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Introgression of resistance to *Alternaria* leaf spot from wild species into susceptible cultivated safflower

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Abstract

Alternaria leaf spot (ALS) caused by *Alternaria carthami* Chowdhury can cause yield loss up to 90% in safflower (*Carthamus tinctorius* L.) under severe conditions. Even though a definite source of ALS resistance is not available in cultivated species, a few of the wild species, *viz. C. palaestinus* and *C. lantaus*, are known to be tolerant to ALS. Therefore, an attempt was made to introgress *Alternaria* resistance from these species into cultivated species. F_1-F_8 generations of crosses (PI537632 × *C. palaestinus*), (*C. palaestinus* × PI537632), ("Nira" × *C. palaestinus*) and ([MS 6(O) × *C. lanatus*] × *C. palaestinus*) were screened against ALS. ALS infection (%) was recorded in field and quantified using Windias Leaf Image Analysis system. Detached leaf technique was used in laboratory to confirm resistance in interspecific selections. Six resistant and 29 moderately resistant interspecific lines resembling mostly cultivated species were developed. Inheritance of ALS resistance indicated involvement of multiple minor alleles having small effects on resistance. The identified resistant lines could provide potential source of resistance to ALS for safflower breeding programmes.

KEYWORDS

Alternaria, Resistance, Safflower, wild species

1 | INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an important edible oilseed crop grown commercially in more than 20 countries. Kazakhstan is the top safflower producing country; Russian Federation, USA, Mexico, Turkey, India and China are the other major safflower producing countries (FAOSTAT, 2019). *Alternaria* leaf spot (ALS) caused by *Alternaria carthami* Chowdhury is the major destructive disease in safflower which can cause yield loss as high as 90% under severe disease conditions (Irwin, 1976). It was reported from all safflower growing countries, *viz.* India (Chowdhury, 1944), Australia, Brazil, USA, Israel, Kenya, Pakistan, former USSR, Italy and South Korea (Alves et al., 2016; Classen, Schuster, & Ray, 1949; Ellis, 1971; Nelen & Vasileva, 1959; Park & Lee, 2003). This disease is prevalent in many safflower growing areas in India (Gud, Murumkar, Shinde & Kadam, 2008). ALS is also a seed-borne pathogen in safflower (Irwin, 1976). Weather conditions especially relative humidity (>80%) and temperature (21–33°C) play a predominant role in determining the intensity of epidemics (Murumkar, Gud, Indi, Shinde, & Kadam, 2008). Age of the crop also determines disease intensity (Suresh, Padmavathi, Varma, & Jagdeeshwar, 2013). ALS causes severe damage when wet cloudy weather prevails continuously for more than a week (Pawar, Ghuge, Gholve, & Sutar, 2017). High occurrence of ALS was reported in India when safflower was sown in August (Ashiq & Virender, 2014; Katkar et al., 2012). Conventional breeding for ALS resistance in cultivated safflower has not been successful so far though some varieties or genotypes have been reported to be moderately resistant to ALS (Bergman, Riveland, Flynn, Carison, & Wichman, 2001). Prasad and Anjani (2008) reported high resistance to ALS in safflower wild species, *viz. C. palaestinus*, *C. lanatus*, *C. creticus* and *C. turkestanicus* and moderate resistance in *C. oxyacantha* and *C. glaucus*. Heaton and Klisiewicz (1981) reported resistance to

ALS in *C. lanatus* and an alloploid derived from (*C. tinctorius* × *C. lanatus*). The wild species are potential sources of resistance to ALS. Cultivation of ALS-resistant cultivars is an effective means to control the disease since chemical control is not very effective. Since no definite source of resistance to ALS was available in cultivated safflower gene pool, the present investigation was aimed to introgress ALS resistance from wild species into cultivated species background and to decipher the genetic architecture of resistance to ALS.

2 | MATERIAL AND METHODS

2.1 | Plant material and crossing programme

The cross (PI537632 × *C. palaestinus*) was made in 2003. After observing variation in disease severity in F_2 of this cross when sown in August 2005, the same cross was attempted again along with three more crosses (*C. palaestinus* × PI537632), ('Nira' × *C. palaestinus*) and (MS 6(O) × *C. lanatus*) in winter season of 2005; *C. palaestinus* (2*n* = 24) and *C. lanatus* (2*n* = 44) are ALS-resistant wild species. 'Nira', PI537632 and MS 6(O) belonging to cultivated species, *C. tinctorius* (2*n* = 24) are highly susceptible to ALS. MS 6 (O) is a genetic male sterile (GMS) line, 'Nira' is a highly susceptible cultivated variety, and PI537632 is an introduction from USA. MS 6(O) was used for ease of crossing between cultivated and wild species; PI537632 was used because it is a high oil (35%) genotype. The F_1 of the cross (MS 6(O) × *C. lanatus*) was crossed to *C. palaestinus* in 2006.

