Screening Wild *Brassica* Species Against *Alternaria Brassicicola* (Schw.) Wiltsh for Breeding *Alternaria* Leaf Spot Resistance in *Brassica* Vegetables

Shrawan Singh (singh.shrawan@yahoo.com)
Indian Agricultural Research Institute
Sushma Sagar
Indian Agricultural Research Institute
Mahesh Rao
National Institute of Plant Biotechnology
Sandhya Saroha
Indian Agricultural Research Institute
Pooja Garg
National Institute of Plant Biotechnology
Lakshman Prasad
Indian Agricultural Research Institute

Research Article

Keywords: Alternaria leaf spot, Wild relatives, Detached leaf inoculation, Breeding strategies.

Posted Date: March 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1375909/v1

Abstract

Alternaria leaf spot (ALS) is a major disease of Brassica crops, and it causes huge economic losses to both the cultivated oilseed-and vegetable- Brassicas . The present study was aimed to develop a non-destructive robust method for screening of wild Brassica species and to find resistant wild species against Alternaria brassicicola (Schw.) Wiltsh available in the germplasm. For this, 38 wild Brassica species were screened at adult plant stage by an in vitro detached leaf inoculation method in three consecutive years i.e. 2019-20, 2020-21 and 2021-22. The new screening protocol provides favourable environment (temperature 25±2 °C; relative humidity >90%) for the pathogen and retained the host leaves in condition (by placing sucrose 5% w/v in petiolar base) for disease development. The consistency in reactions of the species against A. brassicicola during all three years of testing indicates the robustness of the protocol. Further, the multiple parameters were recorded on leaf condition and disease response of the wild species. Complete resistance was observed in Capsella while resistance in Lepidium , Camelina and Biscutella . Both Capsella bursa pastoris (L.) Medik (early) and C. bursa pastoris (late) were symptomless resistant. Camelina sativa (L.) Crantz, Diplotaxis erucoides (L.) DC and Diplotaxis gomez-campoi Mart.-Laborde were found to be resistant against A. brassicicola . The significant correlation between disease parameters indicates the robustness and effectiveness of the screening protocol for wild Brassica species.

Introduction

Alternaria leaf spot of crucifers incited by *Alternaria brassicae* (Berk.) Sacc., *A. brassicicola* (Schw.) Wiltsh., *A. raphani* Groves & Skolko, and *A. alternata* (Fr.) Kreissler. *Alternaria* pathogens are prolific toxin producers that contribute to their pathogenicity and necrotrophic habit (Lawrence et al. 2008). This is also known as *Alternaria* black spot (Brazauskiene et al. 2011) or *Alternaria* blight (Kumar et al. 2014; Meena et al. 2004; Prasad and Vishunavat, 2006) or *Alternaria* curd blight (Sharma et al. 1995). It is one of the major bottlenecks in the global production of oilseed and vegetable *Brassicas*. In oilseed *Brassicas*, *A. brassicae* is the dominant invasive species, while in the *Brassica* vegetables, both species, *A. brassicae* and *A. brassicicola* cause damage. Later occurs mostly at higher temperatures (20-30 ℃) while the incidence of *A. brassicae* appears more at moderate temperature (18-24 ℃) (Nowicki et al. 2012). The pathogen mycelium is septate and always multi-celled. The conidia of *A. brassicae* have prominent beak while it remains rudimentary in *A. brassicicola*. *Alternaria* leaf spot pathogens are aggressive and seed-borne. Since spores remain on the seed coat or mycelium below the seed coat (Prasad and Vishunavat, 2004; Ansar and Ghatak, 2018). It survives on alternate hosts such as *Anagallis arvensis* and *Convolvulus arvensis* and in crops or organic manures. The spores spread through wind, rain-splash and mechanical means and cause symptoms (MacKinnon et al. 1999). The fungi affect all plants' aerial parts, such as leaves, pods, seeds, fruits, and stems. The pathogen makes small to large black concentric ring spots which turn brown and black on leaves. Such spots on the petiolar region can cause the breakdown of leaves, thus impacting the photosynthetic area of the plant. It transmits to edible portion 'curd' of cauliflower and causes curd blight. It causes head rot of cabbage and broccoli, thereby deteriorating the marketable acceptance and eating quality. The use of fungicides at the curding/heading stage leads to chemical residues in the edible portion, thus harmful to the consumers. At the seed stage, it infects siliqua and turn them black, reduces the number of seeds/siliqua and decreases seed yield by 10% to 70% in oilseed *Brassicas* (Downey and Rimmer, 1993) and up to 55.93% in the seed crop of cauliflower (Prasad and Vishunavat, 2006).

Chemical control of *Alternaria* black spot is effective but it has limitations to use up to vegetative stage in non-organic farming practices. Further, the effectiveness and large-scale adoption of biocontrol agents are also doubtful and have limited in use (Ahmad and Ashraf, 2016). Therefore, the use of resistant varieties is the only environmentally benign and consumer-friendly measure to control plant diseases. However, breeding resistant varieties require resistant genotypes/genes to use in conventional or transgenic breeding approaches.

far, no resistance source has been reported for *Alternaria* leaf spot in cultivated *Brassica* species except for some genotypes having moderate level resistance (Singh et al. 2014). More distant wild relatives of *Brassica* namely *Camelina sativa* (L.) Crantz, *Capsella bursa-pastrois*, *Eruca sativa*, *Sinapis alba* L. and *Neslia paniculata* were reported to highly resistant to *Alternaria brassicae* (Conn et al. 1988; Tewari and Conn 1993; Hansen and Earle, 1997; Sharma et al. 2002). Westman et al. (1999) found *Alliaria petiolata*, *Barbarea vulgaris*, *Camelina sativa*, *Capsella bursa-pastoris*, *C. rubella*, *C. grandiflora* and *Erysium cheiranthoides* as resistant to *A. brassicicola*, however, the intra-species and regional variations were also explained in most of these species except *A. petiolata*, *B. vulgaris* and *C. rubella*.

