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Analysis of variability determinants in *Alternaria-Brassica*-interactions

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Abstract

The pathogenic variability in four species of *Alternaria* is reported to be governed by determinant attributes viz., pathological, symptomatological, morphological, cultural, nutritional, biochemical, genetical, molecular, proteome level, thermo, and fungicidal sensitivity. Initially, observations on variability in cultural characteristics and pathogenesis of different isolates of *Alternaria* were made in *A. brassicicola*-vegetables, *A. brassicae–Brassica* and *A. raphani*–radish host –pathosystem. *A. alternata* strains, from Crambe showed differences in their physiological and pathological characteristics; strain B was most virulent, strain A was moderately virulent, and strain C was least pathogenic on Crambe. Three races of *A. brassicae* viz., RM-1, RM-2 and V-3 virulent on rapeseed-mustard group of crops were identified. While race RM-1 was avirulent only on *B. oleracea* var. *Capitata*, race RM-2 was avirulent on both *B. oleracea* var. *Capitata* and *B. oleracea* var. *Botrytis*. Race V-3, from vegetable crops was most virulent on the all host differentials. Thirteen *A. brassicae* isolates evaluated on selected winter rape cultivars differed in their virulence. Three *A. brassicicola* pathotypes infecting siliquae of cauliflower were designated as aggressive, less aggressive, and non pathogenic. *Alternaria raphani* isolates were grouped as "wild Type" and "Variant Type". Three *A. brassicae* isolates designated as A, C and D differed in their morphology, growth, sporulation, and cultural characteristics along with virulence on *B. carinata*. Four *A. brassicae* pathotypes from *B. juncea* were identified and designated as Bj-4, Bj-5, Bj-6 and Bj-7. Pathotypes DLK, RSR-I and GDP of *A. brassicae* were identified on the basis of their reaction on host differentials and symptomatological variations. Isolates of *A. brassicae* from crucifers were genetically similar in the highly conserved ITS region, but differed pathogenically. At molecular level, *Alternaria* isolates from crucifers showed polymorphism by RAPD analysis. Twelve polymorphic microsatellite loci (alleles ranged 2-10 with mean 3.5) were isolated from *A. brassicicola* isolates infecting crucifers. There were differences in the proteome level of virulent and avirulent *A. brassicae* from crucifers. In the absence of standard host differentials, some other parameters including symptomatology, morphology, cultural characteristics, nutritional requirement, biochemical changes, and thermo and fungicidal sensitivity were used to describe number of isolates in *Alternaria* –crucifers interactions without designating pathotypes.

Key Words: *Alternaria, crucifers, pathogenic variability, pathotypes, polymorphism, proteome level, RAPD analysis, Variability determinants*

Introduction

The most dynamic and significant aspects in host-pathogen interactions is that characteristics of individuals within a species are not "fixed" in their morphology, physiology, biochemistry and pathogenicity. During reproduction, all individuals are expected to be different from each other, and from their parents in a number of characteristics, although they retain most similarities with them and belong to the same species (Agrios, 2005; Mehta *et al.*, 2005a). When individuals are produced asexually, the frequency and degree of variability among the progeny are reduced greatly, but even then, certain individuals among the progeny will show different

characteristics. Three categories of such populations are of direct interest to the Brassicalogists of the world:

1. Populations that differ in their ability to attack particular varieties of Brassica hosts.

2. Populations differing in their physiological adaptations to specific environmental conditions, and

3. Populations differing in their ability to tolerate the effect of toxicants.

In Alternaria- Brassica host- pathosystem, following variability categories exist: although the genus *Alternaria* is an imperfect fungus, it shows genetic variability within a species which might be due to the existence of mutation, somatic hybridization, hetero-karyosis, uniform host selection, extensive dispersal, and/or of a cryptic sexual stage.

Historical developments

Initially, variations in cultural characteristics and pathogenesis of different isolates of three *Alternaria* species infecting Brassicaceae hosts were observed during 1952-1953 by Stoll (1952) in *A. brassicicola* - vegetables, by Van Schreven (1953) in *A.brassicae* – Brassica, and by Atkinson (1953) in *A. raphani*– radish host pathosystem. Therefore, it can be considered a beginning of research development on pathogenic variability in *Alternaria*–crucifer's host–pathosystem.

In *A. alternata* strains from Crambe (*Crambe abyssinica*), differences in their physiological and pathological characteristics have been reported. Strain A occurs on leaves, stem and siliquae, whereas strains B and C are mainly found on siliquae and leaves, respectively. In pathogenic ability, strain B has been reported to be most virulent, strain A as moderately virulent, and strain C as least virulent (Czyzewska, 1969, 1971). These strains have different temperature optima for sporulation. Strain A sporulates abundantly at 17-35^{O}C, whereas B requires 20-30^{O}C, temperature, and strain C will sporulates best at 12^{O}C (Czyzewska, 1970).

Alternaria brassicae is generally most virulent on all brassicaceous hosts. Preliminary reports on variability in this species were made from Holland (Van Schreven, 1953) and UK (Mridha, 1983). Isolates of *A. brassicae* from rapeseed (colza) showed differences in cultural growth on cherry agar and differed in their pathogenesis on seedlings. Similarly, Kolte *et al.* (1989, 1991) and Awasthi and Kolte (1989) distinguished three *A. brassicae* isolates viz., A, C and D, on the basis of their morphology, sporulation, growth, and cultural characteristics. On *B. carinata*, these isolates produce distinct types of lesions. Among the three isolates, isolate C is the most sporulating and isolate A the least. Unlike isolates B and C, isolate A produces chlamydospores. In a serological study, Kolte *et al.* (1991) indicated that the Pantnagar isolates A, C, and D resembled the Bihar isolates BHl, BH2, and the Kanpur isolate K, respectively.

