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Review Article
Morphological, Biochemical and Molecular Characterization of *Alternaria brassicicola* and *Alternaria brassicae*: A Comparative Overview

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Abstract

Among different *Brassica*, Indian mustard (*Brassica juncea*), an important and predominant rabi oilseed crop of rapeseed mustard group in India, occupies 90% area of the total hectarage. Mustard encounters number of foliar diseases, among them *Alternaria* blight is the most devastating, causing yield loss up to 35-38%. Chemical properties as well as fatty acid profile govern oil superiority. Besides, the disease undesirably distresses seed quality by reducing seed size, seed discoloration and decline in oil content. In relevance, recently microsatellite locus 'ABS28' has been identified and characterized in terms of cross-transferability to other related and unrelated taxa. Data compiled authenticate that this monomorphic microsatellite marker is greatly particular for *A. brassicicola*, as no magnification signal has been observed from other closely related *Alternaria* species. No false positive or false negative analyses were witnessed with the assays showed the stringency of specific primer set ABS28F and ABS28R, consequently letting precise and sensitive revealing of *A. brassicicola* from cultures and specific plant samples. The assay was noticed to be sensitive, consistently detecting 0.01 ng of genomic DNA per PCR of the target fungi. Thus, marker allows assessing fungal growth from the initial phases of infection, providing a rapid and applied alternative to existing defined markers to differentiate and identify *A. brassicicola* from synchronously occurring fungus, *A. brassicae* and other fungi related to rapeseed mustard. Till date, 14 monomorphic and one polymorphic microsatellite markers have been recognized from the necrotrophic and phytopathogenic fungus *A. brassicicola*. Although, identified polymorphic marker (ABS1) prerequisites to be established to assist a further study on the genetic assembly of natural *A. brassicicola* populations and to match the diversity of isolates initiating from distinct host as well as geographical site. This review provides new insights into further exploration of primer set, ABS28F (50-GCTCCCACTCCTTCCGCGC-30) and ABS28R (50-GGAGGTGGAGTTACCGACAA-30) to probably amplify a more specific amplicon of 380 bp and to prove to be useful as an internal diagnostic marker to differentiate and analyze *A. brassicicola* from *A. brassicae* and other fungi associated with leaves, stem and siliquae of rapeseed-mustard.

Key words: *Alternaria* blight, *Alternaria brassicicola*, *Brassica juncea*, conidia, diagnostic biomarker, host-pathogen interaction

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INTRODUCTION

Brassica is the most economically significant genus of Cruciferae family, which includes oil seed crops like mustard (*Brassica juncea*) and rape (*Brassica campestris*) as well as different vegetables viz., cauliflower *(Brassica oleracea* L. var., *botrytis* L.), cabbage (*B. oleracea* var., *capitata*) consumed all over the world. These crops are heavily infected by *Alternaria brassicae* causing brown to black circular spots on all the above ground parts like leaves, stems, siliquae etc. Among different *Brassica*, Indian mustard (*Brassica juncea*) an important and predominant Rabi oilseed crop of rapeseed mustard group in India occupies 90% area of the total hectarage. Chemical properties as well as fatty acid profile govern oil quality[1]. While studying biochemistry of *Brassica juncea* varieties, like Sarson T42, Lahi T9, Varuna 59 and Rai Pusa Bold more significant alterations in lipid composition of seed oil of Sarson T42 were observed because of infection by *Cuscuta reflexa* (an angiosperm parasite), compared to Lahi T9, whereas Varuna 59 and Rai Pusa Bold varieties showed resistance to infection[1]. Mustard encounters many of foliar diseases, among them *Alternaria* blight is the most devastating, causing yield loss up to 35-38%[2]. Besides, the disease adversely affects seed quality by reducing seed size, seed discoloration and reduction in oil content[3]. The disease also reduces germinability, oil and protein content of seeds. In the absence of resistant donor germplasm of mustard, plant breeding approaches for the development of novel resistant genotypes are still obscure. The most practical way to overcome this situation is to understand inbuilt resistance in the plants. Plants challenged by fungal pathogen exhibit several biochemical defence responses, which include the accumulation of specific metabolites[4].

