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Effect of summer temperatures on survival of *Alternaria brassicae* in infected Indian mustard (*Brassica juncea*) debris and thermal death point variations amongst geographical isolates

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Abstract

During 2010-11 and 2011-12, experiments were conducted to determine the effect of summer temperature on survival of *Alternaria brassicae* (Berk.) Sacc. in Indian mustard (*Brassica juncea* L.) plant debris, and seeds in the field, and growth, sporulation and thermal death point variations (40-65°C) *in vitro* amongst 30 isolates collected from different geographical locations of India. Intact infected plant debris, and leftover seed after harvest from the surfaces of both unploughed and deep-ploughed fields yielded viable spores of pathogen on potato dextrose agar (PDA), and moist blotting paper ranging from 13.3 to 84.5%. The highest per cent viability (84.5%) was obtained on moist blotting paper from infected seeds collected from deep-ploughed (30cm depth) mustard fields. In both years, the maximum summer atmospheric temperature varied between 47-48°C in the experimental area. Survival of *A. brassicae* in high proportion during the study, clearly showed that the primary infection in Indian mustard came from inoculum survived in over-summered (April to June) infected plant debris and seeds. Results of growth and sporulation experiments after exposure of culture for 10 minutes at temperatures ranging from 40-65°C, showed that mycelial growth as well as sporulation decreased significantly with increase in temperature. Very sparse to poor sporulation of 16 isolates at 60°C, and complete absence of growth of 9 isolates at 60°C and all 30 isolates at 65°C, showed that the thermal death point temperature of *A. brassicae* is between 61-65°C. Results of survival and thermal death point could be utilized in eliminating primary inoculum in the farmer's field through appropriate strategy.

Key words: *Alternaria brassicae, oilseed Brassica, survival, thermal death point*

Introduction

Foliar diseases are one of the most important limiting factors for cultivation of oilseed Brassica in tropical and sub-tropical areas in India. *Alternaria brassicae* (Berk.) Sacc. causes heavy economic losses to oilseed Brassica (Latin name) in terms of seed yield up to 34 per cent (Saharan *et al*., 2015a). Severity of disease doesn't only depend upon the favorable weather (Shrestha *et al*., 2005), but also on the available inoculum load on over-summered infected crop residues (stubble and straw) in the field (Verma and Saharan, 1994). The optimum temperature for maximum sporulation of *A. brassicae* and *A. raphani* was between 23-25°C and survived up to the extremes of 37°C (Verma and Saharan, 1994). While, Saharan (1984) reported that *A. brassicae* gets eliminated from seeds of oilseed brassicas during storage in the months, April to September at 25-35°C. Rosette and silique formation stages of plant growth were observed as critical for AB initiation and peak of disease severity, respectively in *B. juncea* (Meena *et al*., 2004).

Information regarding the survival of *A. brassicae* in infected mustard crop stubbles, and seeds over-summered in the field is not clear. Recommendations for deep summer ploughing to reduce the inoculum (Adiver and Kumari, 2010) are also not always effective because the ploughed soil may be the shelter for pathogen survival due to comparatively low temperature. Sunlight expends a lethal effect on all microorganisms located on the surface of soil. This effect may be due to desiccation and/or ultraviolet

(UV) irradiation (Van Donsel *et al.,* 1967). If the ecological surroundings are not favorable for the expansion of disease, even severely-infected seed may develop little or no disease (Cafati and Saettler, 1980). Hence, it may be possible to reduce the chances of survival of the pathogen as a result of direct exposure of plant debris to sunlight at high temperature on soil surface. Crop remains are not only a cause of inoculum of various diseases but it is also a substrate for numerous non-pathogenic micro-organisms (Jenkyn *et al.*, 2004).

Recently, the intensive cultivation also restricts fallow cropping system. The severity of Alternaria blight disease is changing in different regions of India due to either genetic variation in the host and pathogen, or favorable weather conditions (Meena *et al*., 2010; 2012). The pathogen can survive even at high temperatures due to its varying thermo-sensitive nature.