Selected flower heads in each female parent were hand emasculated one day prior to pollination. The pollen from respective male parent was manually brushed on to stigmas of emasculated flower heads of female parent. In case of MS 6(O), no emasculation was done since it is a GMS line (Anjani, 2005; Baydar & Gokmen, 2003). All the female and male flower heads involved in crossing were covered with butter paper bags from preflowering to maturity. *C. palaestinus* being a diploid species produced fertile F_1 plants when crossed to *C. tinctorius*. In F_1 of (MS 6(O) × *C. lanatus*) cross, 98% of the plants were sterile and 2% were fertile, and the fertile F_1 plants were crossed to *C. palaestinus*.

2.2 | Generation advancement

In all crosses, each F_1 plant was self-pollinated and harvested separately and the harvested seed was planted in plant-progeny rows during first to second week of August in the following year. F_2 plants of each cross were screened plant-wise against ALS leaf spot. About 20–25 plants in each F_1 and 127–350 plants in each F_2 were planted. However, in order to study the inheritance of ALS resistance, around 1,475 plants were observed for disease severity in F_2 and 2,965 plants in F_3 of (PI537632 × *C. palaestinus*). In all crosses, the F_2 plants showing <10% ALS severity were self-pollinated and harvested plant-wise. Self-seed of each selected F_2 plant was planted in plant-progeny rows during first week of August in the subsequent year. In this way, progeny showing <10% to 20% ALS disease was advanced progeny-row-wise until the F_8 generation of all crosses; these F_8 generations were further maintained through self-pollination. All the generations were grown under mosquito nets to avoid pollen contamination through honeybee which is the pollinating agent in safflower. From F_2 generation onwards, seeds from each plant were in three progeny rows of 3 m length. The spacing between rows was 30 cm and between plants was 20 cm. The susceptible cultivated safflower varieties, 'A1', 'PBNS-12' and 'Nira', were repeatedly planted after every 10 test rows to determine the uniformity of disease spread in the field. In addition to these susceptible varieties, other susceptible varieties, namely AKS-207, HUS-305, NARI-6, Manjira, Bhima, Co-1, Girna, JSF-1, JSI-7, JSI-73, JSI-97, JSI-99, NARI-38, PBNS-40, Sharada, SFS-658, SFS-708, RVS-113 and the hybrid, NARI-H-15 were planted in 2012–13 in a single row each. Recommended crop management practices, irrigation schedule and fertilization were followed, and plant protection measures were used when required. The experiment has received only a presowing irrigation.

2.3 | Screening against Alternaria leaf spot

2.3.1 | Field screening

Planting of experimental material was taken up in the first to second week of August each year for screening against ALS because sowing during this period ensures very heavy ALS infection at research farm of ICAR-Indian Institute of Oilseeds Research (ICAR-IIOR), Hyderabad, India. The observations for ALS were visually recorded at peak flowering and postflowering stages. In F_2–F_4 generations, ALS incidence was visually recorded for each plant in all progeny rows, and then from F_5 generation onwards, disease was recorded for a total of five plants in each test material. Data were recorded from five lower, five middle and five top leaves. The per cent disease intensity (PDI) was calculated as ([Sum of rating of individual leaves in a plant/Number of leaves examined] × Maximum disease score) × 100. Mean PDI of five plants of each selected line was reported. Mean of PDIs taken at peak flowering and postflowering was reported for each progeny. ALS disease was scored on 0–9 scale indicating the percentage of leaf area covered with leaf spots: 0 = no symptoms on leaf, 1 = small brown spots covering > 1% or less of the leaf area, 3 = lesions small, scattered, brown to black with concentric rings covering 1%–10% of the leaf area, 5 = 11%–25% leaf area affected, 7 = 26%–50% of leaf area affected with lesions enlarging, slightly sunken in the centre with concentric rings and 9 = lesions enlarging up to 10 mm coalescing to form bigger patches covering > 50% area. On 0–9 scale, “0” indicates no disease, “1” indicates high resistance, “3” denotes resistance, “5” indicates moderate resistance while “7” and “9” indicate susceptible and high susceptible reactions, respectively (AICRP on Safflower, 2011). In addition to visual observation of disease infection, ALS disease was quantified in F_6 of ([MS 6(O) × *C. lanatus*] *C. palaestinus*) and F_7 generations of the remaining three crosses in 2012–13 using Windias Leaf Image Analysis System of Delta-T. Leaves from different positions on a plant were collected from the field in Petri dishes for measuring disease

