Chapter

Epidemiology, Genetics and Resistance of *Alternaria* Blight in Oilseed *Brassica*

Subroto Das Jyoti, Naima Sultana, Lutful Hassan and Arif Hasan Khan Robin

Abstract

Alternaria blight is one of the most deadly diseases of oilseed *Brassica*. This recalcitrant disease causes up to 50% yield loss across the globe. The disease is mainly caused by *Alternaria brassicae* and *Alternaria brassicicola*. These pathogens lack sexual stages and survive as conidia or condiospores on the debris of previous crops and susceptible weeds. Developing resistant oilseed *Brassica* cultivars to this disease has become a prime concern for researchers over the years. In absence of resistant oilseed *Brassica* cultivar, identification and introgression of resistance related genes can be a potential source for *Alternaria* blight resistance. As resistance toward *Alternaria* blight is governed by polygenes, intercrossing between the tolerant genotypes and subsequent selection will be the most appropriate way to transfer the quantitative resistance. For that reason, future breeding goal should focus on screening of germplasms for selecting genotypes containing resistance genes and structural features that favors resistance, like thick epicuticular wax, biochemical components such as phenols, phytoalexins and lower soluble sugars, reducing sugars and soluble nitrogen. Selected genotypes should be brought under appropriate breeding programs for attaining *Alternaria* blight resistance.

Keywords: *Alternaria* blight, oilseed *Brassica*, disease resistance, resistance mechanism

1. Introduction

Oilseed crops are one of the crucial pillars of world agriculture, occupying 22% of the world's arable land [1]. Rapeseed-mustard dominates the total oilseed production after soybean globally [1]. *Alternaria* blight is one of the major biotic threats that drastically reduces oilseed production all over the world including Australia, Europe, China and Canada [2]. *Alternaria* blight is a recalcitrant disease caused by the *Alternaria* species primarily *A. brassicae* and *A. brassicicola*, of which *A. brassicae* is the most deadly [3–4]. This disease decreases photosynthetic potential, leads to abnormal growth of the seeds and reduces seed oil content and quality [5]. Disease intensity varies across seasons and regions, and also between crops within an area [6]. Controlling the disease is one of the foremost concerns for researchers for reviving the yield potential of the rapeseed-mustard varieties. Chemical management of this disease is not proposed because maximum foliage

coverage by aerial application of fungicides is hard to achieve. Beside this, application of large amounts of chemicals raises environmental concerns. It is crucial to genetically monitor the disease by breeding for resistance [7]. Despite the immense efforts of breeders throughout the world, no resistant genotypes have been found till date. Combining various breeding tools may be fruitful in defining resistant genotypes in these scenarios. The genetic base of the cultivated oilseed *Brassica* is narrow and resistance governing genes are hard to find. *Alternaria* blight resistance is controlled by additives or polygenes and has been identified in some wild species of oilseed *Brassica* [8]. Easy availability of microarray data led researchers to the identification and understanding of the expression patterns of key genes involved in the *Alternaria* resistance. Another reliable form of plant immunity is Nonhost Resistance (NHR) that is successful against all genetic variants of a pathogen [8–11]. The infected plants also show hypersensitive response by producing reactive oxygen species [12]. Improvement of modern genetic transformation methods is helping scientists to incorporate resistant genes from non-host wild cultivars. Tissue culture method is one of the biotechnological tools that are being used to transfer resistance genes from resistant genotypes to the susceptible ones. Resistant genotypes showed higher phenolic content than the susceptible one, whereas the total soluble sugars, lower sugars and soluble nitrogen levels were lower [13–15]. Apart from all of these conventional methods, exploration and utilization of systematically acquired resistance and *de novo* resistance can be an efficient way to induce resistance in oilseed *Brassica* cultivar. Besides, molecular markers associated with resistance genes may contribute to the successful improvement of the resistance breeding process. This chapter discusses *Alternaria* blight disease with respect to its epidemiology, genetics and possible resistance mechanisms involved in *Alternaria* resistance and revisits earlier work done by oilseed *Brassica* breeders to elucidate future strategies for *Alternaria* resistance breeding.