The collection and conservation of wild species germplasm remain a tedious task particularly in genebanks. It becomes more challenging while screening against seed-borne pathogen such as *A. brassicicola*. This is more crucial for maintaining susceptible species which get killed by the pathogen during the screening process. Also, the pathogen contaminates the seeds of moderate resistant species and makes seeds unfit for conservation or share with other genebanks (s) due to quarantine requirements. Further, these wild species differ in their climatic requirement, plant stature, leaf shape and size, plant growth and development stages, hence their reaction against a pathogen may vary greatly during field screening conditions. Singh et al. (2014) screened germplasm of six *Brassica* species in field condition against *A. brassicae* and Meena et al. (2016) developed a rapid technique for screening against this pathogen at cotyledon stage using *B. juncea*. Deep and Sharma (2012) attempted detached leaf screening method for seedling stage of cauliflower (*B. oleracea* var. *botrytis*), Doullah et al. (2006) in *B. rapa* and Doullah et al. (2016) in cultigans of cultivated *Brassicas*. Sharma et al. (2002) first time showed the effectiveness of detached leaf screening using 38 wild *Brassica* species against *A. brassicae*. These methods were confined to cotyledon or seedling or young stages ((Sharma et al, 2002; Doullah et al., 2006; Deep and Sharma, 2012; Meena et al. 2016) while intensity of disease increases with plants age as reported by Deep and Sharma (2012) in cauliflower and Doullah et al. (2016) in cultivated *Brassica* cultigans, *Camelina sativa* and *Sinapsis alba*. Thus, a robust non-destructive *in vitro* screening protocol for adult plant stage was required for wild *Brassica* species against *A. brassicicola*.

The absence of ALS resistance in the *Brassica* genus invite attention for exploring the wild relative species in *Brassicaceae*, since the new techniques have been evolved to facilitate introgression of genes/QTLs from wild species through embryo rescue followed by backcrossing, bridge species, somatic hybridization (Ryschka et al. 1996; Hansen and Earle, 1997; Kumari et al., 2018) or use of transgenic approach (Mondal et al. 2003).

Since different isolates of *Alternaria brassicicola* cause variable responses during host-pathogen interaction (Shi et al. 2021). However, no attempt has been reported to screen the wild relative species against *Alternaria brassicicola* isolates causing leaf spot and curd blight in cauliflower, particularly in Indian conditions. Therefore, the present study was done to develop a safe, robust and effective screening methods for ALS and also screen the wild species of *Brassicas* against *A. brassicicola* for use in pre-breeding.

Materials And Methods

Raising germplasm in field

A set of 38 species of wild *Brassicas* has grown in 1 m x 1 m plots at the Research field of the National Institute of Plant Biotechnology (NIPB), Pusa, New Delhi, India during the October – February months of each year in 2019-20, 2020-21 and 2021-22. The N:P:K was applied @ 80:40:40 kg/ha. Irrigation was given at weekly intervals and intercultural operations were performed at 30 and 45 days after sowing.

Isolation and purification of *A. brassicicola* isolates from cauliflower

Naturally infected leaves of cauliflower (*Brassica oleracea* var. *botrytis* L.) having typical symptoms of *Alternaria* leaf spot *i.e.* dark leaf spot with concentric rings were collected in October month from cauliflower growing field in Delhi (India) and brought to the laboratory in Division of Vegetable Science, IARI, New Delhi, India. The samples were processed as per the protocol described by Deep and Sharma (2012). In brief, diseased leaf spots with the adjacent healthy area (pieces 0.5-1.0 cm) were surface sterilized by submersion in 70% ethanol for 1 min followed by immersion in 2% Sodium hypochlorite (NaOCl) solution for 30 sec, washing thrice in sterile distilled water and air drying on sterilized filter paper in aseptic condition. The dried leaf samples were then aseptically plated to potato dextrose agar (PDA) medium (3.9% w/v; Himedia Pvt. Ltd.) and incubated at 25±2 °C in biological oxygen demand (BOD) incubator. After 4 days of incubation, each isolate was purified by the hyphal tip method and transferred onto fresh PDA plates. Sporulation was induced by incubating the culture plates of each isolate at 25±2 °C under a 12 h light/dark cycle. The collections were identified morphologically based on the presence of beak as suggested by Corlett and MacLatchy (1996), Pryor and Michallides (2002) and Sharma et al. (2013**)** using a compound microscope (40X) (Ellis, 2001). The single spore culture of all isolates was done and pure culture was transferred into test tubes and kept at at 25±2°C. The four isolates stored at 4°C for further use. Morphological characteristics of the colony and sporulation pattern were determined from single spore colonies. The nature of mycelia growth, colony colour, and shape of conidia was noted. The conidia of *A. brassicae* had long beak (Sharma et al. 2013), *A. brassicicola* had a small or absence of beak. *A. alternata* had 6 to 14 conidial chains in length with abundant secondary and tertiary branches 2 to 8 conidia.

All four isolates were grown on potato dextrose broth (PDB) media in 100 ml capacity conical flask for 7 days at 25 °C in a shaking state on a laboratory shaker. Mycelial mats were harvested by filtering through Whatman™ No.1 filter paper (GE Healthcare UK Limited, Supplier, JP Scientific, India). DNA was extracted according to the modified Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). For this, 200 mg of fungal biomass were ground to powder in liquid nitrogen using mortar and pestle. The fine powder was transferred to 2 ml microcentrifuge tubes containing prewarmed 600 µl CTAB extraction buffer (2%) and incubated at 65 °C in a water bath for 60 min. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v) was mixed and centrifuged (2415 × *g*, 10 min). The upper aqueous phase was transferred to a fresh tube and added an equal volume of Chloroform: isoamyl alcohol (24:1, v/v). mixed well and centrifuged (2415 × *g*, 10 min). The upper aqueous phase was transferred to a fresh tube and added 600 µl of chilled isopropanol and inverted gently for DNA precipitation. It was centrifuged (6708 *× g* for 10 min) and the pellet was washed twice with 500 µl ethanol 70% (v/v) and air-dried at room temperature. The pellet was dissolved in 100 µl 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH- 8.0). DNA extracts were purified by treating with 2-3 µl of 1U of DNase- free RNase for one hour at 37 °C in a water bath. RNase was removed by phenol: chloroform: isoamyl alcohol (25:24:1) as per the extraction step. The extract was treated with 3 M sodium acetate and precipitated in ice-cold isopropanol, centrifuged, dried and dissolved in TE buffer.

Internal transcribed spacer (ITS) region analysis

All the DNA samples obtained from *A. brassicicola* were amplified and characterized using ITS primers namely ITS1 (5-'TCCGTAGGTGAACCTGCGC-3') and ITS4 (5-'TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). The ITS-PCR products (~600bp) were sequenced by Bioserve, Hyderabad. Blast analysis was performed using NCBI database (https://www.ncbi.nlm.nih.gov/) and analysis was done using the database retrieved and sequences of isolates using MEGA software version 5.2 (https://mega.software.informer.com/5.2).