infection using Windias Leaf Image Analysis System of Delta-T. The collected leaves were immediately scanned using Windias Leaf Image Analysis System in laboratory. Ten lower, ten middle and ten top leaves were collected from five plants in each plant-progeny row. The total leaf area and per cent of infection in each leaf area was given by the Image Analysis System. Mean of per cent infection of all leaves in a progeny was considered to categorize the progeny for disease reaction.

2.3.2 | Detached leaf technique under in vitro conditions

A detached leaf technique for screening the ALS disease in safflower was standardized at ICAR-IIOR. *Alternaria carthami* spore suspension from 10-day-old culture grown on potato dextrose agar medium having 1×10^6 spores/ml was prepared and sprayed on mid-stem leaves kept on moist blotters in a Petri dish. The plates were incubated at 26 ± 1°C. Leaf spot symptoms developed within 3–4 days after inoculation, and disease severity was observed visually. The F_6 and F_7 interspecific progeny were screened by this method in 2012–13. The visual observations of the spread of disease on detached leaf were used to confirm the resistant and moderately resistant reactions of 35 interspecific inbred lines selected based on field screening and susceptibility of susceptible varieties.

2.4 | Statistical analysis

R 3.5.3 and RStudio 1.1.456 were used for descriptive statistic, and ggplot2 was used for box plots–point graph.

3 | RESULTS

3.1 | Inheritance of *Alternaria* leaf spot resistance

All plants in F_1 generation of the crosses (*C. palaestinus* × PI537632), (PI537632 × *C. palaestinus*) and ('Nira' × *C. palaestinus*) were fertile and intermediate to both parents in morphology, while the F_1 plants of the cross (MS 6(O) × *C. lanatus*) were both fertile and sterile; the fertile plants were closer to *C. tinctorius*, and the sterile ones were intermediate to both parents in morphology. In F_2 generation of the fertile F_1 plants, some plants were intermediate to both parents in morphology (Figure S1). Meiotic study showed 12^{II} in fertile F_1 plants and their F_2 progeny whereas chromosomal aberrations were present in sterile F_1 plants which did not produce seed upon self-pollination or sib crossing with fertile F_1 plants (Figure S2). The F_1 plants of the cross ([MS 6(O) × *C. lanatus*] × *C. palaestinus*]) were fertile.

F_1 of all four crosses were found to be resistant to *Alternaria* when planted in October. This may be due to the very low occurrence of *Alternaria* in October-sown crop that flowers. The F_2 generation of (PI537632 × *C. palaestinus*), when sown in August, exhibited disease severity ranging from <10% to 100% (Figure 1). Similar pattern of segregation was observed in F_3 generation that was derived from F_2 progeny showing <10% ALS (Figure 2). Less than 2% of plant

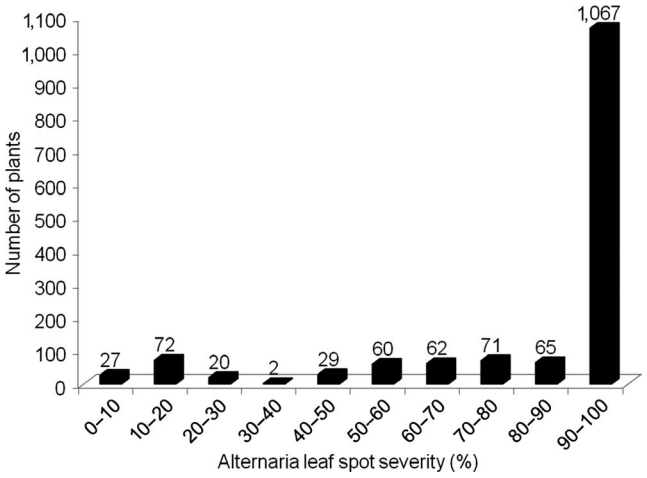

FIGURE 1 Phenotypic variation in response to *Alternaria* leaf spot in F_2 generation of (PI537632 × *C. palaestinus*) cross

in F_2 and F_3 showed <10% disease; even when F_2 progeny with <10% disease were advanced to F_3 generation, there was <10% to 100% disease severity in F_3. Similar pattern of inheritance was also observed in all the three other crosses.