2. Epidemiology

Disease epidemiology provides better understanding of the disease, host and favorable factors that facilitates disease progression. It also creates a better opportunity to control the disease by manipulating different epidemiological factors [16]. Majority of the *Alternaria* species produce asexual spores, as it lacks sexual stage (**Figure 1**; [17]). It survives as conidiospores or conidia under unfavorable conditions [18–19]. It also survives in the susceptible weed and in the infected seeds in temperate regions [20–23]. Although in tropical and subtropical India, the survival of *Alternaria* inoculum in seeds is discarded [24]. At first, symptoms start with black dots. Later, these spots extend and grow into prominent round spots with concentric circles displaying the spot's target board features (**Figure 2**). Many spots coalesce to form large patches which cause the leaves to blight and defoliate [4]. Initially the infection starts from the cotyledonary leaves and forms a basis for the secondary infection. Four hours of leaf wetness is necessary for leaf infection. An increases in leaf wetness duration at 25 °C increases infection and spread of the disease rapidly. Spores attack other parts of the plant upon getting favorable conditions. New lesions arise within four-five days. The pathogen infects the seed by penetrating the pod [25]. The critical factors for spore germination have been reported as darkness or low light intensity (<1000 lux), 25 °C temperature and more than 90% RH in some previous studies [26]. Some studies reported the increase of disease severity with the increase of inoculum concentration [27–29]. The optimal assay temperature of 25 °C and > 90% relative humidity resulted in the highest severity of the disease, regardless of the apparent susceptibility of the

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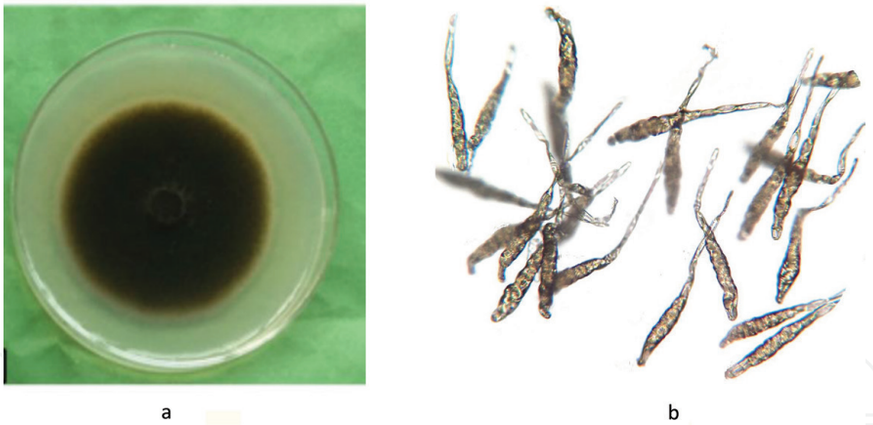

Figure 1.
Cultured spores (a) and conidia of Alternaria brassicae *from the infected field samples (b).*

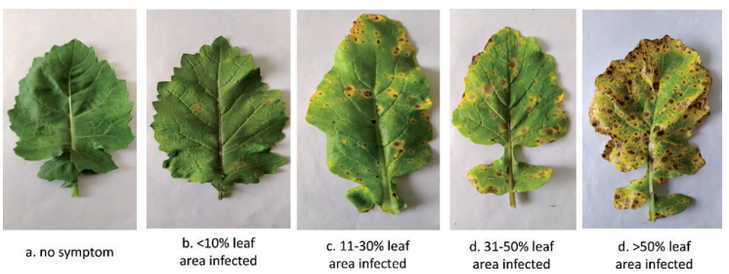

Figure 2.
Symptoms and different level of severity of Alternaria *blight. Symptoms from 'a' to 'e' show gradually higher severity of infection.*

cultigen [27, 30–36]. Previous studies reported that older leaves are more affected by *Alternaria* than the younger ones [27, 30, 37–40]. Weather characteristics such as maximum temperature 18–27 °C and minimum temperature 8–12 °C facilitates *Alternaria* infection on leaves with an average relative humidity more than 92% while on pods, the infection occurs at temperatures ranging from 20–30 °C [41]. Closer spacing (30 × 15 cm), high nitrogen doses (80 Kg Nha^{-1}) and frequent irrigation rapidly increase severity of disease in rapeseed–mustard [12]. Frequent rains are favorable for the initiation and spread of the disease on the leaves of oilseed *Brassica*. In addition, the rate of infection during the flowering and pod phases is the highest [42].

