



## Media Constituents as a Differential Factor for Thermal Stress Susceptibility in *Alternaria brassicicola* Causing Dark Spot Disease of *Brassica*

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### Abstract

The research was conducted during March, 2019 at Bihar Agricultural College, Sabour (Bhagalpur), India to study the effect of culture media and temperature on the mycelial growth and sporulation of fungal pathogen *Alternaria brassicicola* isolated from Indian mustard. The radial growth of the fungi and conidia concentrations (number of conidia ml<sup>-1</sup> of suspension) were assessed on five culture media: potato dextrose agar (PDA), Czapek dox agar (CDA), Richard's agar (RA), Asthana and Hawker's agar (AHA) and Minimal media (MM) at three temperatures viz.; 15°C, 25°C and 35°C. The interaction of these two factors with growth parameters of the fungi was analysed to find alterations in growth patterns and spore production. Among the two factors examined, temperature most significantly influenced both radial growth and sporulation capacity of the pathogen. Among the five tested media, PDA was recorded as the most suitable for *in vitro* growth of *A. brassicicola* at 25°C (85.20 mm), while maximum mycelial growth (34.67 mm) at 35°C was recorded on CDA medium after 7 days of incubation. Cations like Fe<sup>+2</sup> and Mg<sup>+2</sup> were found to be beneficial in sustaining fungal growth at 35°C. Sporulation was best on RA media (14.47×10<sup>4</sup> spores ml<sup>-1</sup>) at 15°C. Mycelial growth was limited at 35°C (24.58 mm) followed by 15°C (30.10 mm) with optimum at 25°C. The results indicate that limiting magnesium and/or iron could inhibit growth of *A. brassicicola* under thermal stress.

**Keywords:** *Alternaria brassicicola*, growth media, temperature stress, sporulation

### 1. Introduction

Fungi are a vivid group of organisms that require several specific nutrient elements in order to grow and proliferate in or on diverse culture media. They are isolated on specific culture medium with the objective of fungal multiplication, preservation or microscopic examination and biochemical and physiological characterization (Rama and Quandt, 2021). A wide range of media are deployed for isolation of different groups of fungi and these variedly influence their vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific culture medium, pH, incubation temperature, light and moisture availability (Nasreen et al., 2017). Culture medium and temperature preferences of fungi are known to vary among species and affect its morpho-cultural characteristics. Some fungi prefer organic media incubated at low or high temperature for growth and sporulation, while others proliferate better on more selective media. Mycelial growth and sporulation are

often abundant on organic substrates rich in carbohydrates which are source of energy for cell metabolism, growth and sporulation of fungi (Pradeep et al., 2013). Studies on extremotolerant fungi have suggested their ability to grow in acidic, metal-rich culture media that simulate the conditions of their natural habitat (Alvarez-Perez et al., 2011). Examination of alternative nutrient sources is therefore an on-going quest to cater to the specificity in requirements of fungal growth (Kuhn and Ghannoum, 2003; Kumara and Rawal, 2008; Basu et al., 2015). In general, the requirements for growth of fungi are less restrictive than that for their sporulation. Several studies have shown that *Alternaria brassicicola* is responsible for leaf spots, blight and fruit rots in various economically important plants around the world (Kolte, 1985; Pattanamahakul and Strange, 1999; Nowicki et al., 2012; Nowakowska et al., 2019). The pathogen *Alternaria brassicicola* infects a wide range of host plants such as cole crops, crucifers, *Brassica* etc. on which it causes grey or black leaf spots (Nasreen et al., 2017; Hazowary et al., 2023). Blagojevic et al. (2020)

investigated four pathogens viz., *Alternaria brassicae*, *A. brassicicola*, *A. japonica* and *A. alternata*, associated with leaf spot disease in rapeseed and concluded that *A. brassicicola* is the most virulent amongst them with the highest sporulation intensity. Thermal stress is known to variedly alter the growth patterns and is quite likely to affect the pathogenicity of plant pathogens (Siebold and Tiedemann, 2012; Damialis et al., 2015). With the alarming rise global temperatures, the geographic range of several plant pathogens is expanding, exposing new areas and plant species to diseases previously limited to warmer climates (Lahlali et al., 2024). Recent report too suggests increase in virulence in *A. brassicicola* under projected warmer temperature regimes pertaining to enhanced activity of cell wall-degrading enzymes (Sinha et al., 2021). Norlia et al. (2020) also showed a significant effect of temperature on growth and aflatoxin production by *Aspergillus flavus*. Besides, in the context of host-pathosystem, there is increasing evidence that the availability, deficiency or toxicity of transition metals might pose a significant impact on plant disease development (Solomon et al., 2003; Fones and Preston, 2013). Nutrients can influence the plant growth and physiology causing alterations in the microclimate and thus affect the infection and sporulation of pathogens (Dordas, 2008). This study was thereby conducted with the objective to contribute to the knowledge of the biology of *Alternaria brassicicola* and reduce the role of cations or anions towards limiting growth under higher temperature..