In vitro experimental condition

Full size three healthy leaves were collected randomly from adult stage plants of each wild species for screening against *A. brassicicola.* A complete procedure of detached leaf screening method is depicted in Fig. 1. In brief, the leaves were gently washed under running tap water. The leaves were arranged in a plastic tray (length × width × height = 45 cm × 30 cm × 10 cm). The base of the tray was provided with two layers of moist germination towel paper. The basal end of the leaf petiole was cut with a surgical blade and immediately fixed with a cotton ball wetted with sterilized sucrose solution (5% w/v) to retain the turgidity and freshness of the leaves. Drop inoculation was done at 2-6 spots by placing drop of 10 µl of *A. brasscicola* inoculum (1×10^5 spores ml^{-1})depending upon the leaf size (Sharma et al*.,* 2004). A susceptible genotype of cauliflower 'DC 1667' was used as the positive control and drops of distilled water only as negative control. The inoculated leaves were placed in an incubator at 25±2 °C under 12/12 hours light/dark cycle. Sterile distilled water was added every day from the sides of the tray to keep the towel paper wet and maintain high humidity (>90%) for disease development.

Preparation of conidial suspension

The isolates of *Alternaria brassicicola* were tested for pathogenicity on detached leaves of cauliflower genotypes DC-1667 as per the protocol described by Deep and Sharma (2012). Most virulent isolate ABC-VEG-SS-2 was used for inoculum preparation by the single-spore culture method (Zhang et al. 2013). For this, the isolate was grown on 2% PDA at 25±2°C under 12 h fluorescent light and 12 h dark cycle for 7 days. Plates were gently scraped using a scalpel and spores were suspended in sterilized distilled water. The spore suspension was filtered through two layers of sterile muslin cloth to remove mycelium debris. The concentration of spores in inoculum was adjusted to about 1×10^5 spore ml^{-1} using a hemocytometer by diluting a conidial stock solution with double sterile distilled water (Kolte, 1985; Doullah et al., 2006).

Observations on leaf colour and disease rating

The inoculated leaves were observed for disease parameters at 2, 4 and 6 days post-inoculation (DPI). The parameters were (i) incubation period *i.e.* days to the occurrence of clear symptoms on inoculated spots in each leaf by visual method, (ii) visual observations on leaf colour *i.e.* green, light green (slight chlorotic regions on leaf), yellow-green (leaves turned predominantly yellow with some visible green area), yellow (completely yellow) and yellow decomposed; (iii) diameter of the lesion (cm) using vernier calliper and (iv) cumulative area of leaf covered by all the lesions (cm). From this, a cumulative score of disease was calculated. The disease rating at 6^{th} DPI was taken for final interpretation of disease reaction of the species, since the leaves lost their freshness or turgidity after this 6^{th} day. Additionally, the disease raring of the genotypes was done on a '0-5' scale depending upon lesion diameter (cm) as suggested by Deep and Sharma (2012) (Fig. 2). Earlier scale of Wheeler (1969), Hansen and Earle (1997) and Doullah *et al*. (2006) for *Alternaria* leaf spot in Brassicas were also referred during refinement of the ALS scale in present study. The disease rating scale and lesion diameter were: 0 = no visible infection and conidia remain non-germinated at inoculation site (inoculum droplet diameter was 0.3 cm), 1 score = upto 0.5 cm, 2 score = 0.51- 1.0 cm, 3 score = 1.1-1.5 cm, 4 score = 1.51-2.0 cm area and 5 = > 2.0 cm. The scale is also shown on leaves of wild *Brassica* species (Fig. 2b). From this, per cent disease incidence (PDI) was calculated as: PDI (%) = (Disease rating × Number of spots observed)/(Maximum disease rating × Total number of spots observed) × 100. The species were categorized as immune (PDI=0), resistant (0.1<PDI>10.0), moderately resistant (10.1<PDI>25.0), moderately susceptible (25.1<PDI>50.0), susceptible (50.1<PDI>75.0) and highly susceptible (75.1<PDI>100.0). It was modified from the scale of Hussain and Thakur (1963), Singh et al. (2008) and Dhingra et al. (2013) to ensure consistency in categories.

Overall disease rating

Further, the rating of the genotypes was done according to Sharma et al. (2002). They used a matrix of assigned weightage to the incubation period, leaf area covered by the lesions and lesion size. The genotypes having higher total score were categorized as susceptible while resistant genotypes had the lowest total score. The details of the categories and their respective scores are given in Table 1. The species were categorized into five broad groups on the basis of the PDI (%) as: Highly resistant (HR; 0.0), Resistant (R; 0.1-20.0), Moderately resistant (MR; 20.1-40.0), Moderately susceptible (MS; 40.1-60.0), Susceptible (S; 60.1-80.0) and Highly susceptible (HS; 80.1-100). The mean value of three consecutive years was used for the categorization of the genotypes for their reaction against *Alternaria* leaf spot.

Statistical analysis

The mean values obtained from three consecutive years *i.e.* 2019-20, 2020-21 and 2021-22 were used for determining the reaction of 38 wild species and 3 cultivated *B. oleracea* against *A. brassicicola*. Each species showed a consistent reaction to *A. brassicicola* in all three years, therefore, mean values were used in analysis of the data. The analysis of variance, standard deviation and correlation were analysed using online OPSTAT software (http://14.139.232.166/opstat/).

Results

Microscopic characterization of *A. brassicicola* isolates

Differences in the conidia of all four *A. brassicicola* isolates were observed to have small, obpyriform, septate, brown-colored spores in the chain having small beak or no beak. Conidia were seen as small, oblong to obpyriform, septate, brown-colored forming in chain (5-6 conidia) having no beak (Fig. 3). More than one species might have infected the plants since different kinds of conidia are clearly visible. However, variaions in shape and size of conidia were observed which could be due to different stages of conidia. Colonies of *A. brassicicola* tend to be dark brown while *A. alternata* seems black in colour. In the case of *A. alternata*, single septate conidia were observed (Fig. 3e).

Molecular characterization of *A. brassicicola* isolates

Characterization of four *A. brassicicola* isolates using ITS1 and ITS4 confirmed the isolates to be of *Alternaria* species (Fig. 4a). The sequence information is given in Fig. S1. Blast analysis using online database available at National Centre for Biotechnology Information (NCBI) and >95% phylogeny between database retrieved and sequences of isolates using MEGA software version 5.2 confirmed the species. All the ITS sequences were submitted to the NCBI database (Table 2). The phylogenetic tree (Fig. 4b) formed two distinct clusters of *Alternaria spp.* isolates. Clade -1 contains three isolates of *Alternaria brassicicola* and Clade -2 contains *Alternaria alternata*.

