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Alternaria Blight of Oilseed Brassicas: Epidemiology and Disease Control Strategies with Special Reference to Use of Biotechnological Apporoaches for attaining Host Resistance

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Abstract

The production of oilseed brassicas, second largest oilseed crops grown in the world, is gravely hampered by the fungal diseases; *Alternaria* blight being one of the most ravaging fungal disease. The pseudo-fungi, not only leads to yield reduction by causing foliar damage to the crop, but also severely deteriorates the oil quality. With no available sources of durable resistance in the presently cultivated varieties of rapeseed-mustard, efforts are being made throughout the world to develop stable disease resistance in oilseed brassicas by utilizing the contemporary tools of biotechnology. The paper gives a comprehensive description of the *Alternaria* blight pathogen and various technologies viz. embryo rescue, somatic hybridization, somaclonal variations, genetic transformation, molecular markers, signal transduction, being used for incorporation of resistance against the pathogen in oil-yielding brassicas.

Keywords: *Brassicas, Alternaria blight, Biotechnological tools, induced systemic resistance*

Introduction

Rapeseed-mustard is an economically prodigious and utile oilseed crop of the world. Contributing to 32% of the total oilseed production in the country, it is the main source of edible oil in India after groundnut. Out of 59.93 m tonnes of rapeseed produced over 30.74 m ha in the world, India produces 7.67 m tonnes from an acreage of 6.51 m ha with a productivity of 1179 kg/ha (GOI 2011). The development of high yielding varieties coupled with improved production technologies has lead to a considerable increase in the productivity of rapeseed- mustard in India during past decade. But despite this increase, the yields of oilseed brassicas in India are much below the global average. A huge sum of money (8000-10000 crores/ annum) is being spent on import to meet the current edible oil demand. The wide gap between the potential and realized yields is largely because of the biotic and abiotic stresses. Among the biotic factors, fungal diseases alone are responsible for severe damages to the crop resulting in yield losses up to 70% on a world wide scale. The severe attack of many fungal diseases not only deteriorates the quality of the seed but reduces the oil content considerably in different oil-yielding *Brassica* species. Amongst the major fungal diseases of oilseed brassicas prevalent in India, *Alternaria* blight caused by *Alternaria brassicae* is the most catastrophic and pervasive disease of oilseed brassicas reported over a wide geographical area all over the world (upto 60% yield loss, Kolte, 2002). The present paper discusses the *Alternaria* blight disease with respect to its distribution, symptoms and physiological changes caused in the host plant, epidemiology, sources and inheritance of resistance, and disease control strategies being used, emphasizing mainly on the biotechnological tools for incorporation of resistance to *Alternaria*.

Alternaria Blight

Alternaria blight, reported in the early 20th century (Fawcett, 1909), is one of the most widespread fungal diseases of *Brassicaceae* crops and exists in almost every country worldwide. Besides India, it is a significant disease in Australia, Canada, Africa, England, Germany, France, Sri Lanka, Spain and Sweden (Sharma and Kolte, 1994; Saharan,

1992). *A. brassicae* and *A. brassicicola*, the causal agents of the disease, are cosmopolitan in occurrence. Both the species are known to damage the vegetable crucifers, *A. brassicae* being most ravaging on oil yielding brassicas. The pathogen is characterized as pseudo-fungi, with absence of an identifiable sexual stage. The key taxonomic features of the pathogen are the production of large, obclavate, olive gray to dark coloured conidia, having longitudinal as well as transverse septae.