Among several fungal diseases, severe damage of the foliage or seed germination in crucifers occurs due to *Alternaria* blight caused by *Alternaria brassicae* (Berk)[2,5-7]. Black spot of different crucifers have been reported in many countries; Italy[8], UK, USA and a number of other European countries[9], Canada[10,11], Iran[12] as well as India[13]. Cauliflower (*Brassica oleracea* var., *botrytis*) and mustard (*Brassica juncea*) are two major crucifer crops of India, which are fronting severe yield and quality loss in production consequent to dark leaf spot disease caused by *Alternaria brassicae* (Berk) Sacc[14]. They can survive in seeds for a number of months at different temperatures as well as relative humidity[15,16]. *Alternaria brassicae* reported to cause loss of about 30 and 47% *in* cauliflower (*Brassica oleracea* var., *botrytis*) and Indian mustard (*Brassica juncea*), respectively[17]. Morphological features of conidia and conidiophores and sometimes host plant interaction, facilitate the major taxonomic measures for restriction of fungal species[18]. Though, due to simple and morphological convergence of conidia and facultative parasitism, the taxonomical arrangement of small spored species, as well as host dependent toxin making fungi has been predominantly jumbled results to an ambiguous host range[19]. Taxonomy of *Alternaria* on brassicas is principally based on morphology and interaction with host plant of each of the species occurring (*A. brassicicola, A. brassicae* and *A. raphani*) that has a different morphology with differentiated shape and size of conidium among *Alternaria* species[20]. All profitable cultivars of brassicas are described to have susceptibility to *A. brassicae*[6]. Also, there is not a single report on resistance amongst the crucifers against *A. brassicae.* For developing cultivars resilient to this species, necessitates extensive awareness of pathogen variations existing in crucifers growing in diverse areas. Intensity of *Alternaria* blight on brassicas differs according to seasons and regions and among crops specified to a region. This may be because of presence of changeability amongst isolates of *Alternaria* species. The existence of morphological changeability in the isolates of other *Alternaria* species has been comprehensively reported[7,21]. Besides, reports on morphological variability in *A. brassicae* isolates from different regions of India have been well documented[22-25].

This review has been aimed to reveal compiled data on the evaluation of *A. brassicae* isolates collected from crucifer crops based on morphological, cultural, molecular correlation parameters. Besides, it pertains to potential information on the variability in *Alternaria brassicae* based on the morphological and genetic level and establishment of modern diagnostic techniques for early, precise and speedy detection of *Alternaria brassicae* and biomarkers generation and validation using different bioinformatics tools.

IDENTITY AND BIOLOGY OF THE PATHOGEN

Berkeley[26] recognized the causal fungus on plants of Brassicaceae family as *Macrosporium brassicae* Berk, renamed later as *Alternaria brassicae*. It was suggested that the genus could be systematized into six groups depending on fundamental features of conidia length, width and septation with each group specified by a typical species. Wiltshire[27] founded the elementary studies on hyphomycetes that reflected to be fundamental to the prevailing concepts of *Alternaria, Macrosporium* and *Stemphylium*. Besides, an extensive study pertaining to taxonomy, parasitism and economic importance of this genus was accomplished[28]. Morphological deviations of *Alternaria* species were explained followed by division into three sections and projected a strategy for determination of the most common species[28-35].

Survival: Contrasting the temperate conditions, existence of the pathogen on host plant debris and/or affected seed in tropical or sub-tropical India has been eliminated[36,37]. In India, harvesting of oilseed *Brassicas* generally take place from February-May, while off-season crops are grown in non-traditional areas from May-September, conditionally coupled with harboring of the fungal pathogen by vegetable *Brassica* crops and alternative hosts (*Anagallis arvensis* and *Convolvulus arvensis*) and probably reasoned for transmission of the *A. brassicae* from one crop-season to another[7,36,38].

Host-pathogen interaction: Multi-layered resistance, multi-component-affinity to host dependent toxin dextruxin B (Fig. 1), qualitative and quantifiable expression of phytoalexins, oversensitive response and Ca^{2+} storage regulates providence of host-pathogen interaction[6]. *Alternaria* tolerant *Sinapis alba* has been found to detoxify dextruxin B[39]. Attempts to transfer resistance from wild crucifers to cultivated oil seed *Brassica* were made[40]. Cellulase enzymes[41] and toxins[42] are known to be produced by *A. brassicae*. Though, their particular role in pathogenesis is still obscure. Principally, *A. napiformae* and *A. longipes* have been reported on interact with rapeseed-mustard, especially in India[43]. Signal transduction and expression analysis have been studied in *Alternaria brassicae* interaction[44].