3. Genetics and genomics of *Alternaria* blight resistance

Identifying resistance mechanisms at the genetic and genomic level has been a prime concern for the researchers over the recent years. Various sources suggest that the resistance against *Alternaria* is polygenic [3, 43–45]. On the contrary, other studies reported that resistance to this disease is mainly controlled by only additive genes or dominant nuclear genes [3, 43–46]. However, Kumar et al. [47] proved that inheritance of *Alternaria* blight resistance is governed by more than one gene and fixable and non-fixable gene effects are vital in the genetic control of *Alternaria* blight resistance. In *Arabidopsis*, six QTLs governing *Alternaria* blight resistance were identified. Among these QTLs, five QTLs were population specific and one was common among all mapping populations. Presence of both common and population specific QTLs indicates that resistance against *Alternaria* blight is quantitative and more than one gene potentially governs the resistance [48].

With the modern development of biotechnology, the discovery of resistance (R) and defense-related genes has opened up new scopes for inducing genetic resistance against different biotic and abiotic stresses [49]. Advances in microarray data processing also ease the process of identifying candidate genes in certain physiological processes. In previous studies, *A. brasscicola* infection contributed to the upregulation of different genes such as *WRKY, peroxidase, p450 oxidases, Chitinase* that modulates defense response in oilseed *Brassica* and *Arabidopsis*. A recent computational study identified vital genes involved in *Alternaria* resistance in *Brassica* by analyzing microarray data of model plant *Arabidopsis thaliana* challenged with *Alternaria* infection [50]. *NHL10, HCHIB* and *XLG2* were identified as major genes and *CZF1, ARF6, WRKY, MP, IAA1, IAA19, AXR3* as candidate genes associated in defense response against *Alternaria* [50]. PR (pathogenesis-related) proteins are a distinct group of molecules which are induced by phytopathogens and signaling molecules linked to defense. They are the vital components of the plant's inherent immune system, particularly systemic acquired resistance (SAR) [51]. Two genes under these proteins namely *Chitinase* and *NPR1* have been characterized in oilseed *Brassica* species. Their high expression level in resistant genotypes compared to the susceptible genotypes suggested that these genes are related to resistance against *Alternaria* blight [52–53]. Another study reported the expression of *PR-3* and *PR-12* only in *Camelina sativa* and *Sinapsis alba* compared with *B. juncea* [54]. This clarifies the involvement of PR proteins in the resistance mechanism of *Alternaria* resistant varieties.

4. Biochemical resistance against *Alternaria*

Biochemical defense is triggered by any stress condition in a plant and is the most important tool of plant defense mechanism. The hypersensitive response is one of the plant's most effective defensive responses against the pathogen [55]. Resistance to *Alternaria* blight in mustard was reported to be linked with the synthesis of phenolic pathway-associated leaf enzymes and higher leaf sugar content [56]. The concentration of phenolic compounds at all stages of plant growth was reported to be high in resistant genotypes compared to susceptible genotypes. Nevertheless, soluble sugars, sugar reduction and soluble nitrogen levels in resistant genotypes were lower [14–15]. Another study reported that, total phenol, total sugar, reducing sugar, o-dihydroxy phenol, chlorophyll content and flavonol contents were higher in resistant genotypes [57]. By activating several defense responses that dissuade the infection process, plants can respond to a pathogen. These include the production of reactive oxygen species (ROS), the accumulation of proteins related to pathogenesis (PR) and phytoalexins and the synthesis of compounds that strengthen the plant cell wall [58]. Moreover the contents of ascorbic acid, total phenol, enzymatic activities of superoxide dismutase and peroxidase, that of cell protecting enzymes such as phenylalanine ammonia lyase and polyphenol oxidases were increased in the resistant genotypes of mustard [59]. *β-Aminobutyric acid* (BABA), a non-protein amino acid has been known to stimulate resistance to a variety of pathogens in a number of plant species [60–61]. Pretreatment of oilseed *Brassica* plants with BABA-mediated resistance to the necrotrophic pathogen *A. brassicae* through enhanced expression of protein genes linked to pathogenesis [62]. The colonization of *A. brassicae* on *Brassica carinata* leaves was substantially inhibited by the foliar application of BABA [63]. A higher and early accumulation of H_2O_2 was observed in resistant *C. sativa* and *S. alba* compared to *B. juncea*. Catalase activity was enhanced in both *C. sativa* and *S. alba*, but the opposite phenomenon was observed in case of *B. juncea* [54].