Alternaria brassicae isolates were collected from different geographical locations covering all oilseed brassica growing areas in India to study the extreme limit of high temperature for survival. It has been reported that the phytotoxin production build upon the nature of individual isolate in addition to an ecological surroundings (Otani and Kohmoto, 1992). Chung (2012) studied that *Alternaria* has developed a remarkable flexibility and uniqueness in the signaling pathways in order to react to different ecological stimuli and to flourish within host plants. Consequently, there may be chances of variability among *A. brassicae* isolates to thermo-sensitivity (Kumar *et al*., 2004; Saharan *et al*., 2015b).

Therefore, in the absence of a durable host resistance, this study was conducted to determine the survival of *A. brassicae* in over-summered infected mustard plant debris, and seeds in the field. Simultaneously, the effect of high temperature exposure *in vitro* on growth, and sporulation of the large number of collected isolates from different geographical regions in India was also determined. Experiment on the effect of *in vitro* exposure of cultures to high temperatures on growth and sporulation to identify the thermal death point of isolates, possibly will conclude the extent of temperature vital to kill the thermo-tolerant isolate of *A. brassicae* in the available population.

Materials and Methods

Experiments on survival of *A. brassicae* on over-summered infected mustard plant debris, and seeds, and effect of *in vitro* exposure of cultures to high temperatures on growth to identify the thermal death point alongwith variations among isolates were conducted at the Indian Council of Agricultural Research-Directorate of Rapeseed-Mustard Research (ICAR-DRMR), Sewar, Bharatpur, Rajasthan, India.

Pathogen isolates

A total of 30 *A. brassicae* isolates collected from different geographical regions in India were obtained from the ICAR-Directorate of Rapeseed Mustard Research, India repository. To obtain a uniform culture of the isolates, a two-step inoculation method was used. *Alternaria brassicae* isolates from the stored collection were initially inoculated onto potato dextrose agar (PDA), and after suitable growth and sporulation, using a 2 millimetre diameter cork borer, a uniform piece of mycelia from the edge of the colony was transferred to the middle of a fresh agar dish using a sterilized needle. Cultures were maintained on PDA with alternate culturing on Brassica leaf broth medium to maintain their virulence (Meena *et al*., 2012).

Survival of pathogen

For pathogen survival studies, samples of over-summered-infected crop debris, including seeds were collected from 10 randomly selected fields during the month of June before the start of rainy season. Plant parts (stubble, threshed straw, whole plant, silique husk, and seeds) were collected from upper surface of ploughed, un-ploughed, and 30cm depth of ploughed fields. The samples were collected including Indian mustard silique husk from mechanically threshed material, plant debris from manual threshed material, plant debris from deep ploughed field surface (15cm depth), threshed silique husk from upper surface, stubbles from unploughed field, and seed from ploughed Indian mustard field (30cm depth) through sieved soil. The samples were kept under laboratory condition till analysis. For

isolation of the fungus, seed and tissue samples were surface sterilized. The infected tissues along with adjacent small, unaffected tissue were cut into small pieces (2–5 mm squares). By using flame-sterilized forceps, plant tissues transferred to sterile Petri-dishes containing sodium hypochlorite (1%) solution for a period of 60 seconds followed by subsequent three washings with sterilized distilled water. The collected seeds and debris samples of mustard were analyzed for the presence of *Alternaria* species by moist blotter method following the International rules for Seed Testing (ISTA, 1996). For isolation of fungi using moist blotting paper (sterile filter plate) method, plates of 90 mm size were autoclaved at 121°C on 15lbs/square inch pressure for 30 minutes after placing three layers of filter paper in the bottom. The filter papers were moistened with 10-15 milliliter sterile distilled water before placing the seed. Two hundred samples were tested for each location maintaining four replications. Twenty-five seeds or pieces of debris were placed on three layers of moist blotting paper in each glass Petri-dishes. The Petri-dishes were incubated at 25±2°C under 12/12 hours light and darkness cycle for 7 days. Each sample was observed under Strereomicroscope in order to record the presence of the fungal colony on 7 days after incubation based on growth habit. In doubtful cases, temporary slides were prepared from the fungal colony and observed under a compound microscope.