Host-pathogen-environment-interaction: Introductory effort accomplished by researchers specifies effects of temperature, Relative Humidity (RH) and sunshine hours on incidence of the blight on the oil bearing *Brassica*[45-47], describing associations between diverse climate factors and *Alternaria* blight occurrence through experimental models. However, there was a need to predict precisely the age of crop at first advent of the disease and the hazard that epidemic of the blight will occur on the crop to enable decisions to be taken regarding optimum time for spray of fungicide by farmers and to avoid unnecessary pesticide application. Intensity of *Alternaria* blight on leaves[48] and pods[49] was observed rather to be elevated in late seeded crops.

Host resistance: Host resistance is one of the significant ingredients of joined disease management. The regular appearance of this disease with no apparent variability among the released varieties of Indian mustard warrants immediate attention for the control of this disease. Amongst the oil seed *Brassica* species, namely, *B. juncea* and *B. rapa* are more vulnerable to *Alternaria* blight than *B. carinata* and *B. napus*[50,51]. A number of sources of tolerance against

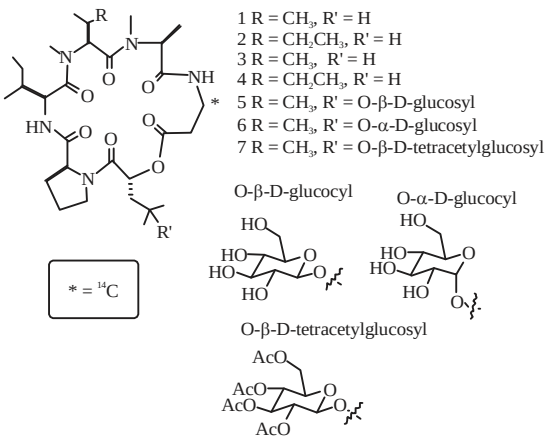

Fig. 1: Chemical structure of dextruxin B

Alternaria blight have been documented[52], including dwarf *B. juncea* cv[53]. Referring to mustard, resistance to *Alternaria* blight has been observed to be connected with leaf enzymes involved in phenolic pathway, e.g., polyphenol oxidase, peroxidase and catalase, higher leaf sugar contents[54]. *Brassica napus*, *B. carinata* and *S. alba* have comparatively more epicuticular wax than *B. rapa* and *B. juncea* and end to be less sensitive to *Alternaria* blight[55]. Higher concentration of phenolics in leaves and lower N[56] has been connected with resistance to *Alternaria* blight that is also known to be linked with features like opposition to conidial retention on plant surface like multiple layering of epicuticular wax forming a physical obstruction as a hydrophobic layer to decrease deposition of water-borne inoculum, decline rate of conidia germination and germ tube formation[56]. Whereas, investigation on machinery of tolerance to *Alternaria* blight has shown influence of polygene or cluster gene[57] with resistance being regulated by nuclear genes of partial dominance, there has also been signal of constituents of resistance being expressively interrelated to each other concerning slow blighting[58] and dominance (h) owing to a predominant role in genetic control of time of expression, additive dominance predominant for other disease development factors viz., AUDPC, etc.[59]. Efforts to breed for resistance for these factors have generated limited success. Since, resistance to *Alternaria* blight and leaf spots are noticed to be regulated by additive or polygenes, breeding for resistance to these diseases could involve pyramiding of minor genes distressing the disease to provide additive polygene resistance against the disease, introgression of genes from material found resistant, reciprocal recurrent selection or di-allele selective mating[57], wide hybridization[21], molecular breeding[60], transgenic expressing *Trichoderma harzianum*