3.2 | Screening against *Alternaria* leaf spot

The high disease pressure experienced in August-sown experiments was ideal for comparing host responses to ALS leaf spot and was certainly more extreme than in normal sowing time in India. Initial infection of ALS disease has appeared at rosette stage which rapidly developed as the crop approaches preflowering stage and reaches very high infection as the crop progressed from flower initiation to full flowering. Maximum disease severity was observed at peak flowering with 100% infection in all highly susceptible individuals and more than 50% in susceptible ones. Not much difference in disease progression was observed in material after peak flowering.

The progeny whose flowering time coincided with the normal duration of check varieties were retained, and the late and very late

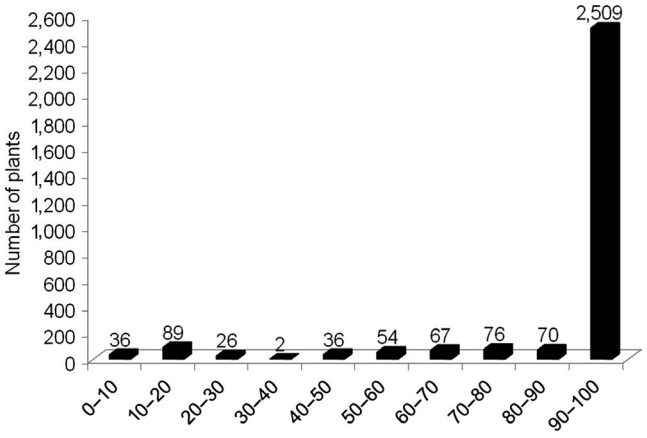

FIGURE 2 Phenotypic variation in response to *Alternaria* leaf spot in F_3 generation of (PI537632 × *C. palaestinus*) cross

progeny were eliminated at every stage to avoid selection of individuals escaping ALS. The resistant types displayed very small size necrotic lesions at low frequencies, whereas the highly susceptible progeny had large necrotic lesions which coalesced and covering 100% of the leaf area and flower heads. All the check cultivars succumbed to ALS with very high disease infection during test years (Figure S3). In all susceptible progeny and cultivars, the entire plant was severely infected while in the resistant and moderate resistant genotypes the infection was observed mostly on the lower leaves and did not progress much towards upper part of the stem and branches where only a few lesions of ALS were observed (Figures S4 and S5). Box plots with point graphs of ALS infection in resistant and moderately resistant lines recorded in the field during 2011, 2012 and 2015 are presented separately in Figure 3. The per cent infection in resistant and moderately resistant inbred lines was on the Y-axis of the figure. The right side box plots in the figure indicate the distribution of disease per cent among the six resistant inbred lines in different years, and the box plots on the left side of the figure depict the disease distribution among 29 moderately resistant inbred lines. Each point plotted adjacent to a box gives exact value of per cent infection in an inbred line. Thus, infection per cent of all the six resistant and 29 moderately resistant inbred lines in different years was depicted by points. The line at the centre of each box indicates the median value of disease per cent among the resistant group or moderately resistant group. The lines drawn on top and lower side of a box indicate the minimum and maximum values of disease per cent in the resistant or moderately resistant inbred lines. In each year, the distribution of disease per cent in resistant and moderately resistant groups was within the stipulated disease scale or range. The trials in 2013 and 2014 have been eliminated due to very low plant stand because of continuous heavy rain fall after sowing; however, the remnant seed of the test material was sown in winter season of 2013 and 2014 to advance to the next generation.

