, less number of branches per plant, and branching from upper one-third of the main stem could be achieved easily by selecting against them. As wilt resistance from both the wild species was a simple inherited trait, it could be introgressed easily into *C. tinctorius*, and the unwanted genes introduced from *C. oxyacantha* and *C. palaestinus* could be detected and eliminated more easily than expected. Our goal of introgression of wilt resistance from wild species has been achieved in direct cross between cultivated and wild species. Backcrosses had less transgressive segregants than F_2; hence, they were not persued vigorously further. Transgressive segregants for yield and other traits were isolated in F_2 populations of interspecific crosses in many crops (de Vicente & Tanksley, 1993; Vega & Frey, 1980; Verma, Ravi, & Sandhu, 1995; Wang, Ulloa, Philip, & Roberts, 2008).

The six resistant interspecific lines giving higher seed and oil yields were also free of any known undesirable traits introgressed from the wild species. Traits associated with yield such as high number of branches per plant, basal branching and high number of capsules per plant have been transferred from *C. oxyacantha* to *C. tinctorius*, resulting in an improvement in seed yield. The genes reshuffling originating from interspecific hybridization might have also produced favourable combinations of genes expressing high yield in both crosses.

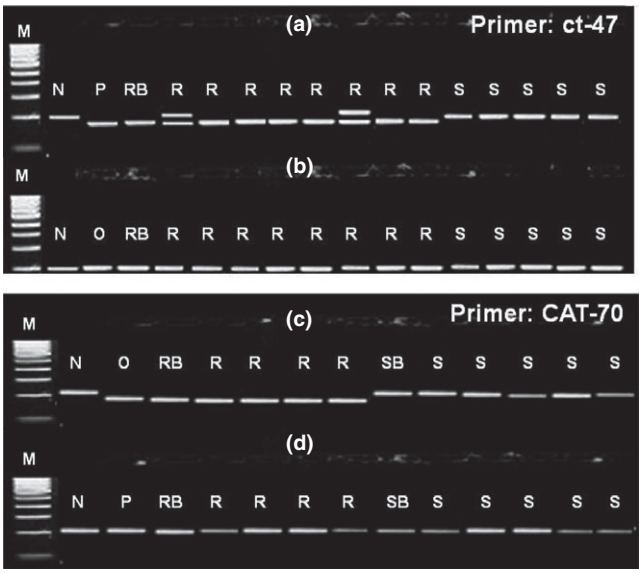

FIGURE 3 Bulked-segregant analysis using SSR markers, ct-47 and CAT-70 in F_2 populations of (‘Nira’ × *Carthamus palaestinus*) and (‘Nira’ × *C. oxyacantha*). (a) ct-47 differentiating resistant and susceptible F_2 individuals of (‘Nira’ × *C. palaestinus*), (b) ct-47 not showing polymorphism in F_2 population of (‘Nira’ × *C. oxyacantha*), (c) CAT-70 differentiating resistant and susceptible F_2 individuals of (‘Nira’ × *C. oxyacantha*), (b) CAT-70 not showing polymorphism in F_2 population of (‘Nira’ × *C. palaestinus*). M, molecular weight marker; R, resistant individual; RB, resistant bulk; RP, resistant parent; S, susceptible individual; SB, susceptible bulk; SP, susceptible parent (λ 100 bp ladder)

The SSR markers linked to Fusarium wilt in (‘Nira’ × *C. palaestinus*) could not differentiate resistant and susceptible individuals in F_2 mapping population of (‘Nira’ × *C. oxyacantha*) (Figure 3a,b), and similarly, the SSR markers linked to Fusarium wilt resistance in (‘Nira’ × *C. oxyacantha*) were not polymorphic in F_2 mapping population of (‘Nira’ × *C. palaestinus*) (Figure 3c,d). These results indicate that the resistance genes present in *C. oxyacantha* and *C. palaestinus*

may be different from each other or these species might have conserved different sequences of Fusarium wilt resistance gene in the long course of evolution of *Carthamus* species.

Effective markers linked to Fusarium wilt resistance are now available. They can be deployed for MAS for wilt resistance in large segregating populations of interspecific crosses, especially the F_2. These markers had facilitated differentiation of heterozygous and homozygous resistant individuals at genotypic level which was otherwise not possible at phenotypic level. The molecular markers have permitted precise identification of resistant individuals at seedling stage itself, which might not be possible sometimes in wilt sick plot because pathogen spread across wilt sick plot may not be uniform due to various factors such as pathogen inoculum levels in wilt sick plot and soil temperature and/or moisture level influence disease development. Using these markers, it is now possible to select precisely the wilt resistant interspecific genotypes in the laboratory itself without going for artificially inoculated wilt sick plot or pots. These markers would certainly assist in simultaneous pyramiding of wilt resistant genes from *C. oxyacantha* and *C. palaestinus* in the susceptible cultivated species for developing safflower varieties with broad and durable resistance to Fusarium wilt. The resistant interspecific lines developed in the present investigation would serve as novel sources for wilt resistance in safflower breeding programmes.

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CONFLICT OF INTEREST

Authors declare that no conflict of interests exists.

AUTHORS' CONTRIBUTION

Anjani, K conceived the study and carried out the entire work with the help of Bhavna, P and Debadutta Mishra in PCR work and Prasad, R. D in Fusarium wilt sick plot maintenance. All authors read and approved the final manuscript.

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