endo-chitinase gene[61] and pollen culture concomitant with sensitivity test to dextruxin B[40]. Speedy developments in techniques concerning tissue culture, protoplast fusion, embryo rescue, genetic engineering have been observed leading transfer of disease resistance traits across wide cross ability obstructions possible. Transgenic disease resistant plants over-expressing diverse antifungal compounds like Pathogenesis-Related (PR) proteins (chitinase, glucanase, osmotin, etc.) and Ribosome Inhibiting Proteins (RIPs) viz., thionins, defensins and phytoalexins[62] to inhibit growth of the pathogen appear less efficacious. Southern analysis of the putative transgenics showed integration of the transgene. Northern and Western analyses evidenced the integrated transgene to be expressed in the transgenics[63]. Microarray studies have directed that different defense signaling pathways converge to form substantial networks, which control and coordinate regulatory interactions[64], inoculating an ecotype of *Arabidopsis thaliana* with *A. brassicicola* (an incompatible interaction) or treated *A. thaliana* with various defense-related signaling molecules, such as Salicylic Acid (SA), Methyl Jasmonate (MJ) and ethylene. Progress in sequencing pathogens or beneficial microflora and the combination of bioinformatics and functional genomics are expected to provide a better understanding of plant-pathogen networks and lead to increase resistance to crop pathogenesis[65]. In a revolution towards study of functional analysis of genes involved in *Alternaria brassicae* interaction, use of linear minimal element is stated to provide high throughput targeted gene disruption[66]. A few expressed sequence tags have been identified in compatible *Alternaria brassicae* interaction[67]. Aminobutyric acid has been documented to induce resistance in *B. juncea* against *A. brassicae*[68].

Biological control: In majority of European countries, organic agriculture has speedily been altered from a farmers' movement to an institutionalized portion of agricultural policy. In accreditation, agreement with published organic standards is corroborated through annual inspections on farms[69]. Spray of soil isolates of *Trichoderma viride* at 45 and 75 days after sowing could administer *Alternaria* blight of Indian mustard (*Brassica juncea*) as efficiently as mancozeb[70], which have been confirmed later in multi-location trials[22]. Botanicals e.g., bulb extract of *Allium sativum has* been described to successfully administer *Alternaria* blight of Indian mustard[71].

Other techniques of *Alternaria* blight administration: Early seeding of well-stored spotless certified seed after deep ploughing, clean crop growing, well-timed weeding and maintenance of optimum plant population, escaping irrigation at flowering and pod formation phases may aid to manage the disease[48]. Though the application of species-group specification does not resolve conclusive species limitations within *Alternaria*, benefits of its use are that it systematizes at the sub-generic level the morphologically diverse assembly of *Alternaria* species and allows the comprehensive discussion of morphologically related species without becoming excessively restricted due to nomenclatural ambiguity. Besides, the species group concept has delivered an important framework for hypothesis testing in cutting-edge studies on *Alternaria* phylogeny.

A comprehensive and comparative account of morphological diversity of certain *Alternaria* species taking place on cucurbitaceous, brassicaceous and solanaceous crops have been described[72-74]. Nevertheless, others had found that on radish leaves, the disease advanced at a temperature range of 15-25°C and 100% relative humidity for 10-12 h[75]. Most of the pathogenic species of *Alternaria* are known to be prolific toxin producers, ultimately facilitating their necrotrophic lifestyle. *Alternaria* produces toxins, likely to be accountable for these lesions. Understanding the mechanism of action, predominantly, Host Specific Toxins (HSTs) offer a better appreciation of host pathogen connections and resistance phenomena. Two methodologies have been employed to study the mode of action of HSTs. A principal one is the investigation of the molecular level concerning host selectivity and the other at the cellular level. It reasons amongst other deviations in chloroplast and mitochondria. Besides, it causes severe infection on most of the family Brassicaceae viz., *Brassica nigra*, *Brassica campestris* Linn, *Brassica oleraceae*, *Raphanus sativus*, *Iberis amara* as a result of which the quality and quantity of crop decreases[36].

Symptoms caused due to *Alternaria brassicae*: Symptoms of the disease have been described by development of spots on stem, leaves as well as siliquae by *A. brassicae*[76], appearing to be generally grey in color rather than black sooty velvety spots created by *A. brassicicola*. Spots created by *A. raphani* appear to be different yellow halos around them. Conversely, the symptoms may vary in accordance to the host as well as environment. Progressively, the disease expresses on middle and upper leaves in the form of smaller sized spots, once defoliation of lower leaves occurs. Subsequently, round black conspicuous spots visualize on stem and siliquae both. These specific spots may combine leading to widespread blackening of siliquae concomitant with wilting of the stem with creation of extended lesions. The infection of *Alternaria* blight on

leaves and silique has been documented to considerably decline the photosynthetic area. The stage of infection on silique unfavorably affects the normal seed development, seed weight, color of seed and percent oil content in seed concomitant with the see quality.