Leaf colour and incubation period

Leaves of all the 38 species were green and healthy at '0' day of inoculation (DPI), while they turned light green to yellow on 4^{th} DPI. Thirteen species turned completely yellow and showed decaying on 6^{th} DPI (Fig. 5). The decaying leaves lost their colour, turgidity and initiated rotting symptoms. The earliest

yellowing was observed in *Lepidium sativum* on 2nd DPI. *Capsella bursa-pastoris*(early), *C. bursa-pastoris*(late) and *Camelina sativa* turned yellow on 4th DPI, however, both did not show infection and conidia remain non-germinated at the point of inoculation.Decaying of leaves was noticed in *Brassica oxyrrhina* (Coss.) Willk., *Enarthrocarpus lyratus* (Forssk.) DC., *Lepidium sativum* L., *Crambe. abyssinica* Hochst. (EC694159) and *Eruca sativa* Mill. (IC62597) on 4th day of inoculation.

The tested species showed considerable variation in the incubation period of ALS, the clear disease symptoms appeared on 2nd day post-inoculation except in *Camelina sativa* (L.) Crantz, *Capsella bursa pastoris* (L.) Medik.(early), *C. bursa pastoris* (L.) Medik. (late), *Diplotaxis gomez-campoi* Mart.-Laborde and *Biscutella didyma* L. *Capsella bursa pastoris* (L.) Medik.(early) and *C. bursa pastoris* (L.) Medik. (late) didn't show infection till 8th day post-inoculation.

Lesion size and changes in post-inoculation period

Overall, the lesion diameter in *Brassica* wild species was ranged from 0.0 to 2.9 cm, 0.0-2.60 cm and 0.0 to 2.63 cm in 2019-20, 2020-21 and 2021-22, respectively. In all three years, the disease ratings of the Cole vegetables (*i.e.* cauliflower, cabbage and broccoli) were recorded in a very high range from 2.80 to 3.3.0 cm for cauliflower 'DC 1667', 2.4 to 3.0 cm for cabbage 'PA-1' and 2.6 to 3.4 cm for broccoli 'DC-Brocco-10'.

The tested species had a significant variation for change in the size of inoculated spots (Fig. 6). No germination of the pathogen was observed in *Capsella bursa-pastoris* (early) and *C. bursa pastoris* (L.) Medik (late). The progress of lesion diameter was lowest in *Biscutella didyma*, *Lepidium sativum* and *Camelina sativa* (L.) Crantz in the same set of screening conditions. The change in lesion diameter over the inoculation spot was highest in *Crambe abyssinica* Hochst. (EC694147) (8.05-fold) followed by *C. abyssinica* Hochst. (EC400058) (6.12-fold), *C. abyssinica* Hochst. (5.54-fold) (Fig. 5). *Brassica oleracea* (cauliflower, cabbage and broccoli) showed clear symptoms after 2nd day post-inoculation and absolute fold change was relatively high *i.e.* 11.66, 10.54 and 9.30, respectively.

Disease rating of *Brassica* wild species and per cent disease incidence

The observations on disease rating of 38 wild species against *A. brassicicola* are presented in Table 4. It was ranged from 0.0 in *Capsella bursa pastoris* to 4.7 in *E. sativa* Mill. (IC341907). Two species *C. bursa pastoris* (Early) and *C. bursa pastoris* (Late) were grouped as highly resistant (DS=0.0) while four species were in resistant category *Diplotaxis erucoides* (L.) DC., *Camelina sativa* (L.) Crantz, *Biscutella didyma* L. and *D. gomez-campoi* Mart.-Laborde. On the basis of PDI value, both *Capsella* species were grouped as highly resistant or immune and all the above four species and *Lepidium sativum* L. were grouped as resistant. Both scales (i.e. disease rating on 0-5 scale or PDI value of 0-100) effectively identified resistant reaction of *Capsella bursa-pastoris* (early) and *C. bursa-pastoris* (late) against *Alternaria* leaf spot disease.

Overall disease rating for *A. brassicicola* reaction

The results of overall rating of wild *Brassica* species on the basis of cumulative values of leaf colour, incubation period, lesion size, leaf area covered by lesion are presented in Table 3. Among the species, *Capsella bursa-pastoris* (early) showed complete resistance against *A. brassicicola*. *Camelina sativa* (L.) Crantz, *Diplotaxis erucoides* (L.) DC. and *Diplotaxis gomez-campoi* Mart.-Labordewere also scored in the resistant category. *Diplotaxis catholica* (Delile) Thell, *Brassica cretica* Lam, *Lepidium sativum* L. and *Sinapis alba* L. and were moderate resistant. Among the tested wild *Brassica* genera, *Capsella*, *Lepidium*, *Camelina* and *Biscutella* showed resistance against *A. brassicicola* with low cumulative disease score and per cent disease incidence (Fig. 7).

Correlation analysis

The correlation analyses between mean values of six parameters observed during 2019, 2020, and 2021 are given in Table 4. Significant correlation was found between leaf colour, disease rating (r^2=0.315) and total disease score (r^2=0.884). Non-significant correlations were observed between incubation period and disease rating, lesion diameter and cumulative disease score. Cumulative disease score had a positive significant correlation with disease rating score (r^2=0.928) and per cent disease incidence (r^2=0.903).

Discussion

Alternaria leaf spot is a serious problem in both the main crop (*i.e.* harvest stage) and seed crop of *Brassica* vegetables (Prasad and Vishnuvat, 2006). It causes huge economic losses to the farmers by attacking the crops at seedling stage, vegetative stage, curding/heading stage (economic harvest) and seed crop in case of vegetable *Brassicas* (Singh and Kalia, 2021). Since, some of the members of *Brassica* coenospecies and wild species of *Brassicaceae* have been reported as resistant against *A. brassicae* (Sharma et al. 2002) and *A. brassicicola* (Westman and Dickson 1999) through *in vivo* and *in vitro* screening studies. However, there was need to devise a proper protocol from isolation of prevalent isolate of the pathogen (*i.e. A. brassicicola*) from the region to categorize the species using a proper scale. Further, almost screening practices were confined to the cotyledon stage (Meena et al. 2016) and seedling or young stage of the plants (Sharma et al. 2002; 2004) while it is important to know the level of resistance at adult stage. Sharma et al. (2004) reported that the level of resistance against *A. brassicicola* in cauliflower decreased from seedling to young and adult stages. Similar cases were also reported in case of downy mildew (*Hyaloperonospora parasitica*) in broccoli (*B. oleracea* var. *italica* L.) (Dickson and Petzoldt, 1993) and cauliflower (Singh et al. 2013). Thus, the screening of wild *Brassicas* at full-grown adult stage was more appropriate for age-independent resistant sources for breeding use unless the resistance is known to be stable across the plant age.