In another method, 3-5 sterilized pieces were aseptically transferred to Petri-dishes containing PDA medium supplemented with streptomycin per Petri-plate and incubated at 25±2°C. A portion of mycelium developing in the medium was transferred to the agar slants for purification and storage for further examination. Fresh subcultures were made by transferring hyphal plugs on to PDA plates, incubated at 25±2°C, and percentages of plant and seed samples yielding *Alternaria* species were calculated. Identification of the pathogen was confirmed by cultural and morphological characteristics under the microscope based on the procedure recommended by Ellis (1971). The results were presented with pooled mean of two years as the percentage occurrence of the pathogen for individual sample by both the methods.

Thermal death point

Experiment on thermal death point was conducted to determine the effects of short-term exposure of high temperature on *A. brassicae* culture in eliminating the pathogen from infected seeds or from debris through pre-sowing soil heating treatments. The thermal death point of *A. brassicae* isolates was determined using the method of Nelson and Wilhelm (1958) with certain modifications. Seven-days fresh culture of all the 30 isolates was exposed for 10 minutes at 40, 45, 50, 55, 60 and 65°C, before a subculture on PDA plates for incubation at 25±2°C by maintaining 12 hour dark and light conditions for 10 days, and determining the mycelium growth f ollowed by sporulation indices using a Neubauer improved haemocytometer. The four sporulation indices used were: + = 1-5; ++ = 6-10; +++ = 11-15; and ++++ = more than 16 conidia/ microscopic field. These four sporulation indices can also be grouped as very poor, poor, moderate and excellent categories. *Alternaria brassicae* isolates were also categorized on the basis of their thermal tolerance among isolates.

Results and Discussion

Survival of pathogen

Maximum percentages of *A. brassicae* survival of 73.3 and 84.5 per cent on PDA and moist blotting paper, respectively, were obtained in infected seeds obtained from ploughed fields after harvest of Indian mustard (Table 1). *Alternria brassicae* survival percentages from Indian mustard silique, and plant debris from threshed material, mustard debris from deep-ploughed field surface and mustard stables from unploughed fields, respectively, were 33.3, 34.4, 41.1, and 25.5. Though, the atmospheric temperature reached up to 48.8°C during summer 2012. Results showed that mustard silique husk from the field surface supported least amount of pathogen survival may perhaps be due to direct contact to the sunlight. Percentage recovery of the pathogen on both PDA and moist blotting paper, generally, was similar. Our findings supported the results of Humpherson-Jones (1989), who suggested that *A. brassicae* over-summered in infected plant debris and seeds act as a source of primary inoculum in the newly ploughed crop. Ansari

Table 1: Survival of *Alternaria brassicae* on plant debris and seed of Indian mustard in summer

Location of samples collected	% Alternaria survival (pooled mean of two years)	
	PDA	Blotting paper
Mustard silique husk from threshed material	33.3	33.3
Mustard debris from manual threshed material	34.4	37.7
Mustard debris in deep ploughed field surface (15 cm depth)	41.1	44.3
Threshed mustard husk from surface	13.8	20.5
Unploughed mustard field stubbles	25.5	13.3
Seed from ploughed mustard field (30 cm depth)	73.3	84.5

Date of sampling : 22 June 11; 15-Jun-12

Table 2: Mycelial growth (mm) of *Alternaria brassicae* isolates exposed for 10 minutes at different temperatures 7-days after incubation