Symptoms and physiological changes in host plant

The pathogen is reported to affect the host plant at all growth stages and infects all the aerial tissues-leaves, leaf petiole, inflorescence, stem, siliqua and seeds. The pathogen causes slow destruction of the host plant by reducing the photosynthetic potential. The symptoms first appear on the lower leaves as minute brown to black dots that rapidly multiply and enlarge to form prominent round spots with concentric rings of different shape, size, colour and intensity depending upon the causal species and host plant under different environmental conditions. As the disease progresses, the complete leaf gets affected and ultimately defoliates. The infection then spreads to middle and upper leaves in the form of smaller spots. At the later stages of plant growth, the spots appear on the stem and siliquae generally in the form of black stripes which subsequently coalesce, turning the siliquae black. The decaying of the seeds can be seen just beneath the black spots of siliqua. Severe infection on the stem and pods leads to premature ripening, resulting in yield losses due to seed shedding (Verma and Saharan, 1994). The disease significantly affects the plant physiology and its cellular organization by altering the cell permeability and thus disrupting the cell organelles. Agarwal *et al*. (1997) reported the presence of degenerated chloroplast and swollen mitochondria, with reduced number of cristae in mustard leaves following severe infection of *A. brassicae*.

Epidemiology

Alternaria has no sexual stage and therefore under temperate conditions is known to survive as mycelium/conidiospores or conidia on the decaying debris of previous year's crop (Humperson-Jones and Maude, 1982; Humpherson-Jones, 1989), as chlamydospores/microsclerotia at low temperatures (Tsuneda and Skoropad, 1977), in susceptible weeds or perennial crops (Chupp and Sherf, 1960; Maude and Humpherson-Jones, 1980a, b) or in the infected seeds for at least a year at room temperature (Shrestha *et al*., 2003, Ahmad and Sinha, 2002). Chattopadhyay *et al*., 2005 studied the time of a ppearance of the pathogen on the host (*B. juncea* var. varuna) and the role of environmental conditions on severity of alternaria blight disease. Highest frequency of occurrence of *A. brassicae* on leaves and pods was found between 67-84 d.a.s (days after sowing) and 67-142 d.a.s respectively. The temperature ranging from maximum 18-27^{0}C and minimum 8-12^{0}C along with an average relative humidity >92% has been reported to favour the initiation and spread of alternaria disease on the leaves. Whereas, temperature ranging from a maximum of 20-30^{0}C, with an average relative humidity >90% along with more than 9 hours of sunshine and 10 hours of leaf wetness favours the disease severity on pods.

Sources and inheritance of resistance

Host resistance forms an integral element of integrated disease management system and therefore demands a thorough study of the host-pathogen responses. *Brassica* species and their varieties have been extensively studied for their response against *A.brassicae* and *A.brassicicola.* Varied degrees of responses ranging from moderate to high susceptibility have been observed in Brassica species, *B. juncea* and *B. rapa* being more susceptible to *Alternaria* blight than *B. napus* and *B. carinata* (Skoropad and Tewari, 1977). Ethiopian mustard (*B. carinata*) has been reported to show high levels of tolerance to *Alternaria* blight (Kolte, 1996), but is not grown on a large scale in India because of its non-desirable agronomic traits. As no known source of transferable resistance is available in cultivated varieties of oilseed *Brassica*, efforts have been made to identify the tolerant/ resistant varieties (Gupta *et al*., 2001). The non-availability of any resistant sources in oilseed brassicas may be because of the fact that the inheritance of resistance to *Alternaria* blight is

governed by the additive genes (Tripathi *et al.*, 1980; Krishnia *et al*., 2000). Kolte et al. (2000) have reported tolerance in *B. juncea* cv. Divya against *Alternaria* blight. Good tolerance has been observed in *B. juncea* cv. RC781 (Tripathi *et al*., 1980). Partial resistance has also been reported in 3 genotypes of *B. juncea* viz. Kranti, PR 8988 and PR 9024 (Kumar, 2008). Apart from possessing high degree of resistance to downy mildew and white rust, *B. napus* strains, EC-338986-2 and EC-338996-1 have shown high tolerance to both *Alternaria* blight and *Sclerotina* stem rot. Also, exotic *B. juncea* strains, EC-399296, EC- 399299, EC-399301 and EC-399313 have shown to be tolerant to *Alternaria* blight (Kolte *et al*., 2008). The exotic strains of *B. napus* – EC 339000 and EC 338997 are also reported to possess high degree of tolerance to *Alternaria* blight (AICRP, 2011). Sources of resistance have also been identified in wild and related species of Brassicas. For instance, *Sinapis alba*, commonly known as white mustard (Hansen and Earle, 1997), *Eruca sativa*, *Brassica spinescenes*, *Camelina sativa*, *Capsela bursapastouris* (Tewari and Conn, 1993), *Diplotaxis* species (Sharma *et al*., 2002) and *Brassica maurorum* (Chrungu *et al*., 1999).