5. Utilization of non-host resistance

Non-host resistance is one of the most useful approaches for attaining resistance against different plant pathogens. Till date, no resistant cultivar is available in oilseed *Brassica* species. Therefore, utilizing the non-host resistance from wild species can be an efficient breeding tool. Plant pathogens manage to affect different species, but they fail to overcome the non-host resistance [64]. Examples of some non-host plants of *A. brassicae* are chickpea, lentil, wheat, sugarcane, barley, tomato, potato [64]. NHR is multilayered and can be splitted into two main forms: the layer of pre-invasion and the phase of post-invasion [65–67]. Preformed defenses may include structural features like abundance of trichomes and spore germination inhibitory chemical compounds [68–70]. Previous studies reported that spore germination occurs at an equal rate in both host and non-host plants [71]. Despite an accurate germination, pathogens might fail to reach the stomata. Stomata in non-host plants may not be correctly recognized by the pathogen because the topography of the surface may vary significantly from that of the host leaf [64]. Another structural feature that can prevent the entry of *Alternaria* is the epicuticular wax [72–74]. Non-host plants may have higher epicuticular wax than the susceptible host plants [64]. The non-host plant is capable of inducing stomatal closure, preventing pathogens from entering and constructing an inducible chemical barrier that suppresses hyphal production and differentiation by the rapid formation of phytoalexins, antimicrobial compounds [75–77]. In a non-host plant, the dietary deficiency and the presence of antimicrobial compounds in the apoplast can also prevent the production of hyphae into mycelium [71]. The pathogen also generates non-host specific or general toxins that might damage plant cells, leading ultimately to necrosis [78–80]. To avoid this, a non-host plant may recognize these toxins and employ defense mechanisms to detoxify these toxins [81]. In *Arabidopsis* and *S. alba* pathogenesis-related genes *PR-1, PR-2, PR-3* were highly expressed compared to *B. juncea* after *Alternaria* infection [82–86]. Furthermore, these two species showed non-host resistance toward *A. brassicicola* [81, 87]. Chitinase enzymes that hydrolyze the fungal cell wall and release fragments of chitin are actively secreted by these two species [82, 88]. The NHR action includes the stimulation by the plant cell of a signal transduction cascade following the detection of a pathogen, which triggers the activation of protein kinases and mitogen-activated protein kinase (MAPK) members and consequently lead to the activation of defensive genes in non-host plants [89]. The expression of *MAPK* was higher in *S. alba* and downregulated in *B. juncea* suggesting its possible role in *Alternaria* blight resistance.

6. Genetic transformation for *Alternaria* resistance

As the resistance of *Alternaria* has not yet been found, identification of resistance genes in non-host plants and transferring them into oilseed *Brassica* species could be a handy tool for resistance breeding. Introgression of genes under PR-proteins have been found effective in many cases. For instance, transgenic Indian mustard was developed with the *chitinase* gene in which the occurrence of disease symptoms was delayed by a duration of 10–15 days compared to control plants [90]. For enhancing resistance against *A. brassicae*, a PR protein-encoding glucanase was introduced from tomato into Indian mustard plants [91]. Glucanase hydrolyzes a main component of a fungal cell wall called glucan and destroys the invading fungal pathogens. In combating *Alternaria* blight disease, a barley anti-fungal class II *chitinase* gene and type I ribosome inactivating protein (*RIP*) gene were co-expressed in Indian mustard [92]. Transgenic mustard plants demonstrated