Alternaria brassicae isolates	Radial mycelial growth (mm) at different temperature (°C) exposure for 10 minute						
	40°C	45°C	50°C	55°C	60°C	65°C	Control (without temperature exposure)
BAB-02	19.1	15.7	14.5	13.1	11.2	0.0	30.8
BAB-03	23.3	21.7	19.8	17.0	18.0	0.0	33.0
BAB-04	22.7	20.3	18.8	16.5	14.7	0.0	31.0
BAB-06	18.7	17.1	16.5	15.8	12.7	0.0	27.4
BAB-08	16.5	11.6	11.2	0.0	0.0	0.0	32.4
BAB-12	18.6	13.2	12.2	15.7	0.0	0.0	30.6
BAB-18	33.8	32.4	31.3	24.5	18.8	0.0	34.8
BAB-19	25.5	22.4	20.4	19.9	19.0	0.0	30.2
BAB-20	13.7	11.3	9.0	7.8	0.0	0.0	15.8
BAB-23	23.6	22.9	21.1	19.5	16.1	0.0	24.1
BAB-24	11.0	10.5	9.0	8.1	0.0	0.0	26.8
BAB-26	10.0	8.0	7.5	7.0	0.0	0.0	24.3
BAB-29	28.3	26.5	25.3	23.3	18.8	0.0	31.4
BAB-39	12.7	11.3	9.5	8.3	6.5	0.0	28.0
BAB-40	19.3	18.8	17.4	16.5	15.5	0.0	26.7
BAB-41	25.9	23.7	21.3	19.8	16.5	0.0	25.8
BAB-42	26.7	24.2	22.3	21.1	19.0	0.0	31.0
BAB-43	32.5	29.7	28.9	22.7	15.5	0.0	24.9
BAB-44	26.5	16.3	16.4	16.8	15.0	0.0	23.6
BAB-45	20.7	15.3	14.3	14.1	13.9	0.0	35.7
BAB-47	24.3	23.7	22.9	21.8	18.6	0.0	31.0
BAB-48	19.1	18.9	18.5	17.3	11.9	0.0	28.4
BAB-49	13.1	10.8	8.8	10.0	0.0	0.0	38.2
BAB-50	25.1	24.7	22.5	20.2	17.2	0.0	35.6
BAB-51	10.0	9.2	8.0	7.3	0.0	0.0	24.0
BAB-52	20.6	17.1	15.1	13.4	12.9	0.0	29.6
BAB-53	20.6	15.6	14.1	10.3	0.0	0.0	30.0
BAB-54	12.8	9.8	9.3	8.8	7.8	0.0	14.7
BAB-55	22.3	20.1	17.3	14.9	12.9	0.0	28.8
BAB-56	19.3	14.6	10.0	8.2	0.0	0.0	28.4

et al. (1989) reported that the pathogen remained viable in diseased plant debris, and seeds of infected plants which served as primary sources of inoculum. *Alternaria brassicae* survived in seeds while stored at room temperatures (11-25°C) for 10 months and at 30°C in an incubator for 6 months (Shrestha *et al*., 2003). Based on the results, the direct exposure of sunlight on soil surface in unploughed fields probably killed the pathogen on infected plant debris. Nowadays, under changing climate situations the plant residues does not decompose in soil as a result of the light rainfall which could carry the pathogen spore for the next crop season. Karavina *et al*. (2008) reported that the soil and crop debris inocula had no effect on disease incidence and severity, but provided inocula for disease development.

Thermal death point

Significant difference in mycelial growth and sporulation indices amongst the all 30 *A. brassicae*

Table 3: Sporulation of *Alternaria brassicae* isolates incubated at 25°C after pre-inoculation exposure for 10 minutes at different temperature