Disease control strategies

Since mid 90's, management and control of *Alternaria* blight disease of crucifers is being performed mainly by two approaches. First is, adhering to common cultural practices of timely sowing, use of clean, healthy seeds and a balanced nutrition, proper field sanitation, destruction of diseased debris and weeds, and crop rotation. Seed treatment with hot water at 50ÚC for 20-25 minutes also eliminates seed-borne Alternaria (Ellis, 1968; Randhawa and Aulakh, 1984). Second and most extensively used is the chemical control by application of fungicides. A large number of fungicides have been reported to be effective in checking the spread of the disease under field conditions viz. Baycor – 0.2%, Blitox 50- 0.3%, Dithane M45- 0.2%, Dithane Z78- 0.2%, Rovral 50- 0.2%, Ridomil MZ (Mancozeb 64% +Metalaxyl 8% WP) etc. (Verma and Saharan, 1994; Khan *et al*., 2007). The chemical control, although found to be effective leads to residual toxicity, detrimental effects in the non-target pathogens and development of resistance in the target organisms. It is also known to affect the oil quality in oilseed brassicas (McCartney *et al*., 1999). Ascribable to all those deleterious effects caused by the use of fungicides, the focus now lays on the development of novel biotechnological tools for production of economically superior, disease resistant/ tolerant crops. Biocontrol is another disease control strategy that's gaining importance these days. Reports are there for the use of Mycostop (formulation of Streptomyces) for seed treatment against *A. brassicicola* (White *et al*., 1990), soil isolates of *Trichoderma virdie* (Meena *et al*., 2004) and bulb extracts of *Allium sativum* (Meena *et al*., 2004; Patni and Kolte., 2006) for *B. juncea*.

Incorporation of fungal disease resistance

Various methods including conventional as well as biotechnological approaches have been utilized either alone or in combination to incorporate the desired traits and to steer the genetic variability into new improved cultivars of oilseed brassicas. The conventional approach involves the identification and selection of disease resistant genotypes and transfer of the desired traits into an agronomically superior genotype by hybridization. Hybridization combined with backcrossing, either singly or in combination with pedigree selection is the most widely used and accepted conventional method to transfer disease resistance in oilseed brassicas (Briggs and Allard, 1953). Till date several germplasm lines and cultivars have been screened for resistance towards *Alternaria* blight and varying degree of response ranging from high susceptibility to moderate resistance has been reported to both these diseases in *B. juncea.* The selected tolerant/ resistant lines have been hybridized with the desired agronomically beneficial lines through both inter- and intraspecific hybridization to produce disease resistant progenies. Among the cultivated species, *B. carinata* identified to be highly tolerant to *Alternaria* blight has been utilized to transfer the same in *B. juncea* through inter-specific hybridization (Sharma and Singh, 1992).

Role of biotechnological tools

Besides utilizing conventional methods of selection and sexual reproduction, biotechnological techniques involving tissue culture and genetic transformation based methods have revolutionized the search for economically promising disease resistant crops. These tools have enabled the researchers to combine the traits from distantly related *Brassica* sp. for development of superior, *Alternaria* blight tolerant varieties of *Brassica*.