The disease also reduces germinability, oil content and protein content of seeds. Variability in the morphological characteristics in *A. brassicae* isolates of different regions of India have been reported[22-24]. Some researchers have studied on cultural variability in *Alternaria* spp. in respect of mycelial growth and sporulation on different temperature, relative humidity, hydrogen ion concentration and light[77] and media[78]. Variability based on morphology, sporulation, growth and other cultural characteristics have also been reported earlier[23]. Sharma *et al.*[14] evaluated 32 representative Indian geographical isolates of *Alternaria brassicae*, the causal agent of *Alternaria* blight of cauliflower (Vegetable) and rapeseed-mustard (Oilseed). All the isolates showed high level of variability *in vitro* in respect of conidial length, width and number of septa. Conidia of Uttar Pradesh (India) isolate (CaAbU4) were smallest in size with lowest number of septa. Cluster analysis of data on cultural variability among 32 *A. brassicae* isolates found a close relationship among isolates of both origins viz., from cauliflower and mustard. Isolates from Uttar Pradesh, Delhi, Haryana and West Bengal were found to be more similar to each other whereas the Rajasthan isolates along with Tamil Nadu and Kerala isolates were distantly related to others. All the isolates were pathogenic in nature but not directly related to the cultural and the morphological characteristics. These isolates were further molecularly characterized by using internal transcribed spacer region where all the isolates were found 56% similar to each other and 99% similar to the *A. brassicae* isolates present in NCBI database[14].

Deviation among the Indian isolates of *Alternaria brassicae* has also been observed[22]. The morphological and cultural characteristics are not sufficient for pathogen characterization as these are easily influenced by environmental factors and unable to emphasize genetic information of isolates[79]. Polymerase Chain Reaction (PCR) has been widely assessed in the field of mycology for diverse genetic analysis, specific detection, phylogenetic studies for identification and taxonomical demarcation. These molecular studies require a number of laborious steps, including preparation of a DNA template by extraction of DNA from fungi[80]. The omission of the DNA extraction procedure, significantly decreases time and cost and also avoid the risk of contamination during the DNA extraction process. Thus, PCR assay has been employed for molecular diagnostic of any pathogens[81].

Variations at DNA level among *A. brassicae* (Berk.) Sacc., *A. brassicicola* (Schwein) Wiltshire, *A. raphani* Groves and Skolko and *A. alternata* (Fr.) Keissl have been established[82,83] by Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD). Insight has recently been gained into genes being expressed during *Alternaria* infection of *Brassica*[84]. The researcher used suppression subtractive hybridization between RNA isolated from spores of *A. brassicicola* incubated in water and on the leaf surface of an ecotype of *A. thaliana* followed by cloning and sequencing of cDNA clones that were differentially expressed.

In recent years, various molecular tools have been brought in practice for delimitation of fungal taxa, which were designated earlier, based on morphological and host range criteria. The PCR is one of the most popular techniques. It opens a new age in genetic analysis on a molecular level[85]. Primer is a significant component of PCR and its design is basically important in PCR based detection approaches. The general criteria for primers are quite simple[86] but it is difficult to choose primers for a given template sequence. Therefore, the computational aid on primer design is a critical issue in bioinformatics[86]. There are several web-based services or standalone software provided to the public for primer design, such as PRIDE, PRIMER MASTER, PRIMO, primer blast and prime. However, most of them only take a single sequence query. Besides, the calculation of the primer annealing condition is often simplified in a short equation of melting temperature™, regardless of the sequence content of the primer itself.