Table 1. Physiological races of *Alternaria brassicae* (Saharan and Kadian, 1983)

Host Differential	Races/ pathotypes		
	RM1	RM2	V3
Brassica juncea	S*	S	S
B. rapa var. *Sarson*	S	S	S
B. rapa var. *Dichotoma*	S	S	S
B. rapa var. *Toria*	S	S	S
Eruca sativa	S	S	S
Raphanus sativus	S	S	S
Brassica oleracea var. *Capitata*	R*	R	S
Brassica oleracea var. *Botrytis*	S	R	S

*R= Resistant; S= Susceptible

None of these workers however, used different *Brassica* host differentials to distinguish *A. brassicae* isolates on the basis of their virulence. Saharan and Kadian (1983) used eight commonly cultivated *Brassica* species to distinguish isolates of *A. brassicae* in India. In a cross-infection study and differential interactions on different hosts, they distinguished three clearly separable isolates and designated them as RM1, RM2 and V3 races. Race RM1 from rapeseed-mustard was avirulent on *B. oleracea* var. C*apitata.* Race RM2 from *B. rapa* (= *B. campestris)* var. Brown and Yellow Sarson and *Eruca sativa,* was avirulent on both *B. oleracea* var. *Capitata* and *B. oleracea* var. *Botrytis.* Race V3 was virulent on all eight host species tested including radish, cabbage and cauliflower (Table 1). This study clearly indicated existence of distinct pathotypes in *A. brassicae* infecting different *Brassica* species. According to Mridha (1983), thirteen UK isolates of *A. brassicae* tested on selected cultivars of winter oilseed rape differed in their virulence.

Alternaria brassicicola is generally more common on vegetable crops than on oil-yielding Brassicas. Stoll (1952) characterized three isolates of this species from siliquae of cauliflower seed crop which showed highly aggressive, less aggressive, and non-pathogenic behaviour. However, highly aggressive isolates were less frequent (7.48%) than the moderately aggressive isolates (56.86%). Cultural and morphological variations in the isolates of this species show no distinction in pathogenic behaviour (Campbell, 1970; Campbell *et al.*, 1968; Changsri and Weber, 1963). Spontaneous occurrence of Albino mutants of this species has been observed (Campbell, 1970; Campbell *et al.*, 1968).

Alternaria raphani is the major pathogen of radish, but also occurs on other brassicaceous hosts. Atkinson (1953) obtained 312 isolates of this species from different geographical areas In Canada , classified them as "Wild Type" and "Variant Type", and found the former as being less virulent than the later. No differences were observed in their nutritional requirements for growth. In a later study, Changsri and Weber (1963) also did not find any variations in the *A. raphani* isolates from *B. nigra, B. napus* and *B. rapa* from different geographical areas of Canada.

Last decade of twentieth century and twenty first century can be considered as boom period for Alternaria-crucifers pathogenic variability research. During first two decades of the present century, pathogenic variability in *Alternaria*–crucifers system has been determined on various aspects including Pathological, symptomatological, morphological, cultural, biochemical, nutritional, thermal and fungicidal sensitivity, proteomic analysis, genetical, and molecular. However, no standard internationally acceptable parameters for selection of host differentials (single gene lines, isogenic lines), and nomenclature of pathotypes have been established. Each researcher has used his own different sets of host differentials and system of pathotype nomenclature. Gupta *et al*. (2004), however, attempted to use *B. juncea* varieties in their set of host differentials and designated pathotypes as Bj-4, Bj-5, Bj-6 and Bj-7. They have also tried to maintain parity in order of discovery of three *A. brassicae* pathotypes, RM-1, RM-2 and V-3 reported by Saharan and Kadian (1983).

Pathological Variations

Out of four species of *Alternaria* known to occur on crucifers, *Alternaria brassicae* (Berk.) Sacc. is more severe and variable (Verma and Saharan, 1994). Preliminary reports on variability in *Alternaria* species from rapeseed (colza) by Van Schreven (1953) in Holland and by Mridha (1983) in U.K. showed differences in cultural growth on cherry agar, and in their pathogenesis on seedlings. Although, pathogenic variability in *A. brassicae* has been observed by various workers (Verma and Saharan, 1994), information on existence of distinct pathotypes using standard host differentials is rather limited (Saharan, 1992 a, b). According to Mridha (1983), thirteen isolates of *A. brassicae* tested on selected cultivars of winter rape differed in their virulence. Similarly, Kolte *et al*. (1989; 1991) and Awasthi and Kolte (1989) also reported variability in *A. brassicae*.

None of these workers used different *Brassica* species to distinguish *A. brassicae* isolates on the

basis of their reaction on host differentials. Using eight commonly cultivated *Brassica* species as differentials, Saharan and Kadian (1983) distinguished three *A. brassicae* isolates and designated them as RM1, RM2 and V3 races which were found to be virulent on rapeseed and mustard group of *Brassicas*. Mehta *et al*. (2003) collected ten isolates from different agro climatic zones of India and cross inoculated them on a set of 17 host differentials. Among the ten isolates, isolate DLK was the most virulent infecting 16 differentials followed by RSR-1 and GDP, which infected 15 host differentials, but isolates could not be differentiated into pathotypes.

Using eleven *B. juncea* genotypes as host differentials Gupta *et al*. (2004) identified four distinct *A. brassicae* pathotypes viz., Bj-4 (BWL), Bj-5 (HSR), Bj-6 (RTK) and Bj-7 (REW). Pathotypes Bj-4 was most virulent infecting all 11 host differentials, and Bj-5 was least virulent infecting only six host differentials (Table 2). Incubation and latent periods also exhibited greater variability for host genotype x isolate interactions. Minimum incubation period of three days was required for pathotypes Bj-4 (BWL) and Bj-6 (RTK) on cultivars Varuna and RH-30. Vishwanath and Kolte (1997) also recorded differential interactions between *Brassica* crop species and *A. brassicae* isolates A and C, and avirulent isolate D. *Alternaria brassicae* isolate A showed significantly higher disease scores than isolate C on *B. napus* genotype PPNS1, *B. juncea* cv. PR15, *B. campestris* var. Toria cvs. PT 303, PT 30, *B. campestris* var. Yellow Sarson cv. T-151; isolate C showed significantly higher disease scores on *B. campestris* var. Yellow Sarson cv. PYST-6, *B. campestris* ssp *rapifera* cv. Turnip red and *B. alba* in comparison to isolate A. *Alternaria brassicae* isolate A is a highly virulent pathotype and isolate C is a moderately virulent pathotype. The toxigenicity study of 3 isolates on leaves of various hosts showed isolate A causing more severe symptoms than the isolates C and D at both 1:10 and 1:100 dilutions. Toxin from isolate D produced maximum symptom severity score on *E. sativa*, but failed to produce symptoms on leaves of other host cultivars. Isolate A toxin support significantly less seed germination and minimum plumule and radical lengths as compared to isolates C and D at 1:10 and 1:100 dilutions. Some differences among different genotypes, however, were observed with respect to

Table 2. Reaction of some *A. brassicae* isolates on *B. juncea* host differentials (Gupta *et al.*, 2004)

Host differentials	Reaction of isolate collected from various locations				
	Bawal (BWL)	Hisar (HSR)	Rohtak (RTK)	Rewari (REW)	Number of VI/HD
EC-129126-1	+	-	-	-	1
EC-322090	+	-	+	+	3
EC-322092	+	-	+	+	3
EC-322093	+	-	+	+	3
Varuna	+	+	+	+	4
EC-287711	+	-	+	-	2
ZEM-1	+	+	+	+	4
RC-781	+	+	+	+	4
RH-30	+	+	+	+	4
RH-8113	+	+	+	+	4
Rajat	+	+	+	+	4
Infectivity size	11	6	10	9	-
Pathotype identified	Bj-4	Bj-5	Bj-6	Bj-7	-

VI/HD= Virulent isolates per host differential; + = Denotes compatible interaction; - = Denotes incompatible interactions

seed germination and seedling growth with respect to toxins produced by the three isolates.