Of the 35 selected inbred lines, six lines showed resistant reaction (<10% disease severity) with a score of 3 on 0-9 scale consistently over years. Four of the resistant lines, *viz.* ISF-15-228, ISF-15-278, ISF-15-282 and ISF-15-283, were derived from ([MS 6(O) × *C. lanatus*] × *C. palaestinus*), and the resistant lines, ISF-15-332 and ISF-15-384, were derived from (PI537632 × *C. palaestinus*) and ('Nira' × *C. palaestinus*), respectively. Twenty-nine lines derived from (*C. palaestinus* × PI537632), (PI537632 × *C. palaestinus*) and ('Nira' × *C. palaestinus*) crosses showed moderate resistant reaction with a score of 5. The disease incidence quantified using Windias Leaf Image Analysis System in the 35 selected interspecific inbred lines is presented in Table 1. The visual disease observations were correlated with the disease severity using the Windias Leaf Image Analysis System (Figure 4). Disease reaction of all the selected inbred lines was further confirmed in laboratory using detached leaf technique (Figures 5 and 6).

4 | DISCUSSION

In the present investigation, inheritance pattern of resistance to ALS indicates polygenic nature of resistance and the role of various minor alleles in the defence against the pathogen (Figures 1 and 2). Some of the alleles might have provided moderate-to-high resistance.

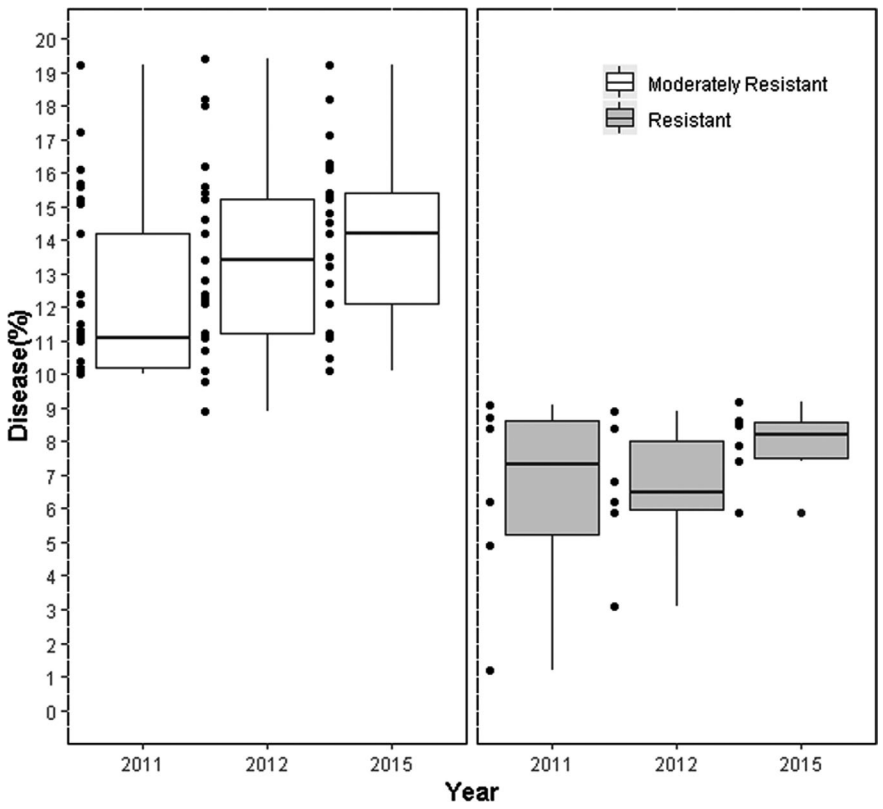

FIGURE 3 Box plots with point graphs of *Alternaria* leaf spot disease intensity (%) in moderately resistant and resistant inbred lines during 2011, 2012 and 2015

TABLE 1 Percent disease intensity of *Alternaria* leaf spot in selected interspecific derivatives quantified using Windias Leaf Image Analysis System