Methods for the localization of molecular markers such as RFLP, RAPD, Amplified Fragment Length Polymorphism (AFLP) and Sequence Characterized Amplified Regions (SCAR) have been used extensively to localization genes of interest and in molecular-assisted plant breeding[87]. Comparison between the rDNA sequence of *Alternaria linicola* strains[88] or *Alternaria* species pathogenic to crucifers[82] revealed variation in the ITS sequence both in base composition and in length, while there is a high degree of homology among species and strains of *Alternaria*. By analysis of the ITS region in common air borne allergenic fungi, including *A. alternata*, *A. brassicicola*, *A. brassicae*, *A. infectoria*, *A. tennuissima* and *A. rapahni*[89] concluded that the inter-species similarity in ITS1 and ITS2 regions will make the design of probes to distinguish between these genera comparatively straight forward. *A. alternata* could be distinguished from other genera present in tomato products by PCR but the specificity of the primers was not verified against other *Alternaria* species and closely associated genera like *Stemphylium* and *Ulocladium*[90]. The

RAPD analysis has also been brought in practice to study the genetic variation in the genus *Alternaria*[33] among *A. solani* and *A. alternata* isolates from potato as well as tomato[91].

Primers called as Universal Rice Primers (URP) were developed from the repetitive sequences derived from the rice genome and universally have been employed in PCR based genomic DNA fingerprinting of various organisms covering plants, animals and microorganisms[92]. The genotype analysis of *Alternaria brassicicola* was accomplished by microsatellite neutral primers, developed by Avenot *et al.*[93] including 16 pairs of specific primers.

Development of diagnostic biomarker for the rapid documentation of *Alternaria brassicae*: It is very difficult to administer the disease, due to no proven source of resistance described till date in any of the hosts[94]. The yield loss due to this pathogen is about 50% in the entire mustard growing area[13]. One of the noteworthy aspects of biology of an organism is the morphological and physiological features of an individual within a species, which are not fixed. This appears to be factual with fungi too, though it is not is not regular feature in asexually created individuals of the progeny. Changeability studies are imperative to verify the variations happening in populations and individuals as inconsistency in morphological and physiological traits highlight the existence of different pathotypes. *Alternaria* blight severity on oilseed *Brassicas* differ season to season, region to region and individual crop to crop in India[50]. This might be due to the presence of variability among geographically similar isolates of *A. brassicae*. The variability is a well-known phenomenon in genus *Alternaria* and may be observed as changes in spore shape and size, growth and sporulation, pathogenicity, etc. Diversity seems even in single spore isolates. Many reports on the existence of changeability among different *Alternaria* species concerning different hosts have been documented by earlier researchers[35,95-97] as also within *A. brassicae* species[23]. Recently, Meena *et al.*[98] studied the assertiveness, diversity and distribution of *A. brassicae* isolates infecting oilseed *Brassica* in India. Deviation in pathogen populations can usually be identified with the aid of certain approaches like morphological, cultural, pathogenic and molecular specificity.

In a recent report, Goyal *et al.*[25] reported variation in conidial morphology, mycelial growth, sporulation of 13 isolates of *A. brassicae* collected from different geographical zones were dependent on temperature and geographical origin. Similarly, variability in the morphological features in *A. brassicae* isolates of different regions of India has been reported[22-24]. Some researchers have worked on cultural variability in *Alternaria* spp. in respect of mycelial growth and sporulation[23,77,78]. Cauliflower and mustard are winter crops and affected by *A. brassicae*. Along these notions, another very recent study has been noticed to understand the variability among the isolates infecting both the crops grown in different parts of Uttar Pradesh, India based on morphology, cultural and molecular level[99]. Significant variation in growth, sporulation and conidial morphology of *A. brassicae* isolates were found on media irrespective of crop and geographical sites.

Information of variability within pathogen inhabitants has pronounced worth in accepting the disease epidemics, its prediction and determining a particular disease management approach[99,100]. *Alternaria brassicae* is an economically significant pathogen of *Brassica* crops in many parts of the world. Variation in *Alternaria brassicae* has been characterized because of growth, cultural physiognomies, sporulation, morphology and reaction of the commercially cultivated *Brassica* species. Nevertheless, these techniques are time consuming and highly influenced by environment[99].

The DNA markers have been established as a powerful tool to study taxonomy and molecular genetics of a number of organisms. A molecular biology technique such as RFLP has been considered to be worthwhile in detecting variation in fungal species[100], though it RFLP is very expensive, laborious and time consuming. Development of RAPD analysis, based on PCR has attracted considerable attention because of its ease of use and rapid generation of reliable and reproducible DNA fingerprints. This technique has already been employed for detecting variation in various pathogenic fungi. The RAPD permits rapid assessment of genetic changeability and has been brought in practice to study inter- and intra-specific changeability among the isolates of numerous fungal species. Certain reports have been documented concerning the genetic distinction intra- and inter-*Alternaria* spp., through RAPD molecular markers[95,97,101,102].