Fifteen *A. brassicae* isolates from rapeseed and mustard collected from different locations in Haryana (India) showed pathogenic diversity on seventeen host differentials under green house conditions (Kumar *et al*., 2003). Isolates CHR-I, CHR-II, JND-II, JHR, and SRS were most virulent infecting all 17 host differentials, followed by REW, RTK and SPT causing infection on 16 host differentials. Isolates BWL, BHI and KTL-II infected all differentials except *E. sativa,* and *B. alba,* whereas isolate HSR is least virulent producing symptoms only on thirteen host differentials. Out of seventeen host differentials, six differentials of *B. juncea, B. carinata, B. nigra, B. oleracea* var. *Botrytis*, *B. rapa* and *B. alba* differentiated 15 isolates in to 8 pathotypes/races. The eight pathotypes identified, CHR-I, CHR-II, JND-II, JHR and SRS were grouped in the first group; CHR-III, JND-I, and KTL-I in the second group, and BHI and KTL-II in third group. However, isolates BWL, HSR, REW, RTK, and SPT formed as individual group of pathotypes, respectively (Kumar *et al.* 2003).

In a cross inoculation study on 17 Brassica differential hosts and ten *A. brassicae* isolates from different locations of India, Mehta *et al*. (2003) revealed that each isolate behaved differentially. Isolate DLK was the most virulent one infecting 16 host differentials; RSR-I and GDP infected 15 host differentials. Isolates HSR-I, HSR-III, RSR-II, and isolates HSR-II, GNR, LDH and KNR, respectively, infected 14 and 13 differentials. The comparative study revealed that all differentials were susceptible. The genotype *B. alba* was susceptible to only four isolates, whereas *B. oleracea* var. *Botrytis* genotype was susceptible to only five isolates. The incubation period although varied from 3-13 days in isolates, most took 3-5 days to produce infection (Mehta *et al.*, 2003).

Analyzing virulence pattern of twenty four *A. brassicae* isolates on a set of 17 *Brassica* host differentials, Sangwan and Mehta (2007) reported varied virulence pattern. Isolates BTD, BBK, DSA, GNR, HSR and PNT had very wide virulence pattern infecting all seventeen-host differentials. Isolates BHP, BRT, GDP, HSRP, JPR, NGN, *B. alba* and Midas-1 and isolates *B. chin* and VRN infected 16 and 13 host differentials, respectively. *Brassica alba* variety 'Local' was the least susceptible as it was infected only by twelve isolates. In terms of incubation period, majority of the isolates required 3-5 days to initiate infection; isolates ASM, BHP, FRD, JPR and RSR required longer incubation period to produce symptoms on cruciferous vegetables. Eight host differentials differentiated all twenty-four isolates into fourteen pathotypes/races. Meena *et al.* (2012) measured aggressiveness of *A. brassicae* isolates in the form of lesion size, and discovered that *B. alba*, *B. juncea* (PAB, EC-399299), *E. sativa*, *B. carinata* and *B. napus* host differentials produced the least lesion size.

Pathogenic variability of 98 *A. brassicae* isolates was studied to identify virulent pathotypes for screening oilseed *Brassica* genotypes for resistance (Singh *et al.*, 2013). Eight variants were grouped on the basis of resistant and susceptible reactions, incubation (IP) and latent period (LP), lesion size, and disease severity. Isolates from Rewari and Fatehabad districts were able to infect all host differentials followed by Bhiwani district isolates with 10, and Rohtak isolate with only 7 differentials. The Rewari district isolate had the shortest IP and LP of 4-5 and 6-7 days, respectively, compared to 6-8 and 8-10 days, of Rohtak district isolates. Maximum Alternaria blight severity (24.6%) and maximum lesion size were also produced by the Rewari district isolates (group-1) compared to Rohtak district isolates (group-6). *Brassica juncea* var. Varuna contracted the highest disease severity (24.6%) and *B. alba* the least (2.9%). Rewari district isolates can, therefore, be used for screening oilseed *Brassica* germplasm. Alternaria blight tolerance of *B. alba* genotypes can be harnessed as donor parent for breeding resistance/ tolerant variety (Singh *et al.*, 2013).

Symptomatological Variations

The symptom variability exhibited by *A. brassicae* isolates on leaves of different *B. juncea* host

differentials is generally in the form of medium size, circular, greyish brown spots, 6-8 mm in diameter, with three regular concentric raised rings of dark brown colour; no yellow halo is produced (BWL). Another type of symptoms produced by other isolate are in the form of large, circular lesions (8-10mm), light green in center with concentric rings containing dark yellow halo around the spot (RTK). Third type of isolates produced large, irregular spots of 10 mm in diameter, dark brown in centre and grayish around the margins with concentric rings without yellow halo (HSR). In the fourth type no yellow halo is produced, medium size spots, light green in colour with only one concentric ring and very light pale ring around the spots (REW). The symptoms produced by four different isolates are stable and distinct on *B. juncea* host differentials; each distinct pathotype produces its own characteristic symptoms. This shows that symptom variability is a function of specific pathotype rather than host differentials genetic variations (Gupta *et al.*, 2004). These pathotypes are designated as Bj-4 (BWL), Bj-5 (HSR), Bj-6 (RTK) and Bj-7 (REW) in order of their discovery as suggested by Saharan and Kadian (1983). Kolte *et al.* (1991) identified three pathotypes A, C, D on the basis of virulence and some spot characters viz., spot colour, periphery colour, presence or absence of concentric rings, and central region of the spot. Goyal *et al.* (2013) reported pathogenic variability among *A. brassicae* isolates on host genotypes, on the basis of many qualitative characters including spot, and periphery colour, central point and its colour, presence or absence of concentric rings, yellow halo region, and one quantitative character viz., % disease severity. Three characters i.e., central point colour, presence or absence of central point, and yellow halo region of the spot should be used to study variability among *A. brassicae* isolates. Pathogenic variability test revealed that all the isolates from rapeseed-mustard were pathogenic or aggressive at different rates on all twelve host differentials, and produced different types of spots on different hosts.