	Interspecific inbred line	Alternaria leaf spot[a] (%)	Disease reaction
C. palaestinus × PI537632[b]	ISF-15-13	15.0 ± 0.45(3.94)	MR
	ISF-15-293	11.0 ± 0.24 (3.39)	MR
PI537632 × *C. palaestinus*[b]	ISF-15-51	14.6 ± 0.31 (3.89)	MR
	ISF-15-69	13.4 ± 0.42 (4.35)	MR
	ISF-15-79	15.6 ± 0.29 (4.01)	MR
	ISF-15-295	13.2 ± 0.35 (3.70)	MR
	ISF-15-297	12.5 ± 0.34 (3.61)	MR
	ISF-15-298	10.7 ± 0.42 (3.35)	MR
	ISF-15-305	10.5 ± 0.25 (3.32)	MR
	ISF-15-305-1	14.0 ± 0.24 (3.81)	MR
	ISF-15-84	13.5 ± 0.35 (3.74)	MR
	ISF-15-88	14.1 ± 0.42 (3.82)	MR
	ISF-15-89	12.5 ± 0.47 (3.61)	MR
	ISF-15-310	16.5 ± 0.21 (4.12)	MR
	ISF-15-94	14.7 ± 0.27 (3.90)	MR
	ISF-15-95	10.8 ± 0.28 (3.36)	MR
	ISF-15-97	11.1 ± 0.34 (3.41)	MR
	ISF-15-99	12.8 ± 0.31 (3.65)	MR
	ISF-15-100	10.5 ± 0.41 (3.32)	MR
	ISF-15-101	11.6 ± 0.45 (3.48)	MR
	ISF-15-102	12.9 ± 0.29 (3.66)	MR
	ISF-15-311	14.1 ± 0.31 (3.82)	MR
	ISF-15-313	15.2 ± 0.37 (3.96)	MR
	ISF-15-320	10.7 ± 0.34 (3.35)	MR
	ISF-15-321	16.3 ± 0.35 (4.10)	MR
	ISF-15-328	14.1 ± 0.19 (3.82)	MR
	ISF-15-332	6.1 ± 0.22 (2.57)	R
	ISF-15-334	13.4 ± 0.17 (3.73)	MR
	ISF-15-368	11.4 ± 0.18 (3.45)	MR
[(MS 6(O) × *C. lanatus*) × *C. palaestinus*][c]	ISF-15-228	6.5 ± 0.17 (2.65)	R
	ISF-15-278	5.1 ± 0.2 (2.37)	R
	ISF-15-282	4.7 ± 0.19 (2.28)	R
	ISF-15-283	6.6 ± 0.21 (2.66)	R
'Nira' × *C. palaestinus*[b]	ISF-15-381	18.9 ± 0.34 (4.40)	MR
	ISF-15-384	4.9 ± 0.21 (2.32)	R
Susceptible checks	A1	100 (10.02)	HS
	PBNS-12	100 (10.02)	HS
	"Nira"	100 (10.02)	HS

Abbreviations: HS: high susceptible with 9 score on 0-9 scale; MR, moderately resistant with 5 score; R, resistant with 3 score.
[a]Percent Disease Intensity (PDI).
[b]F_7 generation.
[c]F_6 generation; ±: standard error of mean; Figures in parentheses indicate square root transformed values.

The expression of resistance in a given individual might have also relied on additive and/or epistatic genetic interactions of multiple alleles. The inheritance has clearly indicated that the accumulation of various minor alleles in one genetic background is required to capture the additive effect of alleles to mount an effective resistance. Through conscious progeny-wise selection of resistant to moderate

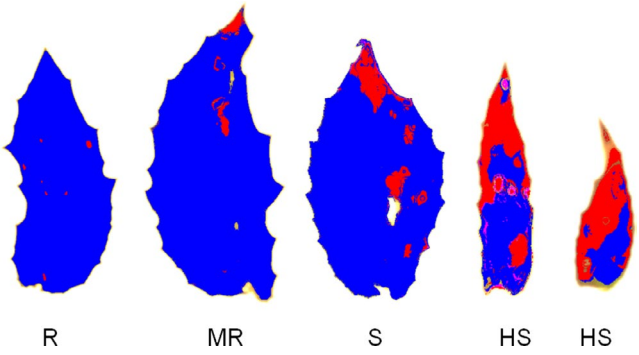

FIGURE 4 Windias Leaf Image Analysis System images of middle leaves depicting reactions of different inbred lines against *Alternaria*. R: resistant; MR: moderately resistant; S: susceptible; HS: highly susceptible. Blue colour indicates healthy leaf area and red indicates diseased leaf area

resistant progeny, we were able to isolate progeny possessing some alleles contributing to resistance. Finally, six resistant inbred lines (<10% disease severity) and 29 moderately resistant (<20% disease severity) inbred lines were isolated (Figure 3). Polygenic inheritance pattern was reported for resistance to *Alternaria solani* in tomato (Thirthamallappa & Lohithaswa, 2000; Chaerani & Voorrips, 2006) and *Alternaria* leaf blight (*Alternaria dauci*) in wild and cultivated carrot (Arbizu, Tas, Simon, & Spooner, 2017). Involvement of several minor QTLs in resistance to *Aleternaria* blight was reported in *Arabidopsis thaliana* (Rajarammohan, Kumar, Gupta, Pental, Pradhan & Kaur, 2017).