In vitro embryo rescue

Embryo rescue, documented for the first time in 18th century, forms one of the most successful techniques for the production of interspecific and intergeneric hybrids from naturally incompatible crosses that do not result in the production of viable offspring. The technique involves excision of the ovary/ ovule/ embryo resulting from an interspecific cross, its culturing and maturity in suitable media for production of a hybrid plant with desired traits. The technique has proven to be very efficient for transferring *Alternaria* blight tolerance in oilseed brassicas. *In vitro* ovary culture was used to transfer the resistance against *Alternaria* blight from *B. tourneforti* to *B. juncea* cv. RH 30 (Yadav *et al*., 1991). Agnihotri *et al*. (1991), developed intergeneric hybrids of *B. campestris* and *B. spinenscens* through sequential ovary, ovule and embryo culture. The hybrid plants were then multiplied using somatic embryogenesis. The resistance trait from *Sinapis alba* cv. Carine was transferred to *B. napus* cv. Brutor using *in vitro* fertilized ovary culture (Chevre *et al*., 1994). Intergeneric hybrids with *Alternaria* blight resistance were developed between *Erucastrum cardaminoides* and *B. oleracea* var. alboglabra using sequential ovary and ovule culture (Mohanty *et al*., 2009). Gupta *et al* (2010) used interspecific hybridization in combination with *in vitro* ovule culture to incorporate high tolerance to *Alternaria* blight and white rust from *B. carinata* cv. Kiran to low erucic acid TERI (OE) M21 lines. The hybrids were reported with high tolerance to *Alternaria* blight and white rust with negligible amount of erucic acid (1.25%) and high oleic acid (42.50%).

Somatic hybridization

Somatic hybridization refers to the enzymatic removal of the cell wall and isolation of protoplasts which are then fused together to produce hybrids. Due to the absence of the cell wall, there occurs a non-specific fusion between the two protoplasts and thus there is no barrier to intergeneric/ interspecific crosses. It also bypasses both the pre and post fertilization barriers. This technique has been utilized effectively in brassicas, but the studies are limited to *B. napus* and *B. oleracea*. Efforts have been made to transfer Alternaria resistant trait from *Moricandida arvensis* to *B. oleracea* (Toriyama et al., 1987), and from *Sinapis alba* to *B. napus* (Primard *et al*., 1988). Sharma and Singh (1992) attempted the transfer of alternaria resistance trait from *B. carinata* to *B. juncea*. Leaf mesophyll protoplasts from *M. arvensis* and *B. napus* were hybridized to produce disease resistant hybrid plants (O' Neil *et al*., 1996). Jourdan and Salazar (1993) resynthesized *B. carinata* by protoplast fusion between *B. nigra* and *B. oleracea*. The hybrids thus obtained were fertile and grew into robust plants. Hybridization between *S. alba* and *B. oleracea* and between *Camelina sativa* and *B. oleracea* has also shown to produce resistant hybrids (Hansen and Erle, 1997; Hansen, 1998). Sigareva and Erle, (1999) developed somatic hybrids between *S. alba* x *B. oleracea* by protoplast fusion followed by embryo rescue and recovered four highly resistant hybrid progenies after repeated back crosses.

Somaclonal variations

These are regarded as the impulsive, inheritable changes in gene expression that appear under *in-vitro* conditions especially in case of tissue culture experiments. Studies have shown the use of somaclonal variations for the incorporation of disease resistance/ tolerance against *Alternaria* blight (Sharma and Singh, 1995). Resistance to susceptible varieties of *B. juncea* were achieved by inducing variations through mutagenesis; by exposing the seeds to gamma radiations (Verma and Rai, 1980) and by treating the microspores/microspore derived embryos with chemical mutagens (Agnihotri *et al*., 2009), ethyl methanesulfonate (EMS) and ethyl nitrosourea (ENU).