Morphological and Cultural Variations

Mehta *et al.* (2003) identified various isolates collected different locations on the basis of the size of *A. brassicae* spores by designating them with the place of collection viz., Hisar (HSR-I)–*B. juncea* (var. RH-30); HSR-II- *B. campestris* var. Yellow Sarson (var. YSPb-24), and HSR-III-*B. tournefortii* (var. Local); Sriganganagar (GNR)-*B. juncea* (var. Kranti); Ludhiana (LDH)-*B. juncea* (var. RH-30); Kanpur (KNR)- *B. juncea* (var. RH-30); Dhaula Kuan (DLK)-*B. juncea* (var. RH-30); Gurdaspur (GDP)-*B. juncea* (var. RH-30), and R.S. Pura (RSR-I)- *B. juncea* (var. RH-30), and (RSR-II)- *B. juncea* (var.RH-30). The morphological characteristics of each isolate including size (length and breadth), number of septa, beak length, beak septa etc. were recorded from 15 days old culture. Based on spore length, isolates were categorized into four groups i.e. small (<100µm); medium (101-150µm); long (151-200µm); and very long (>200µm). The group-1 includes GDP; group-2- includes HSR-I, HSR-III, GNR, KNR, RSR-I; group 3- contains LDH, DLK, and group 4- includes HSR-II and RSR-II isolates.

The longest spore length was observed in the case of HSR-II and RSR-II (>200 µm), and the shortest in case of GDP (94.45µm). The breadth ranged from 13.5 to 36.0µm with the maximum spore breadth in GNR, and minimum in case of HSR-II. The number of horizontal septa varied from 5-13, with maximum being in case of HSR-II, and minimum in case of GDP. The maximum beak length was observed in case of HSR-II, and minimum in case of GNR (Mehta *et al.*, 2003). The number of septa in beak varied from zero to six (Plate-1). Conidial size variations in the *A. brassicae* isolates is due to nutrition rather than a characteristic pathological variation (Saharan and Kadian, 1983). However, glaring differences in conidial size are noticed among the isolates even when same medium is used for the growth of the isolates. It can be assumed that variation in the isolates may be inherent since isolates were collected from diverse agro climatic zones. It is evident from the data that each isolate differed in their conidial size. Hence, these variations in the conidial size indicate the existence of variability in this pathogen in India (Mehta *et al.*, 2003).

Conidial/spore measurement recorded on each isolate from Haryana (India) revealed that isolates

differed in their conidial size. The average conidial length varied from 118.62 to 194.52µm, being maximum of isolate RTK and minimum of isolate HSR. The range of conidial size varied from 81 to 300µm. The average breadth of conidia varied from 14 to 23µm, the thickest being of isolate JND-I and the thinnest of isolate CHR-III. The horizontal septations varied from 3 to 12 and vertical from 0 to 6. Some variations in beak size were also recorded. The average beak length varied from 39.99 to 119.07µm. The longest beak was of isolate RTK and smallest of JND-I. The average beak septations varied from 1.9 to 5.8 (Plate 2). These observations revealed that variation in the conidial size existed in Haryana, India (Kumar *et al*., 2003b).

Variations in morphology and cultural characteristics among 13 different geographical *A. brassicae* isolates in India were analyzed by Goyal *et al.* (2011). All the isolates showed high level of variability *in vitro* in respect to conidial length, width, beak length, and number of septa. Conidia of Nazirhat isolate (SS 04) are smallest with lowest number of septa. Substantial variations amongst the isolates were found in mycelial growth, and sporulation in different nutrient media, and artificial environmental conditions including temperature, relative humidity, light, and hydrogen ion concentration. Different optimum temperature ranges were found for mycelial growth (25-30^{O}C) and sporulation (15-35^{O}C). All thirteen isolates grew bested at 100% relative humidity. However, they sporulates the most at different relative humidity (40-100%). This reflects the adaptation of the respective isolates to the ambient conditions in the different cropping areas, which also may have induced the cultural variability. All the isolates did not grow and sporulate abundantly on the same nutrient medium. Asthana and Hawker's media were generally, better for all the isolates. Variation in optimum pH and light conditions for mycelial growth and sporulation were also observed. Cluster analysis of data on cultural variability among thirteen *A. brassicae* isolates found a close relationship among isolates from Uttar Pradesh, Uttaranchal and Haryana, but distantly related to other states (Goyal *et al*., 2011).

Morphological characteristic of different *A. brassicae* isolates revealed variation in growth, shape and pigmentation of colony, conidial measurements, and number of septa. Conidial length varied from 106.7 to 285.9µm, width from 33.5 to 57µm, and beak length from 41.4 to 180.0µm. Number of horizontal septa varied from 3.2 to 8.0 and vertical septa from 0.3 to 1.4. Different synthetic media showed profound variation in mycelial growth and sporulation indicating that the degree of sporulation in *A. brassicae* isolates is a function of nutrition. Pathogens, aggressiveness demonstrated the existence of considerable variations in the level of tolerance of *Brassica* species to *A. brassicae* (Meena *et al*., 2012).

Variations in morphology and cultural characteristics were observed among 32 representative Indian geographical isolates of *A. brassicae* from cauliflower and rapeseed-mustard (Sharma *et al*., 2013). All the isolates showed high level of variability *in vitro* in respect to conidial length, width, and number of septa. Conidia of isolates from Uttar Pradesh (CaAB U4) were the smallest with lowest number of septa. Substantial variation among isolates was also observed in mycelial growth, and sporulation on different nutrient media.