## 2. Materials and Methods

### 2.1. Sample collection, isolation and identification of fungi

Infected leaves of Indian mustard (*Brassica juncea*) were collected from the mustard field, Bihar Agricultural College, Sabour (Bhagalpur), India during March, 2019. Geographically, Sabour is situated at 87°2'42" East longitude and 25°15'40" North latitude, at an altitude of 46 meters above mean sea level (MSL) in the middle of the Indo-Gangetic plains of North India. A single isolate of *Alternaria brassicicola*, the causal organism of Alternaria leaf spot of mustard was used in all the investigations. Isolation of the fungi was carried out on potato dextrose agar medium (PDA) supplemented with streptomycin at 300 mg l<sup>-1</sup> at 25±2°C for 7 days (Agostini and Timmer, 1992). Slides of the culture were prepared in lactophenol and cotton blue and examined under compound microscope (Olympus BX-41). On the basis of morphology of conidia and conidial chains, as described by Simmons (2007), the pathogen was identified as *A. brassicicola* (Figure 1). After confirming the pathogenicity, the virulent culture was maintained on PDA slants for further studies. The culture was periodically transferred at one-month interval to fresh PDA slants and stored in a refrigerator at 4 °C for further studies.

### 2.2 Effect of culture media and temperature on growth and sporulation of *Alternaria brassicicola*

Growth characteristics of the fungi were evaluated on potato

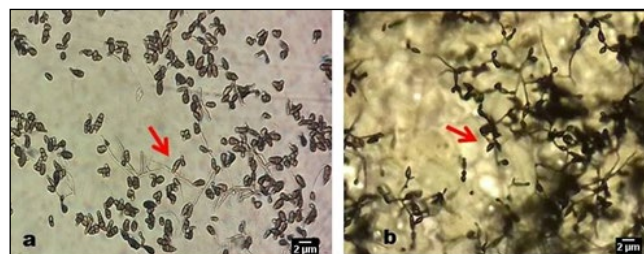


Figure 1: (a) Germinating spores of *A. brassicicola* in vitro (b) Chains of *A. brassicicola* spores on potato dextrose agar media at 25°C

dextrose agar (PDA), Czapek dox agar (CDA), Richard's agar (RA), Asthana and Hawker's agar (AHA) and Minimal media (MM). The media were prepared based on previously available protocols and composition (Dhingra and Sinclair, 1985; Pradeep et al., 2013; Poloni et al., 2009). A volume of 20 ml sterilized molten medium was aseptically poured in each Petri plate and allowed to solidify. Then, 5 mm- diameter plugs were taken with the help of a cork borer from the margin of 7 days old culture (maintained at 25±2°C on PDA), and placed at the centre of each set of Petri plates containing different media. Petri dishes were incubated at 15, 25 and 35°C. The average radial growth (mm) of colony was recorded after 7 days of incubation.

After 10 days of incubation 5 mm bits were excised from growing edge of colony subjected to each treatment combination to assess the sporulation capacity of the pathogen. The spore suspension was prepared by shaking mycelial bits in 1.5 µl distilled water amended with 0.02% Tween 20 and the spore count per ml was estimated using a Haemocytometer (Improved Neubauer). The number of spores ml<sup>-1</sup> was calculated using standard formula derived for haemocytometer-Number of spores ml<sup>-1</sup>=Number of spores counted per square (average of four corner squares and central square)×10<sup>4</sup>.

Factorial Completely Randomized Design was followed with three replications for each treatment combination (temperature×media). Correlation coefficients were computed between radial growth and sporulation capacity at each temperature under study.

### 2.3. Evaluation of inorganic media constituents for thermal susceptibility in *A. brassicicola*

Among the five different media tested for growth of *A. brassicicola* at the three incubation temperatures, the one which significantly favored growth of the fungus at 35°C was further evaluated for its individual constituents. Experiment was laid in completely randomized design with three replications, each treatment comprising of an eliminated constituted. The original media composition served as control. The experiment was repeated twice and the results were analyzed statistically. A volume of 20 ml sterilized molten medium was aseptically poured in each Petri plate and allowed to solidify. After inoculation with 5 mm diameter plugs from

the margin of 7 days old culture (maintained at  $25\pm 2^\circ\text{C}$  on PDA), the Petri plates were incubated at  $25^\circ\text{C}$  and  $35^\circ\text{C}$ . The average radial growth (mm) of colony was recorded after 7 days of incubation (DAI).