Isolation of four resistant inbred lines from the cross ([MS 6(O) × *C. lanatus*] × *C. palaestinus*), and only two resistant lines and 29 moderately resistant lines from the other three crosses where only one wild species was involved, might be due to accumulation of more resistance-conferring alleles from two wild species, *C. lanatus* and *C. palaestinus* in progeny of ([MS 6(O) × *C. lanatus*] × *C. palaestinus*).

FIGURE 5 Reactions of resistant inbred line (left leaf) and highly susceptible check, PBNS-12 (right leaf) against *Alternaria* leaf spot in detached leaf technique in the laboratory

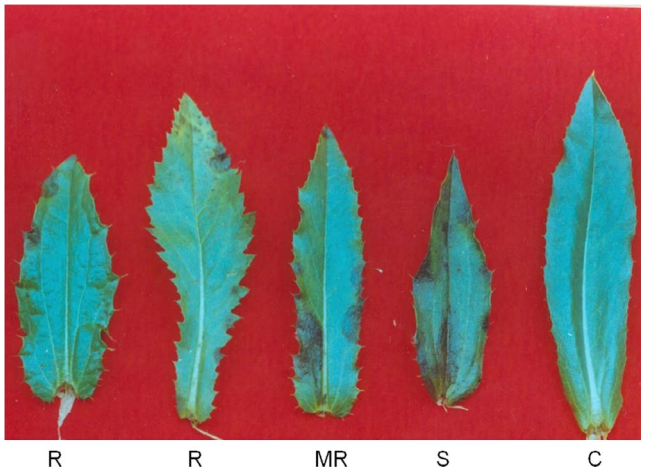

FIGURE 6 Reaction of different interspecific individuals against *Alternaria* in detached leaf technique. R: resistant; MR: moderately resistant; S: susceptible; C: control

C. lanatus and *C. palaestinus* were reported to be resistant to ALS (Prasad & Anjani, 2008). However, low frequency of resistant derivatives in all interspecific crosses might be also due to elimination of many progeny possessing wild species' traits such as late to very late flowering, very tall, deeply serrated leaves that might have led to simultaneous elimination of some resistant alleles. Finally, only a few resistant progeny which resembled mostly *C. tinctorius* morphologically were retained. Interspecific transgressive segregations between cultivated chickpea and wild species of Cicer were found resistant to ALS (Adak, Sari, Sari, & Toker, 2017).

In conclusion, the results of this study indicated that the wild species, *C. palaestinus* and *C. lanatus*, are potential sources of resistance to ALS which could be successfully introgressed into susceptible cultivated species. Furthermore, the resistance contributing alleles from these wild species could be pooled in a single genetic background. The six resistant inbred lines provide a unique source of resistance to ALS for safflower breeding. Multiple crossing among the resistant inbred lines might accumulate more resistant alleles without the problem of introgression of undesirable traits of wild species since these inbred lines resembled *C. tinctorius*. To accumulate more and more resistant alleles in one genetic background, development of a population using the resistant and even the moderately resistant inbred lines is suggested for developing inbred lines possessing broad ALS-resistant genetic base from the population.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. Each author has participated sufficiently in the work to take responsibility for appropriate portions of the content. The article has been scrutinized critically by our institute's publication committee with respect to authenticity, accuracy and integrity of research work, contribution of each author and conflict of interests among the authors and others. The authors have agreed to be accountable for all aspects of the work.

AUTHOR CONTRIBUTIONS

A. Kammili has conceived the idea and involved in *Alternaria* resistance breeding activities, cytogenetic studies and *Alternaria* data recording in the field as well using Windias Leaf Image Analysis System in the laboratory from 2003 to 2015 and still maintaining the resistant and moderately resistant inbred lines. M. Pallavi and P. Bhavna have helped the senior author in *Alternaria* data recording in the field during 2004–2006 and 2011–2013, respectively. R. D. Prasad has screened the material against *Alternaria* using detached leaf technique under in vitro conditions and guided the senior author in Alternaria data recording in the field during various years and C. Sarada has involved in making box plots presented in Figure 3.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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