All the isolates do not grow and sporulate abundantly on the same nutrient medium. However, Potato Dextrose agar, cauliflower (host) agar, and carrot potato agar were suitable for all isolates. Cluster analysis of data on cultural variability among thirty-two *A. brassicae* isolates found a close relationship among isolates of both host viz., cauliflower and mustard. Isolates from Uttar Pradesh, Delhi, Haryana and West Bengal are found to be similar to each other whereas the Rajasthan along with Tamil Nadu and Kerala isolate are distantly related to others. All the isolates are pathogenic in nature but directly related to the cultural and morphological characteristics. These isolates are further molecularly characterized by using internal transcribed spacer region where all the isolates are found 56 % similar to each other, and 99% similar to the *A. brassicae* isolates present in NCBI database. *Alternaria brassicae* colonies varied in their cultural behaviour ranging from

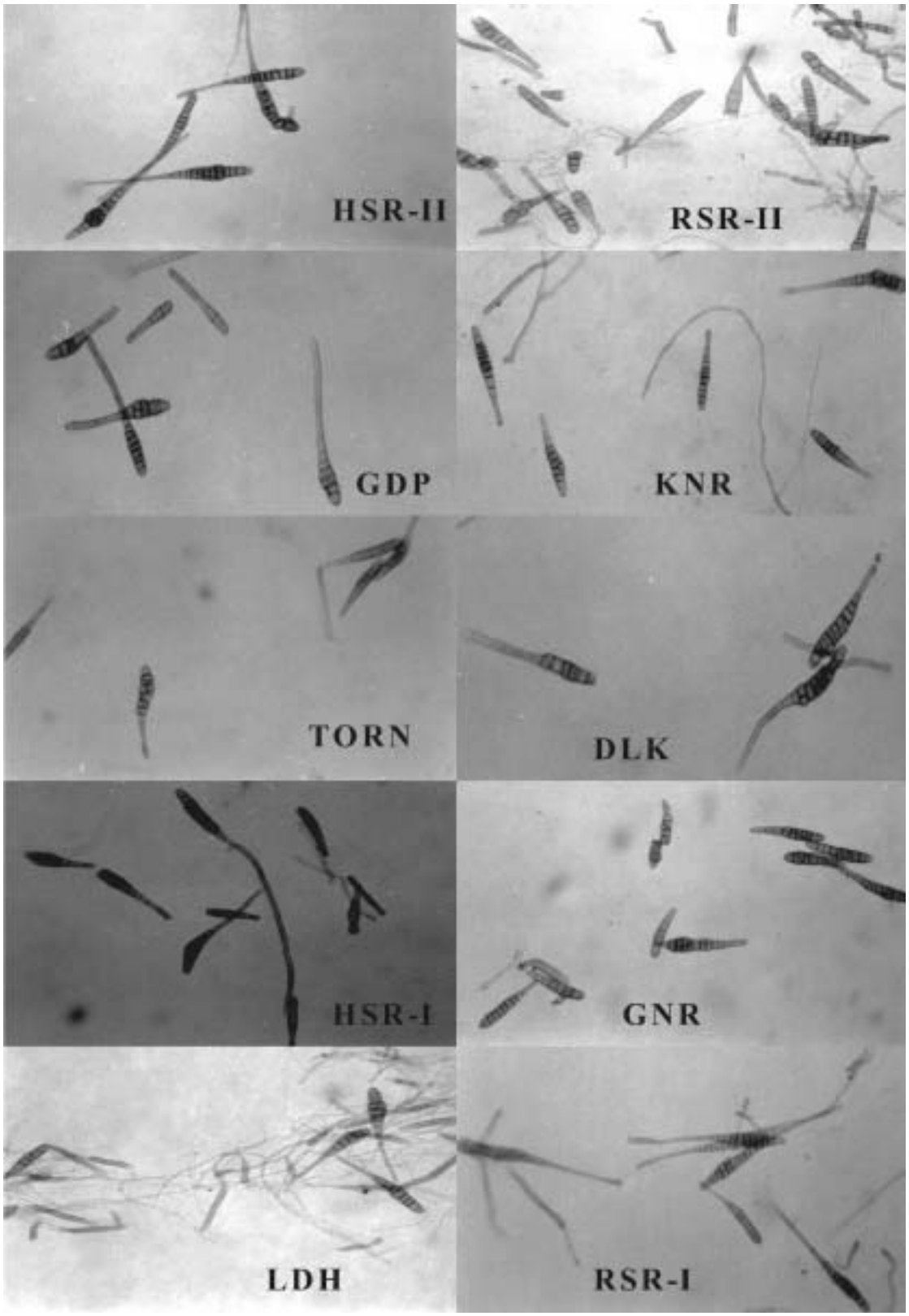

Plate 1: Morphological variations in conidia of *Alternaria brassicae* from India (Mehta *et al.*, 2003)

cottony, flurry to feathery, with smooth to rough margins, and white, off white to light brown in colour. The growth rate varied from slow, medium to fast, with fastest being in isolate KM and slowest in isolate JD. Significant morphological variations in conidial length, width (105 to 135 x 10 to 20 µm), and number of horizontal septa were observed. Isolates exhibited variations in disease severity, number and size of lesions. The dendogram analysis based on molecular (DNA, RAPD) basis reveals two groups at 14 % similarity coefficient. Group I composed of seven isolates namely VR, DV, P7, LM, P10, KR and ND with 18 % similarity (82% dissimilarity) while group II contained only three isolates namely JD, KA and AS with only 24% similarity (76% dissimilarity) (Pramila *et al.,* 2014).

Genetic Variability

Genetic variability in nucleotide sequence of ITS region of four *Alternaria* species *(A. brassicae*, *A. brassicicola*, *A. raphani*, *A. alternata*) infecting crucifers has not been recorded so far (Jasalavich *et al.*, 1995). Cluster analysis of pathogenic variability data reveals a close relationship between Nazirhat (SS 04), Jaipur (SS 07), Sachha Khera (SS-10) and Samalakha (SS-11) isolates. Use of 100 Random Amplified Polymorphic DNA decamer primers indicates genetic variability among thirteen *A. brassicae* isolates. Almost all the isolates show relationship according to their geographical origin except Sachha Khera (SS-10) and Hatikhuti (SS-05) isolates. Pantnagar isolate (SS-09) was found closely related to Sachha Khera (SS-10) isolate. No variability could be located among the *A. brassicae* isolates by Internally Transcribed Spacer–Amplified Fragment Length Polymorphism Molecular marker. Hence, pathogenic variability does exist among the isolates at the genomic level, but not in the highly conserved region of the genome of the pathogenic *A. brassicae* isolates (Goyal *et al*., 2013). However, Internal Transcript Spacer analysis done by Sharma *et al.* (2013) shows that all isolates are 90-100 % similar to each other, indicating genetic similarity among different *A. brassicae* isolates that vary pathogenically. Analysis of 26 RAPD primers revealed a high level of genetic variability among ten isolates of *A. brassicae* from different *B. juncea* cultivars (Promila *et al.,* 2014).