The *A. brassicicola* isolate from cauliflower was used to screen the wild *Brassica* species. During the survey of the prevalent species of *Alternaria* in cauliflower in 30 random samples from leaf, curds and siliqua in Delhi region, it was observed that *A. brassicicola* was the most prevalent while infection of *A. brassicae* and *A. alternata*. The identification of the species was done on the basis of conidia morphology and sequence analysis. A similar approach was also followed by Sharma et al. (2002) while screening *Brassica* coenospecies against *A. brassicae*. Since, variation of host response against the pathogen also depends upon the isolates. Shi et al. (2021) tested 175 isolates of *Alternaria* and tested their pathogenicity by detached leaves on Chinese cabbage with the disease incidence and disease index ranging from 73.3% to 100.0%.

Further, leaf epicuticular wax is reported to have role in plant defence particularly by creating a hybrophobic surface that decrease the reaction of water-borne inoculum, reducing germination of conidia and causing fewer germ tubes to be produced (Conn and Tewari, 1989). Thus, in present study, the wax layer of the leaves was kept intact while screening the genotypes.

Since, leaf colour and turgidity are important indicators for freshness, thus, both were monitored during the screening process. A moist cotton ball (5% sucrose solution) was effective to retain the leaf of most of the species turgid till 6^{th} day of inoculation, however, the chlorosis was independent of this supplement. The *A. brassicicola* is a necrotroph and it can grow on yellow or dead leaf tissues. Thus, reaction of the species was not affected by the change in colour, however, occurrence of other saprophytes on decaying leaves influence lesion progression and observations.

Sharma et al. (2002) suggested for a cumulative score from observation on incubation period, disease rating and lesion size. While, Sharma et al. (2004; 2012), Singh et al. (2008), and Meena et al. (20016) used disease rating or PDI for categorization of genotypes in different disease reaction groups. Significant correlation between disease rating and PDI (r^2= 0.903) and cumulative disease score (r^2=0.928) indicates for the effectiveness of different disease parameters.

Change in lesion size is an important indicator of genotype response to the host-pathogen (Sharma et al. 2002; 2004). Significant variation among the species for increase of the lesion diameter on 6^{th} day post-inoculation indicates that these species have some resistance mechanism that is activated following infection and inhibits or slow down the growth of fungus as also indicated by Sharma et al. (2002) while screening *Brassica* coenospecies against *A. brassicae*.

The appearance of observable symptoms after inoculation of leaves with *A. brassicicola* is an important indicator at the initial phase of host-pathogen interaction. There was significant variation among species for the incubation period during *in vitro* challenge inoculation which could be due to role of defence substances such as camalexim produced during the germination and establishment of the pathogen (Browne et al. 1991).

Vishwanath and Kolte (1999) reported that detached true leaf inoculation as the most efficient and reliable method for host screening for resistance to *Alternaria brassicae* in rapeseed mustard. It has no interference from local growing conditions (Hong et al., 1996; Scholze, 2002; Shrestha et al. 2005), the suboptimal developmental stage upon pathogen incidence, or presence of other strains and or pathogens under natural epidemiological conditions (Michereff et al. 2012) and length of growing period (Sharma et al. 2002). Nowakowska et al. (2016) also in the view that this procedure is equally effective as of seedling stage screening to discriminate resistant and susceptible genotypes. It has been validated in different crops *i.e.* oilseed *Brassicas* (Vishawanath et al., 1999; Meena et al., 2016) and wild *Brassicas* (Sharma et al. 2002), cabbage and cauliflower (Sharma et al., 2004). This reduces the chance of variation in disease rating in field conditions which could arise due to differences in growing and infection conditions. Further, we suggest the detached leaf inoculation method for screening wild *Brassica* species for reasons: (i) it is always better to do primary screening against a pathogen which have a wide host range in fully controlled condition, (ii) it is an effective way to screen large germplasm with limited resources available, (iii) avoid contamination of germplasm since it is a seed-borne disease, (iv) it avoids the escape of pathogen in a new environment, (v) field screening requires special arrangements to contain the pathogen, (vi) growing wild species in field condition may disastrous since there are possibilities to escape and became a weed or may contaminate/pollinate the common *Brassica* crops or minor *Brassica* weed species in the region and (v) Doula et al. (2006) reported a significant positive correlation between *Alternaria* leaf spot disease rating in detached leaf test and seedling test.

Only moderate resistance was reported in cauliflower by Nowakiwaska et al. (2016), in red cabbage (PI291998) and cauliflower (PI291565 and PI441510) and broccoli (IHRGRU04, 003571 and IHRGRU04,004712), however, dealing with such devastating necrotrophic parthogen, it there is urgent need to find stable and robust resistance source(s). Besides, in vegetable *Brassicas*, the most devastating impact is during their economic maturity (*i.e.* edible parts) since infection during this phase directly affect the market price and consumer acceptance. No robust donor sources reported so far in the primary gene pool (GP1) of cultivated *Brassicas*, hence it is imperative to search the available germplasm of wild relatives (GP2 and GP3) of *Brassicaceae* for use in resistance breeding. Buchwaldt and Green (1992) screened 19 species of *Brassicaceae* against A. brassicase and its potential phytotoxin destruxin. Later, Sharma et al. (2002) screened 38 species of *Brassica* coenospecies against *A. brassicae* and identified *B. desnottessi*, *Camelia sativa*, *Coincya pseuderucastrum*, *Diplotaxis berthautii*, *D. catholica*, *D. cretica*, *D. erucoides* and *Erucastrum gallicum* as resistant. In present study, *Capsella bursa pastoris* (early) and *Capsella bursa pastoris* (late) showed symptomless resistance during all three years. The resistance in *Camelia sativa* and *D. erucoides* against *A. brassicae* (Sharma et al. 2002) and *A. brassicicola* as observed in present study indicates their broader prospect in resistance breeding.