The genetic linking among the isolates of *A. brassicae* has been effectively analyzed by random primers to create reproducible polymorphisms[99]. All amplified products revealed banding designs that directed the presence of genetic multiplicity. The RAPD has been employed earlier to course inter- and intra-specific genetic distinction in fourteen isolates of *Alternaria brassicae*, which were gathered from different geographical regions of the world[102]. Further, the data obtained by Singh[99] showed genetic divergence among the 21 isolates of *A. brassicae* isolated from mustard. The genetic polymorphism within an *Alternaria* species has been positively studied employing RAPD molecular markers by many researchers[101,102]. Genetic polymorphism among *A. brassicae* isolates from different geographical regions around the world was studied and low intra-regional variation

among Indian and Canadian isolates of *A. brassicae* with 75% resemblance among them was documented[102]. This genetic changeability within an *Alternaria* species might be due to the existence of heterokaryosis, mutation, somatic hybridization, host selection, extensive dispersal or of a cryptic sexual phase.

Genetic changeability was observed among 21 isolates of *A. brassicae* isolated from different cultivars of mustard growing in different parts of Uttar Pradesh, India. This could be the possible reason behind extreme and different disease reactions of genotypes at maximum locations. Several reports are accessible on the genetic variation within and between *Alternaria* species by RAPD molecular marker[95,97,99102]. Correspondingly, RAPD has been employed earlier to investigate inter- and intra-specific variation in 32 *Alternaria brassiae* species, which were isolated from cauliflower (*Brassica oleracea* L. var., *botrytis*) and mustard (*Brassica juncea* L. var., Czern) growing areas in India[14].

Expressed Sequence Tags (ESTs) are the source of microsatellites, which can be employed to develop molecular markers for studies on the population genetics of *A. brassicicola* and closely associated *A. brassicae*, accountable for leaf spot of rapeseed-mustard. Usually, success rate of EST-SSR primers (percentage of SSR primers producing discrete amplification products) were observed to range from 50-100% between species within plants' genera[103,104] and cross species transmission of microsatellites within genera in fungi was more than 30%[105]. Forty-five percent amplification of EST derived microsatellites loci in *A. brassicicola* population obtained in the present investigation is in corroboration to the findings of Benichou *et al*.[106]. In cross-species amplification assay, 45% microsatellites loci of *A. dauci* showed amplification signal for *A. bataticola, A. solani* and *A. zinnia*. Similarly, Dracatos *et al*.[107] tested 55 primer pairs designed from the *Puccinia coronata* f.sp., *lolii* ESTs to amplify the DNA from various fungal species (*Puccinia coronata* f.sp., *avenae*, *Puccinia striiformis* f.sp., *tritici*, *Neotyphodium lolii*, *Blumeria graminis*, *Aspergillus nidulans* and *Penicillium marneffei*) and found that 22-53% amplified. This recommends that microsatellites developed from EST sequences are extremely transferable to other associated species. The more closely connected the organisms, the higher the rate of transferability because of more closely related species sharing more homology in microsatellite loci. Another probability for high rate of success in the amplification of EST-SSRs may be the consequence of numerous factors, such as the sequences from which the primers were derived, the adequate criteria used for primer design and use of the species of same genus for the design and amplification of the primer set[99,108]. A fascinating aspect of present study is the presence of high percentage of monomorphic SSR loci in *A. brassicicola* population. This may be due to environmental and survival based adaptations of fungus, which vary from habitat to small niche. There could be more explanations for the presence of monomorphic microsattelite in *A. brassicicola*, (i) It reasons leaf spot on mustard and cauliflower, both have the same cropping period and environmental conditions and appears very late in season with low intensity, probably leading to less variability in population, (ii) The number of isolates assessed was small and the genetic analysis was of low resolution, (iii) There were repeated bottlenecks and/or single founder population/pathogen mating systems, causing in homogenizing effect, (iv) Microsatellite recognized may be located in the conserved region of the *A. brassicicola* genome and (v) The incidence of *A. brassicicola* is less common over *A. brassicae* in India. All these arguments are reasonable, as evidenced by highly clonal nature with no known teleomorph of *A. brassicicola*[109]. Configurations of cross-species SSR amplification in fungi are beginning to emerge, though there are still few studies, which systematically explore microsatellite transferability out there closely related genera[92,105,110]. Five EST-SSR markers established from *A. brassicicola*, amplified *A. brassicae* isolates and showed very low levels of polymorphism[99]. Analysis of limited number of *A. brassicae* isolates might be one of the probable explanations for low level of transferability of microsatellites[99]. Additionally, the wide distribution of *A. brassiscae* in India[14,98-100] over all mustard growing areas may lead to wide changeability in the population that may be possible reason for low level of transferability of microsatellites from *A. brassiscicola*. It is worth mentioning here that a small number of markers (4 of 25) have also been designated as transferable from linked *Uredinales* species to *Hemileia vastatrix*[111]. The primary results obtained in the recent study accomplished by Singh *et al*.[100] come to an agreement with previous reports, describing a smaller fraction of cross-species transfer of microsatellites within fungal genera[112]. Nevertheless, there might be a high probability of transferability of *A. brassicicola* derived markers to *A. brassicae*, which necessities further investigation and statistical validation using large set of EST-derived SSRs. The results also established wide species transferability of developed EST primers and demonstrated that they may represent a set of well-conserved loci across the species. This may be consequent to the transfer of lineage specific genomic regions in fungi[113,114]. It has been noticed from the study that the distribution of microsatellite in the *A. brasssicola* genome is not random. Tri-nucleotide repeats (AAC, GAA and TAC) have been found to be common feature in EST derived microsatellites. High occurrence of these repeats in coding