Molecular Techniques

RAPD analysis is easy, efficient, fast and reproducible than RFLP analysis in the detection of intra-specific variation in *A. brassicae*, *A. brassicicola* and *A. raphani* pathogenic to crucifers. Polymorphism within an *Alternaria* species by RAPD molecules marker has been described by many workers (Sharma and Tewari, 1995, 1998; Kumar *et al*., 2008). Observing polymorphism among *A. brassicae* isolates from different geographical regions of the world, Sharma and Tewari (1995, 1998), however, found low intra-regional variations among Indian and Canadian isolates with 75 % similarity. However, RAPD analysis of *A. brassicae* isolates from different geographical regions of India using more than one hundred primers suggested a high degree of polymorphism among isolates. The dendograms from both pathogenic and molecular analysis seem to indicate that the Pant Nagar (SS-09) and Hatikhuti (SS-05) isolates are quite different from the others, and the two dendograms follow the same trend (Goyal *et al.,* 2013). BLAST analysis of the ITS of 32 *A. brassicae* isolates conducted by Sharma *et al*. (2013) showed high similarity among the isolates available at the NCBI database.

Proteome Analysis

Two isolates of *A. brassicae* with significant differences in virulence have been characterized at the proteome level. The morphological observations indicated the Ontario isolate to be more virulent by virtue of increased disease severity score as compared to the UAMH7476 isolate. This was further confirmed through histological observations that showed extensive colonization of the host tissue by the highly virulent isolate. Mycelial protein profiles of the two differentially virulent *A. brassicae* isolates were compared using two dimensional gel electrophoresis (2DE) and mass spectrometry (MS) in order to identify proteins that may be responsible for the differences. Several differences in the mycelial proteomes of the two isolates were recorded. The proteins that were significantly abundant in the more virulent isolate included a protein with conserved actin related protein2/3 domain, enolase, malate dehydrogenase and serine

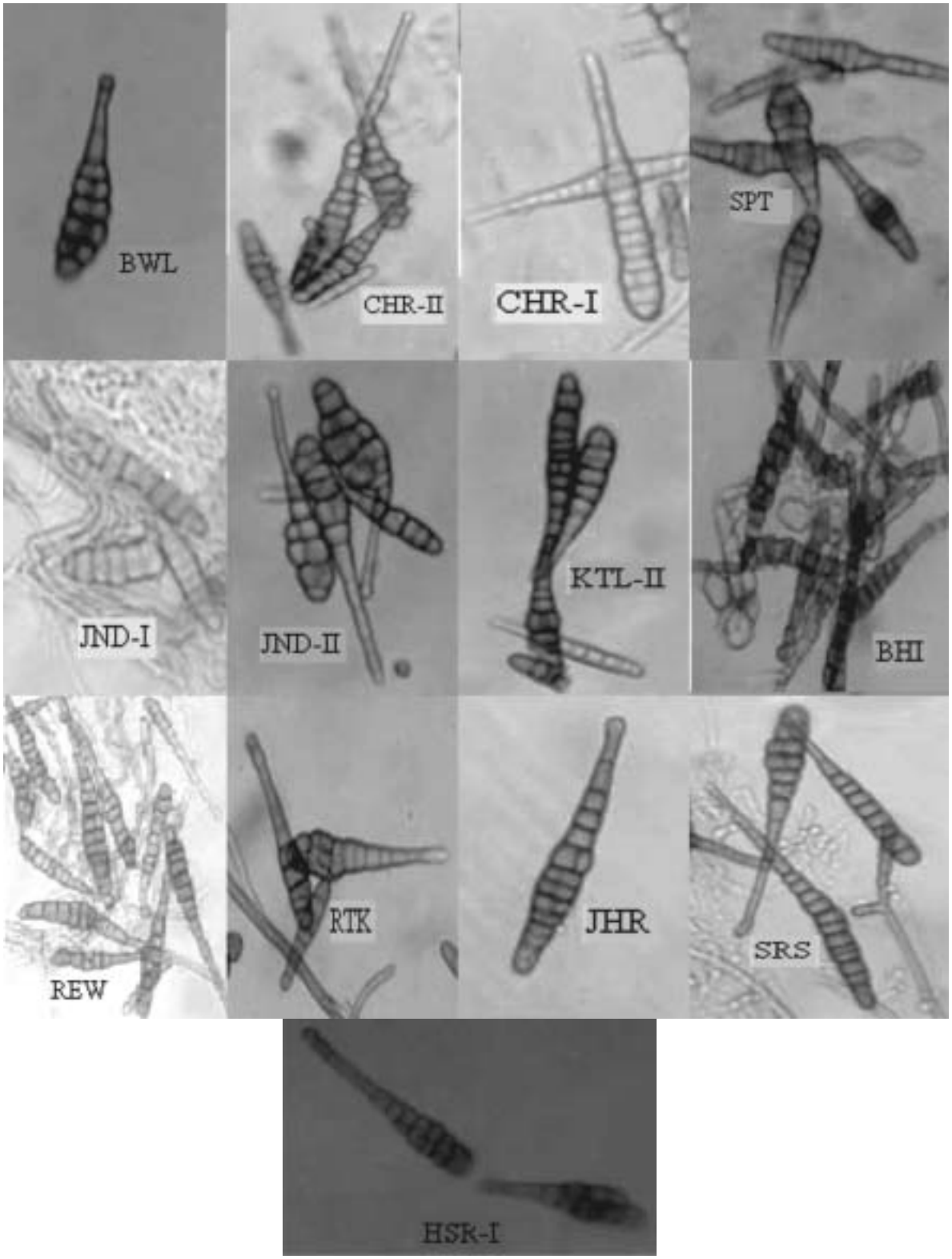

Plate 2: Morphological variations in conidia of *Alternaria brassicae* from Haryana (India) (Kumar *et al.,* 2003b)

protease. The differential protein expression pattern can be exploited to identify putative virulence and pathogenicity factors in *A. brassicae* (Sharma *et al*., 2010).