A. brassicae	Sporulation index* at different temperature (°C) exposure for 10 minute						
isolates	40°	45°	50°	55°	60°	65°	Control
BAB-02	+	++	+	++	+	No growth	++
BAB-03	+++	++	+++	+++	+++	No growth	+++
BAB-04	+++	+	+++	+++	+++	No growth	+++
BAB-06	++	++	++	++	+	No growth	+
BAB-08	+++	+	+	+	-	No growth	+
BAB-12	++	+	+	+	-	No growth	++++
BAB-18	+++	++	++	+++	++	No growth	++++
BAB-19	+++	+++	+++	+++	+++	No growth	++++
BAB-20	++	+	++	+	-	No growth	+
BAB-23	+++	+++	+	++	+	No growth	++
BAB-24	++	+	+	+	-	No growth	+
BAB-26	+	+	+	+	-	No growth	++
BAB-29	+++	++	++	+	+	No growth	+++
BAB-39	++	++	+	+	+	No growth	+
BAB-40	++	++	+++	++	+	No growth	+++
BAB-41	++	+++	++	++	+	No growth	++
BAB-42	+++	++	++	+++	++	No growth	+
BAB-43	+++	++	++	++	+	No growth	+
BAB-44	+++	+	+	+	+	No growth	++
BAB-45	++	++	++	+	+	No growth	+++
BAB-47	+++	+++	++	++	+++	No growth	+
BAB-48	++	+	+	+	+	No growth	+++
BAB-49	+	++	++	+	-	No growth	++
BAB-50	++	+++	+++	++	+++	No growth	+++
BAB-51	+	+	+	+	-	No growth	++
BAB-52	++	++	+	+	+	No growth	++++
BAB-53	++	+	+	+	-	No growth	+++
BAB-54	+	+	+	+	+	No growth	+
BAB-55	++	++	+	++	+	No growth	+
BAB-56	++	+	+	+	-	No growth	+

*+ = 1 to 5; ++ = 6 to 10; +++ = 11 to 15; ++++ = above 16 conidia/ microscopic field

isolates were observed not only in the temperature-exposed, but also in the unexposed control cultures (Table 1). Poor mycelial growth of eight isolates (BAB-08, BAB-20, BAB-24, BAB-26, BAB-39, BAB-49, BAB-51, BAB-54 and BAB-56) indicated highly sensitive response to temperature at 40°C over the control (without exposure). Five isolates (BAB-18, BAB-29, BAB-43, BAB-47 and BAB-50) indicated tolerance to temperature with maximum mycelial growth up to 60°C exposure. While, Kumar *et al*. (2003) reported that only 5% spores survived of two isolates of Haryana at 55°C.

Sporulation indices of five isolates (BAB-03, BAB-04, BAB-19, BAB-47 and BAB-50) upto 60°C, and five isolates (BAB-03, BAB-04, BAB-18, BAB-19 and BAB-42) upto 55°C, were same indicating virtually no effect of high temperatures on survival. Total five isolates (BAB-03, BAB-04, BAB-19, BAB-47 and BAB-50) producing moderately high sporulation indices (+++), significantly decreased with increase in exposed temperatures; numbers of such isolates were BAB-06, BAB-09 BAB-12, BAB-15 and BAB-16 at 40, 45, 50, 55, and 60°C, respectively. Complete absence of growth of all 30 isolates at 65°C suggests that the thermal death points of 21 remaining isolates are between 61° to 65°C. Our findings supported the results of Weimer (1924) who reported survival of *Alternaria* species in infected crucifer seeds for 8 months in the laboratory, but the death of the pathogen with hot water treatment at 56°C in 10 minutes. It is a function of time and temperature relationships, in which the temperatures commonly reach up to 95°F to 140°F (35-60°C) depending on soil type, season, location, soil depth and other factors. Contrarily, Shivpuri and Siradhana (1989) reported that the mustard seeds showing 42% infection of *A. brassicae* in April became completely free when stored at 45 and 35°C for two months, respectively. Of the 30 isolates tested, death of 9 at 60°C and all the 30 isolates at 65°C, raise the questions regarding the thermal death point which needs to be further investigation. Contrarily, Kumar *et al*. (2003) reported that the pathogen cannot survive during summer months in northern India. Such variable thermal death points could be utilized in eliminating or reducing the inoculum load from the infected seeds or plant debris in the field will also require more studies.

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