Symptomless resistance was observed in both genotypes of *Capsella bursa-pastoris* during 2019-20, 2020-21 and 2021-22 years. The observations are in agreement with the findings of Conn et al. (1988) and Buchwaldt and Green (1992) who had reported resistance in *C. bursa-pastoris*, *Camelina sativa*, *D. erucoides*, *Sinapis alba* and *Thilaspi arvense* against *A. brassiciae*. Sharma et al. (2002) also reported resistance in wild relatives of *Brassica*s namely *B. maurorum* (Chrungu et al., 1999), *B. desnottesii*, *Coincya pseuderucastrum*, *Diplotaxis berthautii*, *D. catholica*, *D. cretacea*, *D. erucoides* and *Erucastrum gallicum*. Westman and Dickson (1999) identified *Camelia sativa* and *Capsella bursa-pastoris* as highly resistant to *Alternariabrassicicola*. Few members of

Brassica coenospecies *viz*., *C. sativa*, *S. alba* and *D. berthautti* were also have been found to be resistant against *A. brassicae*, to another major pathogen of *Alternaria* leaf spot (Sharma et al. 2002). The durable resistance in these wild species to *Alternaria* leaf spot could be attributed to the co-evolution of resistance since these species remain in wild or weed state and adapted to prevalent biotic and abiotic stresses. Significant correlation among the observations from all three years indicates the robustness of the screening procedure. A poor correlation between leaf colour and disease rating supports the necrotrophic habit of the *A. brassicicola* which multiplies on dead tissues also.

Conclusion

Two wild *Brassica* species namely *Capsella bursa-partosis* (early) and *Biscutella didyma* were found to be completely free from *Alternaria brassicicola*. Although, high level of resistance was also observed in *Capsella bursa pastoris* (late), *Diplotaxis assergens*, *D. gomez-campoi* and *D. muralis*. This pool of resistance sources provides an opportunity for breeders to select the source of resistance to use in a pre-breeding for introgression of ALS resistance in *Brassica* vegetables. A novel disease scale and a non-destructive, fast and easy method of *in vitro* detached leaf screening for screening of ALS in wild *Brassicas*.

Declarations

Acknowledgments

The authors are grateful to DST-SERB for financial support for the project 'Mapping and marker assisted breeding for *Alternaria* leaf spot resistance in Indian cauliflower (ECR/2017/003136) and the director, IARI, New Delhi for facilities.

Conflict of interest

Authors declare no conflict of interest.

Contribution

SS1 conceptualization, supervision and draft preparation; SS2 performed screening experiment and draft preparation; MR maintained and provided wild *Brassica* species and edited manuscript; SS3 and PG managed plants and brought samples; LP helped in inoculum preparation and edited manuscript.

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Tables

Table 1. Scoring pattern for categorization of disease reaction of Wild Brassica species against *Alternaria brassicicola*.

Trait	Disease rating pattern	Remark
Incubation period (DPI) (max. score = 10) *(1)*	No disease upto 8th DPI (0), upto 6th (2), upto 5th (4), upto 4th (6), upto 3rd DPI (8), upto 2nd DPI (10). (Observed everyday till 8th day post inoculation)	Modification of Sharma et al. (2002)
Leaf colour (visual) (max. score = 10) *(2)*	Green (0), Intermediate (5), Yellow (10)	New addition
Average lesion size (cm) (max. score – 30) *(3)*	No infection (0), 0.1-0.5 cm (6), 0.51-1.0 cm (12), 1.1-1.5 cm (18), 1.6-2.0 cm (24), >2.0 cm (30)	Modification of Sharma et al. (2002)
Cumulative leaf area covered by all inoculated lesions on a leaf (cm) (max. score = 50) *(4)*	No infection (0), 0.1-3.0 cm (10), 3.1-6.0 cm (20), 6.1-9.0 cm (30), 9.1-12.0 cm (40), >12.0 cm (50)	Modification of Sharma et al. (2002)
Cumulative score (DPI) (max score=100) *(1+2+3+4)*	Highly resistant (0.0), Resistant (0.1-20.0), Moderately resistant (20.1-40.0), Moderately susceptible (40.1-60.0), Susceptible (60.1-80.0) and Highly susceptible (80.1-100).	Modification of Sharma et al. (2002)

Table 2. The accession number of *A. brassicicola* isolates (submitted to NCBI)

Seq. Name	Organism	Identity (%)	Acc. No.	Origin
IARI-VEG-SS-1	*A. brassicicola*	98.68	MW365462	India (IARI)
IARI- VEG-SS-2	*A. brassicicola*	100	MW365463	Iran
IARI- VEG-SS-3	*A. brassicicola*	100	MW365464	India (IARI)
IARI- VEG-SS-4	*A. alternata*	99.61	MW365465	India (IARI)

Table 3. Mean performance of wild *Brassica* species against *Alternaria brassicicola* during 2019-20, 2020-21 and 2021-22.