Genetic transformation

Production of transgenic plants through genetic transformation forms another biotechnological approach to integrate the disease resistance genes from resistant/tolerant genotypes to the economically important susceptible ones. The genetic transformations in *B. juncea* have been reported to achieve delayed onset of the *Alternaria* blight. Kanrar *et al*. (2002) isolated cDNA sequence for "hevein-rubber tree lectin" from *Hevea brasiliensis* and transferred it to *B. juncea* cv. RLM-198 through binary vector pBinAR. The transgenic plant thus produced was shown to support delayed onset of *Alternaria* blight with considerably low disease intensity. Mondal et al. (2003) identified the role of "chitinase" in plant defense responses and transformed *B. juncea* (cv. RLM198) with chitinase gene tagged with over expressing promoter 35S CaMV. The transformed plants resulted in delayed disease onset and about 12-56% reduction in fungal colony size. Osmotin protein, known for its role in signal transduction, when transferred to *B. juncea* also showed tolerance to *A. brassicae* in transgenic plants (Taj et al., 2004). In another study, transgenic Indian mustard expressing "class I glucanase" gene under the control of CaMV 35S promoter was developed (Mondal et al., 2007). For undertaking the genetic transformation, cDNA encoding class I glucanase gene was isolated from tomato and cloned into *Bam*HI-*Sal*I restriction site of binary vector, pBinAR flanking CaMV 35S constitutive promoter and an *ocs* terminator region. The resulting recombinant $pBinG_B$ clone was transferred into *Agrobacterium tumefaciens* strain GV 2260, which was further used to transform *B. juncea* (cv. RLM198). Recent studies (Verma et al., 2012) have reported the introgression of cysteine rich-antimicrobial peptide, PmAMP1 from *Pinus monticola* into *B. napus*, thus conferring enhanced protection against *A. brassicae*, *Leptosphaeria maculans* and *Sclerotinia sclerotiorum*. Combined expression of type I ribosome inactivating protein (RIP; AAA32951) and Barley class II chitinase gene in *B. juncea* has shown to reduce the growth of fungal hyphae by 44% and it also affected the onset of disease, thus generating protection against the pathogen. Efforts have also been made to study the signal transduction pathways in *Brassica* species. Taj et al (2011) reported the interaction of MAPK3 and Lox genes during pathogenesis of *A. brassicae* which is suggested to play a role in biosynthesis of jasmonic acid and jasmonic acid induced expression of defense genes in *B. juncea*.

Molecular Markers

As the efficient management of plant diseases relies on the accurate identification of the plant pathogen, knowledge of the variability in the pathogen population, both at the pathological as well as genetic level is very important. Characterization of variability in the pathogens including Alternaria sp. has been earlier done using traditional methods of morphological markers (Goyal et al., 2011), biochemical tests (Vishwanath and Kolte, 1997), cultural and pathogenic assays (Goyal et al., 2011; Singh et al., 2011). These markers differentiate among the pathogens based on their growth behavior, physiological characters and microscopic appearance; however, these are influenced by different factors like, age and quality of the inoculum, age and type of host, culture media used and incubation conditions. Also, these techniques are more laborious, time consuming and often give non-reproducible data. To overcome these limitations of traditional markers, detection of genetic variability through DNA fingerprinting technology by the use of molecular markers has come to the aid of plant breeders and pathologists. The main advantage of using these fingerprinting techniques is that, these do not require any prior knowledge or availability of variable sequences in the database, as they deal with the genome-wide biodiversity.

Various molecular markers being used nowadays for assessing variability include, 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), Microsatellite, sequence tagged sites (STS), single nucleotide polymorphism (SNPs) etc. Only a few studies, probably due to the presumed dependency on traditional characterization, sare reported on molecular

characterization of *Alternaria* species pathogenic to oilseed brassicas. The ITS regions are conserved regions in the fungal genome considered the most popular locus for DNA based mycological studies at species level. Chou (2002) and Berbee (2003) along with their coworkers studied the ITS regions of rDNA of *A. brassicae* and *A. brassicicola* to evaluate the phylogeny of the pathogen. Molecular techniques like RAPD, that do not require any prior knowledge of the DNA sequences have been efficiently used to analyse the genetic variations in *Alternaria* species (Cooke *et al*., 1998; Sharma and Tewari, 1995, 1998; Gherbawy, 2005). However, due to the limitation of reproducibility faced by RAPD, the assaying of genetic variability in *Alternaria* species has shifted to more sensitive techniques like AFLP (Bock *et al*., 2002) and Microsatellite markers (Avenot *et al*., 2005). Though, the use of molecular tools have revealed huge amount of genetic variations in *Alternaria* at the sub-generic as well as sub-species level, this field still remains largely unexplored and demands an extensive study into the variability at different geographic levels among different hosts.