a 44% reduction in *A. brassicae* hyphal production relative to the control plants. When transgenic events were sprinkled with fungal spores through greenhouse screening, the late onset of the disease and a lower number of lesions with reduced size distribution were recorded. In addition, *Chitinase* gene was transferred from *Streptomyces griseus* HUT6037 to Indian mustard [93]. A previous study transformed *B. juncea* with the *osmotin* gene and documented resistance to the purified *A. brassicae* toxin in the transformed calli [94]. *B. juncea* was modified to add resistance to *Alternaria* blight and stem rot diseases with the *MSRA1* gene [95]. Bioassays after *Alternaria* infection *in vitro* showed that transgenic *B. juncea* lines inhibited the growth of *Alternaria* hyphae by 44–62% and reduced infection ranging from 69–85%. The *lectin* gene of chickpea was transferred to Indian mustard cv. Varuna to induce resistance against *A. brassicae* in transgenic lines [96]. Another study incorporated *B. juncea* with the gene *MPK3* and examined its role in providing tolerance against *A. brassicae* [97]. In transgenic plants, both ascorbate peroxidase (APX) and guaiacol peroxidase (GP) activity and proline content were higher, leading to the scavenging of ROS in transgenic plants developed as a result of infection with *Alternaria*.

When an *endochitinase* gene '*echh42*' from the *Trichoderma virens*, a fungal species used as a bio-control agent, was introduced to *B. juncea*– the transformed plants showed 7-fold higher endochitinase activity compared to the non-transformed plants based on fluorimetric analysis [98]. These results indicated that the *endochitinase* gene '*ech42*' could be a major gene that may provide resistance to oilseed *Brassica* plants against the *Alternaria* blight. In previous studies, the transgenic broccoli plants also showed expression of *chitinase* gene of *Trichoderma harzianum* [99–101]. Moreover, the synthetic *chitinase* gene (*NIC*) showed broad-spectrum resistance to the transgenic lines of *B. juncea* including *A. brassicicola* [102]. Further research utilizing RT-PCR validated that these *chitinase* genes were induced after wounding and exogenous treatments of jasmonic acid and salicylic acid similar to *Alternaria* infection [103]. A recent review summarized that the chitinases, glucanases or cry proteins provide broad-spectrum resistance against some major diseases including *Alternaria* blight and blackleg [104].

7. *De novo* resistance

It is assumed that the disease can be successfully managed by inducing protection inducers in plants. Some novel fungicides may mimic the action of different plant hormones that activate the plant's internal immune response. Jasmonic acid (JA) mediated defense response to *A. brassicae* fungus can prevent necrotrophic colonization mode. The JA receptor, coronatine insensitive 1 (COI1), is one of the possible targets to activate JA-mediated immunity via JA signal interaction [105]. It is understood that Jasmonates and its functional analogs play a crucial role in systemic defense, likely serving as the initiating signal of acquired systemic resistance [106]. It has been shown that necrotrophic fungal pathogens are the primary activators of JA-dependent defenses via COI_1 receptor activation [107]. A previous study identified some JA mimicking molecules that might be helpful in *de novo* resistance induction [108].

8. Tissue culture techniques in *Alternaria* resistance

Tissue culture is one of the most effective tools of modern biotechnology. Somaclonal variation provides an opportunity to extend the genetic variation of