Wild species code	Code	Disease rating score (0-5)	Per cent disease incidence %)	Leaf colour score (Max. 10)	Incubation period (Max. 10)	Cumulative leaf area covered by all inoculated lesions on a leaf (Max. 50)	Average lesion size (Max. 30)	Total score (Max. 100)	Overall disease rating (0-5 score)
				1	2	3	4	(1+2+3+4)	
Brasscia fruticulosa Cirillo-1	WS-1	3.5±0.50	70.0±8.2	8.7±1.15	8.0±0.00	40.0±0.00	22.0±9.17	78.7±9.45	S
B. fruticulose Cirillo-2	WS-2	3.3±0.29	60.0±.8.2	5.3±2.31	8.0±0.00	40.0±0.00	22.0±9.17	77.3±11.72	S
B. oxyrrhina (Coss.) Willk.	WS-3	4.5±0.50	82.5±3.4	9.3±1.15	8.7±1.15	43.3±5.77	22.0±3.46	84.0±7.21	HS
B. tournefortii (RBT 2002)	WS-4	3.2±0.35	64.0±5.7	4.0±0.00	7.3±1.15	36.7±5.77	16.7±	68.0±13.11	S
B. tournefortii (RBT 2003)	WS-5	3.5±0.50	60.5±13.8	4.7±1.15	7.3±1.15	36.7±5.77	14.0±3.46	65.3±2.31	S
Camelina sativa (L.) Crantz	WS-6	1.0±0.00	6.1±2.7	6.7±2.31	1.3±1.15	3.3±5.77	2.0±3.46	16.0±8.72	R
Capsella bursa pastoris (L.) Medik.(early)	WS-7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HR
C. bursa pastoris (L.) Medik. (late)	WS-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HR
Diplotaxis assurgens (Delile) Thell	WS-9	2.5±0.50	46.7±4.7	1.3±1.15	5.3±1.15	26.7±5.77	22.0±3.46	62.7±11.02	S
D. catholica (Delile) Thell	WS-10	2.2±0.29	33.3±6.2	4.7±1.15	4.7±1.15	10.0±0.00	6.0±0.00	29.3±2.31	MR
Brassica cretica Lam	WS-11	2.5±0.87	38.8±9.8	0.7±1.15	6.7±1.15	13.3±5.77	8.0±3.46	36.7±5.03	MR
Diplotaxis erucoides (L.) DC.	WS-12	0.7±0.58	10.8±8.2	4.7±1.15	3.3±1.15	7.3±	6.0±0.00	26.0±3.46	R
Diplotaxis gomez-campoi Mart.-Laborde	WS-13	1.0±0.00	10.3±7.6	0.7±1.15	1.3±1.15	10.0±0.00	6.0±0.00	24.7±1.15	R
D. muralis (L.) DC.	WS-14	2.7±0.76	45.8±10.1	4.0±0.00	6.7±1.15	33.3±5.77	16.0±3.46	62.7±7.02	S
D. siettiana Maire	WS-15	3.8±0.29	71.8±6.1	2.7±2.31	7.3±1.15	36.7±5.77	20.0±3.46	72.0±9.17	S
D. tenuifolia (L.) DC	WS-16	4.3±0.29	63.3±24.9	4.7±1.15	9.0±	43.3±5.77	20.0±3.46	76.3±5.51	S
D. viminea (L.) DC	WS-17	4.2±0.29	62.4±14.3	5.3±1.15	7.3±1.15	36.7±5.77	22.7±1.15	71.3±6.43	S
Enarthrocarpus lyratus (Forssk.) DC.	WS-18	4.5±0.50	86.2±13.1	5.3±2.31	8.7±1.15	43.3±5.77	20.7±3.06	81.3±7.02	HS
Erucastrum abyssinicum (A.Rich.)O.E.Schulz	WS-19	4.0±0.87	80.0±14.1	0.7±1.15	7.3±1.15	36.7±5.77	24.7±5.77	78.0±12.17	S
E. canariense Webb & Berthel.	WS-20	4.5±0.50	87.3±11.6	4.0±0.00	8.7±1.15	36.7±5.77	28.7±2.31	82.7±5.03	HS
E. cardaminoides (Webb) O.E. Schulz	WS-21	4.2±0.58	68.3±1.2	4.0±0.00	7.3±1.15	36.7±5.77	20.7±2.31	74.0±8.72	S
E. gallicum (Willd.)O. E. Shulz	WS-22	3.2±0.29	60.5±7.6	7.3±1.15	7.3±1.15	28.3±7.64	16.0±3.46	60.3±9.61	MS
Lepidium sativum L.	WS-23	1.5±0.50	5.2±3.4	9.3±1.15	5.3±1.15	10.0±0.00	8.0±3.46	33.3±2.31	MR
Sinapis alba L.	WS-24	3.2±0.58	50.3±14.0	4.7±1.15	7.3±1.15	10.0±0.00	11.3±5.03	36.0±5.29	MR
Crambe abyssinica Hochst. (EC400058)	WS-25	4.0±0.50	70.0±16.3	4.0±0.00	8.0±0.00	40.0±0.00	24.0±6.00	82.0±6.00	S

C. abyssinica Hochst. (EC694071)	WS-26	4.2±0.29	59.4±14.9	0.7±1.15	8.0±0.00	40.0±0.00	20.7±3.06	78.0±4.00	S
C. abyssinica Hochst. (EC694138)	WS-27	4.0±0.50	74.8±15.0	0.7±1.15	4.7±1.15	33.3±5.77	24.0±5.29	70.7±8.33	S
C. abyssinica Hochst. (EC694144)	WS-28	4.2±0.29	52.6±25.9	0.7±1.15	8.7±0.58	43.3±5.77	20.0±8.72	81.3±12.01	S
C. abyssinica Hochst. (EC694145)	WS-29	3.7±0.76	73.3±12.5	4.0±0.00	8.0±0.00	40.0±0.00	22.7±6.11	80.3±5.69	S
C. abyssinica Hochst. (EC694147)	WS-30	4.0±0.00	66.7±18.9	9.3±1.15	7.3±1.15	36.7±5.77	15.3±4.62	69.3±6.11	S
C. abyssinica Hochst. (EC694159)	WS-31	3.7±0.29	61.7±19.3	9.3±1.15	8.0±0.00	40.0±0.00	15.7±9.71	73.7±9.71	S
Eruca sativa Mill. (IC341907)	WS-32	4.2±0.29	80.0±8.2	7.3±1.15	8.0±0.00	40.0±0.00	28.0±3.46	84.0±2.00	HS
E. sativa Mill. (IC341907)	WS-33	4.7±0.58	80.8±15.3	4.0±0.00	9.0±1.00	43.3±5.77	18.7±4.62	80.3±4.73	S
E. sativa Mill. (IC62597)	WS-34	3.7±0.29	73.3±4.7	6.0±0.00	8.0±0.00	40.0±0.00	20.7±7.57	77.3±6.43	S
E. sativa Mill. (IC62599)	WS-35	3.5±0.50	58.6±10.0	6.0±0.00	8.0±0.00	40.0±0.00	16.0±6.93	72.0±8.72	S
E. sativa Mill. (IC62713)	WS-36	3.7±0.29	66.7±4.7	5.3±2.31	8.7±1.15	43.3±5.77	11.3±10.97	72.7±9.50	S
E. sativa Mill. (IC62733)	WS-37	4.0±0.50	80.0±8.2	10.0±0.00	6.7±2.31	33.3±11.55	20.0±8.00	69.3±22.30	S
Biscutella didyma L.	WS-38	0.7±0.58	9.0±7.0	1.3±1.15	2.0±0.00	10.0±0.00	8.0±3.46	22.7±4.62	MR
Cauliflower 'DC-1667'	CFL	5.0±0.00	94.4±7.9	5.3±1.15	8.0±3.46	43.3±5.77	28.0±3.46	88.0±10.0	HS
Cabbage 'PA-1'	CB	5.0±0.00	96.3±5.2	5.3±1.15	8.0±3.46	40.0±0.00	28.7±2.31	85.3±3.06	HS
Broccoli 'DC-Brocco-10'	BRO	5.0±0.00	96.3±2.6	6.0±0.00	8.7±2.31	46.7±5.77	28.0±3.46	92.0±10.39	HS
CD @5%		0.7	10.4	1.9	2.0	7.2	7.8	12.8	

Table 4. Correlation in disease parameters (from mean values of three years) on wild *Brassica* species against *A. brassicicola.*

Observations	Leaf colour score (Max. 10)	Incubation period score (Max. 10)	Leaf area covered by lesion score (Max. 50)	Lesion size score (Max. 30)	Cumulative score (Max. 100)	Per cent disease incidence (%)
Incubation period (Max. 10)	0.315*					
Leaf area covered by lesion score (Max. 50)	0.912**	0.395*				
Lesion size score (Max. 30)	0.915**	0.299NS	0.889**			
Cumulative score (Max. 100)	0.884**	0.198NS	0.772**	0.854**		
Disease rating (0-5 scale)	0.945**	0.298NS	0.900**	0.981**	0.928**	
Per cent disease incidence (%)	0.960**	0.313*	0.857**	0.895**	0.903**	0.937**