regions could be as a result of mutation and selection procedure for specific amino acids. The abundance of tri-nucleotide repeats EST-SSR is likely due to suppression of other kind of repeats in the coding region that reduces the frame-shift mutations in the coding regions[115]. Additionally, there is a possibility that these tri-nucleotides in the coding region are translated into amino acid repeats (histidine, glutamic acid, threonine, serine, arginine, asparagine, glycine, etc.), which possibly contribute to the biological function of protein[100,116,117]. Specific and speedy detection is very significant to monitor and quantify the occurrence of plant pathogens for efficient management of plant diseases. Presently, most of the assays used in *A. brassisicola* disease diagnostic rely on visual assessment of the symptoms, lesion diameter measurement or spore counting[118].

Recently, microsatellite locus 'ABS28' was recognized and characterized in terms of cross-transferability to other associated and dissimilar taxa. Data confirm that this monomorphic microsatellite marker was highly specific for *A. brassicicola* because no amplification signal was observed from other closely connected *Alternaria* species. No false positive or false negative results were observed with the assays revealed the stringency of specific primer set ABS28F and ABS28R, thus allowing specific and sensitive detection of *A. brassicicola* from cultures and plant samples. The assay was sensitive, reliably detecting 0.01 ng of genomic DNA per PCR of the target fungi. Consequently, the marker allows assessing fungal development from the initial stages of infection and eventually provides a fast and practical alternate to currently designated markers to differentiate and diagnose *A. brassicicola* from synchronously occurring fungus, *A. brassicae* and other fungi connected with rapeseed mustard[99,100,110,119].

CONCLUSION

Fourteen monomorphic plus one polymorphic microsatellite markers have been recognized from the necrotrophic phytopathogenic fungus *A. brassicicola*. Although the identified polymorphic marker (ABS1) needs to be developed to enable a further study of the genetic conformation of natural *A. brassicicola* populations and to match the diversity of isolates originating from different host as well as geographical location. Furthermore, primer set, ABS28F (50-GCTCCCACTCCTTCCGCGC-30) and ABS28R (50-GGAGGTGGAGTTACCGACAA-30), intensified a specific amplicon of 380 bp and would be advantageous as an internal analytical marker to differentiate and diagnose *A. brassicicola* from *A. brassicae* and other fungi linked with leaves, stem and siliquae of rapeseed-mustard.

SIGNIFICANCE STATEMENTS

- The study provides complete knowledge of *Alternaria* blight on *Brassica juncea*
- This review provide comparison of *Alternaria brassicicola* and *Alternaria brassicae* and their effect on *Brassica juncea*
- It also provides the most updated information on morphological, biochemical and molecular, studies carried out on *Alternaria* blight

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