crops, i.e. the variation caused by cell and tissue culture. By applying *in vitro* selection process, the efficiency of selection can be increased [109]. Somatic hybrids were produced through PEG-mediated symmetric and asymmetric protoplast fusion, in which *S. alba*, *B. nigra* and *B. juncea* were found to be the most effective resistance donor to *Alternaria* pathogen [110]. Through protoplast fusion, a previous study developed three hybrids between *B. juncea* and *S. alba* [111]. Among the hybrids, two of the hybrids were symmetric, while the third was asymmetric and had greater similarity to *B. juncea*. *Alternaria* resistant lines were developed through interspecific hybridization between *S. alba* and *B. juncea* [112]. *Alternaria* blight resistance was transferred from *B. tourneforti* to *B. juncea* cv. RH 30 through *in vitro* ovule culture [113]. Intergeneric hybrids of *B. campestris* and *B. spinenscens* were generated through sequential ovary, ovule and embryo culture [114]. The resistance trait was transferred to *B. napus* cv. Brutor from *S. alba* cv. Carine following *in vitro* fertilized ovary culture protocol [115]. *Erucastrum cardaminoides* and *B. oleracea* var. alboglabra were used to develop intergeneric hybrids with *Alternaria* blight resistance following sequential ovary and ovule culture procedures [116]. Previous studies reported transfer of *Alternaria* resistance through somatic hybridization such as, from *S. alba* to *B. napus* [117] and *Moricandida arvensis* to *B. oleracea* [118]. A research group in India transferred *Alternaria* resistance trait to *B. juncea* from *B. carinata* [119]. Disease resistant hybrid plants were produced from the hybridized leaf mesophyll protoplasts of *M. arvensis* and *B. napus* [120]. *B. carinata* was resynthesized by protoplast fusion between *B. nigra* and *B. oleracea* [121]. The hybrids thus obtained were fertile and grew into robust plants. Previous studies conducted hybridization between *S. alba* and *B. oleracea* and between *Camelina sativa* and *B. oleracea* for producing resistant hybrids [122–123]. Another study developed somatic hybrids between *S. alba* and *B. oleracea* by protoplast fusion followed by embryo rescue and managed to recover four highly resistant hybrid progenies after repeated backcrosses [124]. By inducing variations through gamma-irradiated mutagenesis the resistant varieties were obtained in *B. juncea* [125] while another study achieved the similar results by treating the embryos with chemical mutagens [126]. It is plausible to say that proper utilization of tissue culture techniques can be a successful means of incorporating *Alternaria* resistance into oilseed *Brassica* cultivars.

9. Molecular markers and *Alternaria* blight resistance

In any disease resistance breeding program, the primary approach is to quickly screen all the available germplasm including local races, improved variety and exotic genetic stocks. The traditional approach of screening of genotypes can be costly, time and space consuming, laborious, and involves large sample sizes [127]. The limitations of conventional approach can be solved through molecular markers. By utilizing molecular markers, economically important major genes and quantitative trait loci (QTLs) can be identified [128]. Pre-selection using molecular markers can minimize the size of a population and facilitate early detection of desirable genotypes [127]. Various molecular markers are being used nowadays for assessing genetic variability against *Alternaria* blight. For example, internal transcribed spacer regions (ITS), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSRs), microsatellites (SSR), sequence tagged sites (STS), single nucleotide polymorphism (SNPs) etc. The ITS regions are the preserved areas in the fungal genome that are considered as the most common loci to study DNA based mycology at the species level. Berbee and co-workers

studied the ITS regions of rDNA to determine the pathogen's phylogeny [129]. RAPD technique was used successfully to examine the genetic differences in *Alternaria* infected species [130–132]. Later on, the assessment of genetic variability in *Alternaria* species has moved to more sensitive techniques such as AFLP [133] and microsatellite markers [134] due to the constraints of reproducibility of RAPD. Simple sequence repeats have been isolated and characterized from *B. napus*, *B. nigra*, and *B. rapa* [135, 136]. Moreover, SSR marker libraries have been developed for *B. rapa* those are being used to produce a genome map for *B. rapa* [137]. Recently, SNP markers have taken the supremacy over SSR as they are unique and plentiful in high and ultra-high-throughput and are able to find polymorphism within a single base pair [138].

10. Conclusions

Alternaria blight is one of the major diseases of oilseed *Brassica* causing enormous yield loss every year. In order to reduce the use of chemical fertilizers and to save the environment, breeding is important to attain resistance against *Alternaria* pathogens. Since the resistance against *Alternaria* blight is governed by additive or polygenes, molecular breeding for resistance could be more effective. All possible sources including wild relatives and non-host plants should be brought under the selection process for identifying ideal resistance donors. QTL mapping and continuous hybridization between resistant genotypes should be performed for better results. Emphasis should be given on functional analysis of PR proteins for engineering *Alternaria* resistance more effectively. In addition, accurate modeling of plant's internal defense responsive pathways can provide new insights on *de novo* and systematically acquired resistance.

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Conflict of interest

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