Nutritional Variability

Fourteen isolates of *A. brassicae* causing Alternaria blight in rapeseed–mustard were characterized by their responses to various carbon and nitrogen sources, as well as to pH. All the isolates behaved differentially in growth and sporulation in relation to different carbon and nitrogen sources. Isolates KTL-I, and BWL showed significantly higher growth on all the carbon sources, whereas isolate CHR-I yielded minimum growth and responded differentially to different carbon sources. Similarly, isolate REW showed more variation in sporulation than SPT, and HSR isolates. Amongst the nitrogen sources evaluated, growth was maximum on sodium nitrate followed by potassium nitrate, ammonium nitrate and glycine. Irrespective of the nitrogen source, Isolates KTL-I, and CHR-II produced the maximum whereas, SRS, and JD-II, the minimum radial growth. Isolates BWL, CHR-I, CHR-II, RTK, KTL-I, and SPT responded best on KNO_3 - amended medium, whereas, REW poorly on glycine; isolates BWL, CHR-II, JD-I, SRS, and SPT sporulated best on KNO_3, and HSR very poorly on glycine. All isolates grew better at pH 7.5, but sporulated best at 5.0 after 21 days of incubation. On the basis of nutritional behaviour, all the isolates were placed into two major groups: isolates BHI, JD-I, JHR, REW, RTK, SRS, CHR-I, CHR-II, JD-II, and HSR were placed in group-I, whereas, isolates KTL-I, KTL-II, and BWL formed the second group (Mehta *et al.,* 2005b).

Biochemical Variations

Biochemical constituents of *A. brassicae* isolates differ significantly (Khurana *et al*., 2005b). The isolate BWL contains the maximum and KTL-II the least ortho-dihydric phenols (O.D. phenols); isolate REW contains the highest amount of total phenols. The differences in the amount of total and ortho-dihydric phenols indicates the existence of variation in isolates. The isolate JD-II contains the maximum, and RTK, the minimum amount of both reducing and non-reducing sugars. The amount of reducing sugars also varied significantly between isolates. The isolates BWL, CHR-II, CHR-I, and RTK have significantly lower reducing sugars than other isolates. Vishwanath and Kolte (1997) reported that *A. brassicae* isolate containing high amount of carbohydrate is an indicator of virulence. The estimation of total RNA content revealed significant differences among the isolates; isolate KTL-II contained the maximum amount of RNA while SPT the minimum. On the basis of RNA contents, isolates can also be categorized in to three groups.

The protein content also differed significantly among isolates. Isolate CHR-I, and JD-I contained the maximum, and isolate KTL-I the least amount of proteins. Similarly, there are significant differences among the isolates in their free amino acids content; isolate JD-I, and SPT contained the highest, and lowest amount of free amino acids, respectively. *Alternaria brassicae* isolates containing higher amount of proteins generally contain moderate amount of free amino acids, and RNA, while those containing higher amount of RNA often contain moderate amount of proteins and free amino acids. On the basis of similarity in biochemical composition, all isolates were grouped into three categories: maximum, moderate, and minimum. Isolates BHI, JHR, KTL-I, SRS, CHR-I, and SPT formed the first group with maximum amount of bio-chemicals; isolates BWL, RTK, CHR-II, HSR, KTL-II, and REW were in the second group with moderate amount, while isolates JD-I, and JD-II were in the third group with the minimum amount of bio-chemicals (Khurana *et al*., 2005b).

Fungicidal and Plant Extracts Sensitivity

The variation in efficacy of fungicides in controlling *Alternaria* diseases of crucifers may be due to response of pathotypes prevalent in a region. According to Vishwanath and Kolte (1997), isolated A of *A. brassicae* showed more tolerance to Ziram, and Ridomil-72 at 50, and 100 ppm as compared to isolate C. Similarly, isolate A exhibited maximum tolerance followed by isolates C and D against mancozeb and iprodione. Efficacy of several fungicides and neem products were evaluated against fifteen *A. brassicae* isolates collected from different locations in Haryana, India. The

fungicides Kitazin was highly effective against all isolates in inhibiting the spore germination, which was followed by Dithane M-45 and Ridomil MZ-72. Similarly, amongst four neem products, Achook and Bio-neem were quite effective compared to Furpume and Nimbicidine. Variations were also observed among isolates in their sensitivity against fungicides. Isolates BHI, CHR-I, and CHR-III were sensitive to all the fungicides whereas JHR was sensitive only to Dithane M-45, Kitazin, and Bavistin. In case of neem products, HSR isolate was not sensitive to Achook whereas Bio-neem proved to be effective against CHR-I, CHR-III, HSR, KTL-I, KTL-II, REW, and SPT isolates. Based on their sensitivity against fungicides and neem products, all isolates fell more or less in the same group. Variations in the sensitivity of 14 *A. brassicae* isolates to extracts of Bougainvillea, garlic, Lawsonia, neem, mint, and Eucalyptus have also been observed (Kumar *et al.,* 2004; Khurana *et al*., 2005b).

Evaluation of efficacy of ten fungicides against *A. brassicae* isolates from various parts of India showed that Emisan-6, in general, proved most effective in inhibiting spore germination followed by Ridomil MZ -72; Sulfex, and Blitox proved least effective (Sangwan and Mehta, 2006). Isolates PNT, BHP, CAUL and *B. alba* were highly sensitive where as isolates FRD, *B. chin*, and ASM were the least sensitive. Spore germination among isolates varied from 17.1 to 43.59 per cent (Sangwan and Mehta, 2006). Differential behaviour of various isolates also indicated that isolates BHP and *B. alba* were more sensitive followed by CAUL, TRN, PNT, and HSR. The isolates RC-781, FRD, B. chin, ASM, GRN, and GDP responded similarly.

Thermal Sensitivity

Differences among *A. brassicae* isolates in relation to their sensitivity to different temperatures have been reported. In general, there is no significant differences in spore germination in the temperature range of 20-30^{O}C. As the temperature rise, the viability of the spores declines, and in most isolates, except KTL-I and KTL-II, spore loose their viability at 55^{O}C. In isolates KTL-I, and KTL-II, 5% spores remain viable even at 55^{O}C. Based on spore germination, isolates BWL, CHR-II, and JND-II are more resistant to high temperatures. In most isolates, only 10% spores germinated at 45-50^{O}C as compared to 35-43% in KTL-II, and SPT isolates. The drastic reduction in spore germination at 45^{O}C indicates that the pathogen cannot survive during summer months in northern India. Spores of only two isolates KTL-I, and KTL-II germinate at 55^{O}C indicating their capability to withstand high temperature, which can have a significant implication on their survival. The isolates, which withstand highest temperature probably, have genetic resistance to high temperature. On the basis of their thermal sensitivity, the isolates were grouped into three categories by Kumar *et al.* (2003a). Isolates BWL, CHR-II, and JND-I formed the first group since they lost 90 percent spore viability at 45^{O}C; isolates BHI,CHR-I,CHR-III, HSR, JND-I. JHR, REW, RTK, SRS, and SPT formed in the second group as they lost 90 percent spore viability at 50^{O}C; and isolates KTL-I, and KTL-II formed the third group, where only 5% spores survived at 55^{O}C.