*NS- Non-significant, *&** for significant at 1 & 5%, respectively.*

Figures

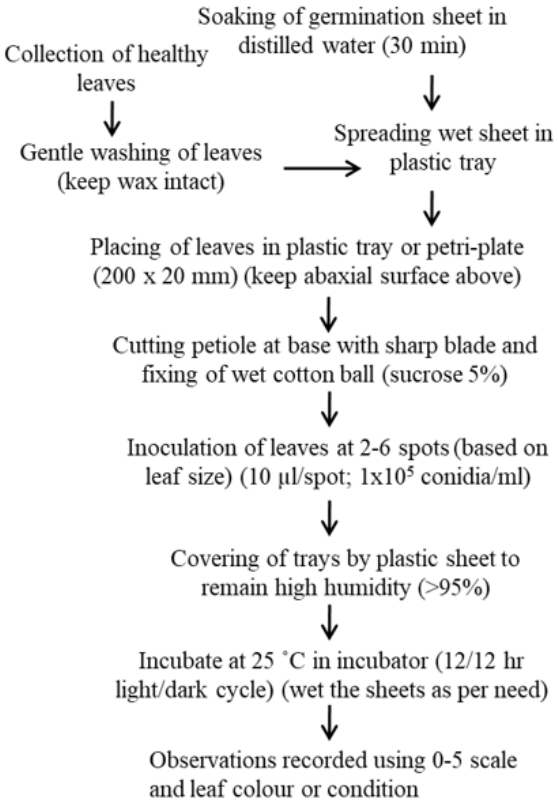

Figure 1

Detached leaf protocol for screening wild *Brassica* species against *Alternaria brassicicola.*

Figure 2

See image above for figure legend.

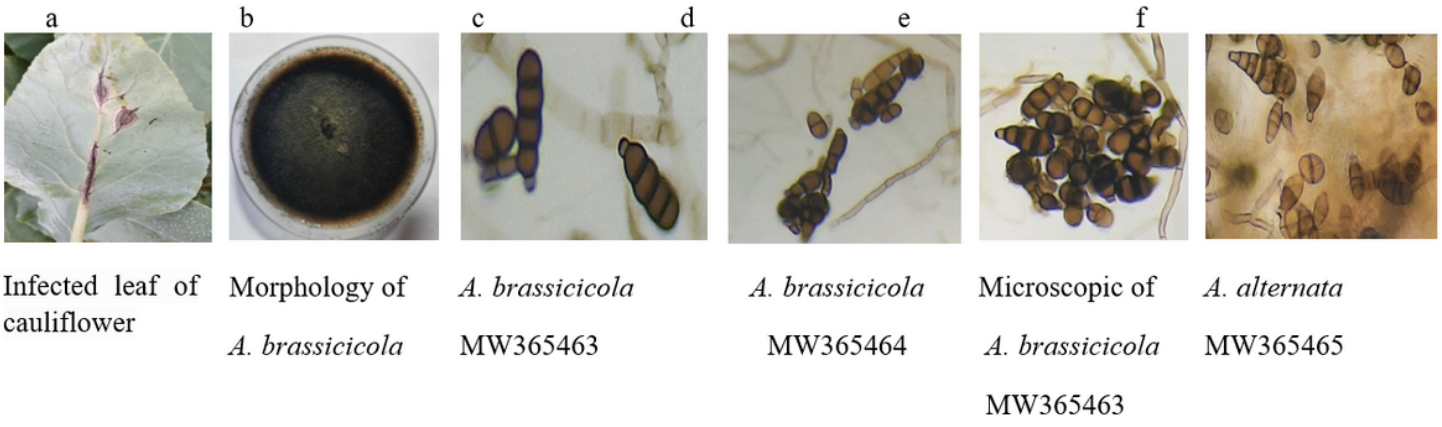

Figure 3

Morphological and microscopically characterization of *A. brassicicola* isolates.

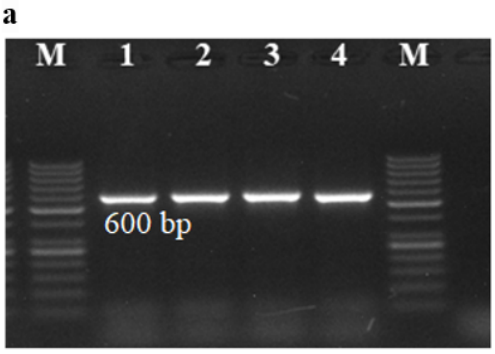

Fig. 4**a**. Amplification of *A. brassicicola* isolates using internal transcribed spacer (ITS) region of the isolates collected from cauliflower. Lane M: 50bp ladder. 1, 2, 3 and 4 are *A. brassicicola* isolates.

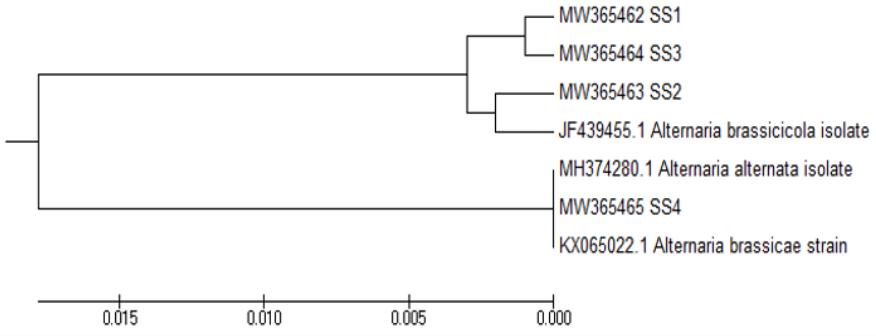

Fig.4b. Dendrogram showing variability in *A. brassicicola* isolates through ITS sequence analysis by MEGA 5.2 software.

Figure 4

See image above for figure legend.

Figure 5

Leaf colour of wild *Brassica* species during *in vitro* screening in detached leaf method.

Figure 6

Absolute fold change in lesion size in wild *Brassica* species after artificial challenge with *A. brassicicola* (mean of 2019-20, 2020-21 and 2021-22).

Figure 7

Variation in disease reaction parameters of different genera of wild *Brassica* species against *A. brassicicola* (mean of 2019-20, 2020-21 and 2021-22).

Supplementary Files

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- ALSSuppl2022.docx