Induction of systemic resistance

Induced host resistance refers to activation of resistance in otherwise susceptible plants, through biotic as well as abiotic agents (Stitcher *et al*., 1997; Kessman *et al*., 1994), without changing their basic genetic make-up. The resistance/ defense related genes in the vulnerable plants can be activated by inoculating the plant either by an avirulent form of the pathogen or by limited inoculation with the pathogen (Deverall, 1995). The infecting avirulent pathogen triggers natural defense responses in the plant through the release of the elicitors which then result in the expression of novel anti-pathogenic proteins. In order to harp the benefits of induced host resistance and build up a stable, long term resistance mechanism in the host plant against the pathogen, there is a need to identify the pathogen and understand its behavior under diversified conditions. In case of *Alternaria* blight, considerable morphological and pathological variations have been observed in *A. brassicae* under different environmental conditions (Goyal *et al*., 2011; Kaur *et al*., 2007; Singh *et al*., 2007; Patni *et al*., 2005; Meena *et al*., 2005, 2012; Ansari *et al*., 1989; Awasthi and Kolte., 1989). Vishwanath *et al*. (1999) reported the induction of resistance in susceptible *B. juncea* cv. PR-15 against extremely virulent *A. brassicae* isolate A (AbA) and reasonably virulent isolate C (AbC) from *B. carinata* cv. PPCS-1 by inoculating with avirulent isolate D (AbD) isolated from same cv. Around 60% reduction in the disease severity was observed against AbA and AbC by the induction of resistance. However, due to the limited studies in molecular characterization of the pathogen in India, we are still way behind to explore its diverse behavior.

In view of the above, the work has been initiated at our laboratory, to screen *A. brassicae* isolates from different geographical regions, and their characterization based on aggressiveness to specific host species, morphological and molecular characteristics. Variations have been recorded in colony morphology; diameter ranging from 32-68mm, surface texture from velvety to wooly, color from cream to dark olive green and sporulation varying from very less to intense. The host-pathogen interaction studies using four varieties of *B. juncea* and two of *B. rapa* have shown pathological variation among the isolates that differ in their aggressiveness from avirulent (few) to highly virulent (Aneja *et al*., 2012). Work is in progress for studying the molecular polymorphism through molecular markers. A thorough knowledge of diversity in the pathogen will not only help in characterizing and analyzing it phylogenetically, but also aid in inducing durable resistance against *Alternaria* blight, which can act as an environment friendly substitute to the traditional use of fungicides.

Conclusions

With the growing demand for edilble oil crops like brassicas and the immense losses caused by the fungal pathogens like *Alternaria*, there is an urgent need to curb this pressure and bring about the production, cultivation of disease free, high yielding, agronomically superior brassica crops. The advent of biotechnology has revolutionized the classical breeding process and has enabled the combining of traits from distant species for development of better germplasm. As discussed, a large number of

studies have reported the incorporation of disease resistance/ tolerance traits against *Alternaria* blight in oilseed brassicas but still there are areas that need exclusive attention and thorough research. There is a need to identify the variability of the pathogen at a larger scale covering different geographic locations and correlate the morphological, pathological characters with the molecular diversity of the pathogen. Emphasis has to be laid on studying the signal transduction pathways of Brassica-Alternaria interaction in order to understand the host response to the pathogen attack and identify the genes activated during pathogenesis. The identification of defense related proteins released in response to the *Alternaria* blight disease in other crops will allow the scientists to develop Alternaria resistant/tolerant transgenic crops and remarkably reduce the consumption of harmful fungicides being used. Overall the scenario demands for bringing together the traditional methods of disease control along with the modern biotechnological tools to develop integrated disease management strategies for building stable, disease resistant varieties of oilseed brassicas with superior agronomic traits that are not only high yielding but are also environment friendly.

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