Identification and Nomenclature of Pathotypes

Physiologic races or pathotypes of plant pathogens are identified on the basis of infection types produced by them on specific set of cultivars called "Differentials". The procedures and problems involved in the collection of diseased samples, isolation and purification of cultures, maintenance of specific isolates, techniques of inoculation, and scoring of infection types have been described (Verma and Saharan, 1994). In biotrophs host-patho-system (*Puccinia*–wheat, *Melampsora*–flax) norm and standards of selection of host differentials acceptable at international level has been followed, but it has not been met in the studies conducted in *Alternaria* – crucifers system. Selection of standard host differentials consists of a set of host varieties termed "differentials", supplemented differentials (additional host varieties), single gene lines, and near isogenic lines. Use of such a set of host differentials can clear the picture of presence, and identification of pathotypes in *Alternaria* spp. infecting crucifers.

Nomenclature of a race or pathotype has been done

Table 3. Determinants of variability in *Alternaria* infecting crucifers

Determinant attributes	*Alternaria* species	Host	Pathotypes /races	Reference
Pathological	*A. alternata*	Crambe	A, B, C	Czyzewska, 1969; 1971
	A. brassicae	Crucifers	RM-1, RM-2, V-3	Saharan and Kadian, 1983
	A. brassicae	Rape	13	Mridha, 1983
	A. brassicicola	Cauliflower	3	Stoll, 1952
	A. raphani	Radish	Wild Variants	Atkinson, 1953
	A. brassicae	*B. juncea*	Bj-4, Bj-5,Bj-6, Bj-7	Gupta *et al.*,2004
	A. brassicae	*B. juncea*	DLK, RSR-1, GDP	Mehta *et al.*, 2003
	A. brassicae	*B. juncea*	A,C,D	Vishwanath and Kolte, 1997
	A. brassicae	*B. juncea*	8	Kumar *et al.*,2003
	A. brassicae	*Brassica* spp.	10	Mehta *et al*., 2003
	A. brassicae	*Brassica* spp.	14	Sangwan and Mehta, 2007
	A. brassicae	*Brassica* spp.	8	Singh *et al*., 2008
Symptomatological	*A. brassicae*	*Brassica* spp.	Bj-4, Bj-5,Bj-6, Bj-7	Gupta *et al*., 2004
	A. brassicae	*Brassica* spp.	A,C,D	Kolte *et al.*, 1991
	A. brassicae	*Brassica* spp.	12	Goyal *et al*., 2013
MorphologicalCultural	*A. brassicae*	B. carinata	A,B,C,D	Kolte *et al*., 1989; 1991
and Nutritional	*A. brassicae*	*Brassica* spp.	4	Mehta *et al*., 2003
	A. brassicae	*Brassica* spp.	4	Goyal *et al*., 2011
	A. brassicae	*Brassica* spp.	5	Meena *et al*., 2012
	A. brassicae	Cauliflower and Rapeseed-mustard	2	Sharma *et al*., 2013
	A. brassicae	*B. juncea*	2	Pramila *et al*., 2014
	A. brassicae	Colza	-	Van Schreven *et al*., 1953
	A. brassicae	Rapeseed-Mustard	2	Mehta *et al*., 2005b
Biochemical	*A. brassicae*	Rapeseed-Mustard	3	Khurana *et al*., 2005b
	A. brassicae	Rapeseed- Mustard	3	Vishwanath and Kolte, 1997
Genetical	A. species (4)	Crucifers	Genetical similarity in ITS region	Jasalavich *et al*., 1999; Goyal *et al*., 2013
	A. brassicae	*B. juncea*	Vary pathogenically	Sharma *et al*., 2013
	A. brassicae	*B. juncea*	Isolates genetically variable	Pramila *et al*., 2014
Molecular	*A. brassicae*	Crucifers	Polymorphism in isolate by RAPD analysis	Sharma and Tewari, 1995; 1998
	A. brassicicola	Crucifers	-do-	Goyal *et al*., 2013
	A. raphani	Crucifers	-do-	Kumar *et al*., 2008
Proteome level	*A. brassicae*	Crucifers	Variation in protein level of virulent and avirulent isolates	Sharma *et al*., 2010
Thermo Sensitivity	*A. alternata*	Crambe	A, B,C	Czyzewska, 1970
	A. brassicae	*B. juncea*	3	Kumar *et al*., 2003
Fungicidal sensitivity	*A. brassicae*	Crucifers	A, C,D 6	Vishwanath and Kolte, 1997; Sangwan and Mehta , 2006
	A. brassicae	Crucifers	8	Kumar *et al*., 2004; Khurana *et al*., 2005

earlier as:1). Arbitrary numbers:- races are generally designated as number, or letters in an arbitrary manner, generally in order of their discovery e.g., cereal rusts; 2). Black's nomenclature:- A race is designated on the basis of its virulence on a host resistance gene, e.g., *Phytophthora*-potato system, race R1, R2, Race R1, 4 etc.; 3). Virulence formulae:- It is based on a race virulent and avirulent of particular gene for resistance e.g., the formula 6, 7, 10/5, 8, 9a, 11 for a race of *Puccinia* virulent on Sr 6, Sr7, Sr10, but avirulent on Sr5, Sr8, Sr9a and Sr11; 4). Habgood nomenclature, and 5). Virulence analysis. However, out of these criteria of nomenclature, *Brassica* researchers have adopted first method in isolation (not taken in account earlier reports) giving their own arbitrary numbers not keeping parity with others and order of discovery. Even researchers had hesitation in designating of pathotypes of *Alternaria* species except Saharan and Kadian (1983), and Gupta *et al.* (2004). The procedure and method of *Alternaria* species pathotypes designation adopted by Gupta *et al.* (2004) seems to be logical since it is based on interaction of a pathogen isolates with one specific genotype of a host species. Apparently, it meets gene for gene hypothesis in the absence of standard monogenic/ isogenic host differentials sets. The determinant attributes used by different workers for identification of pathotypes of *Alternaria* species infecting crucifers are given in Table 3.

The utility and advantages of race/pathotype identification in *Alternaria* can boost *Brassica* production through: 1). Development of resistant cultivars; 2). Identification of new genes for resistance; 3). Development of multigene resistant cultivars, and 4). Identification of favourable gene combinations (Singh and Chand, 1983).

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