### 3. Results and Discussion

#### 3.1. Effect of culture media and temperature on growth and sporulation of *Alternaria brassicicola*

Both the factors, i.e., temperature and culture media significantly influenced the fungal radial growth after seven days of incubation. The colour and the colony morphology

of the *A. brassicicola* mycelium varied from one medium to another (Figure 2). Among the five different media evaluated, the average radial growth of *A. brassicicola* was found maximum on PDA medium (85.20 mm) followed by RA (62.60 mm), both at  $25^\circ\text{C}$ . The least growth was recorded on MM (17.80 mm) at  $35^\circ\text{C}$  which was statistically at par with PDA at  $35^\circ\text{C}$  (19.93 mm) and AHA at  $15^\circ\text{C}$  (21 mm). Maximum colony diameter at  $35^\circ\text{C}$  was observed on CDA medium (34.67 mm). The mean radial growth was highest at  $25^\circ\text{C}$ , followed by  $15^\circ\text{C}$  and  $35^\circ\text{C}$  (Table 1).

Sporulation capacity was also significantly influenced by

Table 1: Effect of temperature and culture media on radial growth (7 DAI) and sporulation capacity (10 DAI) of *Alternaria brassicicola*

Culture Media	Radial growth (mm)			Sporulation capacity ( $10^4 \text{ ml}^{-1}$ )		
	$15^\circ\text{C}$	$25^\circ\text{C}$	$35^\circ\text{C}$	$15^\circ\text{C}$	$25^\circ\text{C}$	$35^\circ\text{C}$
Potato dextrose agar	$47.83\pm 3.52$	$85.20\pm 4.69$	$19.93\pm 3.59$	$9.80\pm 2.27$	$10.50\pm 1.00$	$5.75\pm 0.66$
Richard's agar	$34.07\pm 5.61$	$62.60\pm 2.46$	$22.87\pm 4.50$	$14.47\pm 5.20$	$10.00\pm 0.29$	$5.20\pm 0.10$
Czapek dox agar	$23.33\pm 1.07$	$49.30\pm 4.26$	$34.67\pm 9.41$	$4.60\pm 0.20$	$12.60\pm 0.10$	$3.60\pm 0.10$
Asthana & hawker's agar	$21.00\pm 2.52$	$40.97\pm 1.58$	$27.63\pm 5.77$	$6.53\pm 0.75$	$11.80\pm 0.21$	$5.00\pm 0.06$
Minimal media	$24.27\pm 1.76$	$37.83\pm 3.75$	$17.80\pm 6.90$	$6.10\pm 0.26$	$8.10\pm 0.10$	$4.36\pm 0.02$
LSD (0.01) media $\times$ temperature	10.37			3.32		

DAI: Days after inoculation

temperature and culture media. After 10 days of incubation, the best sporulation of *A. brassicicola* was recorded on RA incubated at  $15^\circ\text{C}$  ( $14.47\times 10^4$  spores  $\text{ml}^{-1}$ ). It was followed by CDA ( $12.6\times 10^4$  spores  $\text{ml}^{-1}$ ) and AHA ( $11.8\times 10^4$  spores  $\text{ml}^{-1}$ ) at  $25^\circ\text{C}$ . Least spore concentration was observed on CDA at  $35^\circ\text{C}$ . Temperature affected the concentration of fungal spores produced on all five culture media. Temperature of  $25^\circ\text{C}$  best favoured sporulation in the pathogen followed by  $15^\circ\text{C}$  and  $35^\circ\text{C}$  (Table 1). The correlation between radial growth and sporulation capacity was found to be significantly positive (0.60) at  $15^\circ\text{C}$  and significantly negative (-0.61) at  $35^\circ\text{C}$ . Spore concentration increased with increasing mycelial growth till  $25^\circ\text{C}$ , and then decreased significantly at  $35^\circ\text{C}$  on all evaluated media except RA.

#### 3.2. Evaluation of inorganic media constituents for thermal susceptibility in *A. brassicicola*

Culture medium significantly influenced the radial growth of the fungi under different incubation temperature. Czapek dox agar media best favoured the mycelial growth of *A. brassicicola* incubated at  $35^\circ\text{C}$ . It was observed that both M3 and M5 lacking  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  and  $\text{FeSO}_4$  respectively could not support mycelial growth of *A. brassicicola* at warmer incubation temperature of  $35^\circ\text{C}$  as compared to control. In treatments M2 and M4, the mycelial growth was localized around the inoculum plugs. No significant influence of  $\text{NaNO}_3$  was observed on the mycelial growth of *A. brassicicola* at  $35^\circ\text{C}$  as compared to the control (Table 2).

Table 2: Radial growth of *Alternaria brassicicola* on modified CDA after 5 days of incubation at  $35^\circ\text{C}$

Media	Radial growth (mm)
M1 (CDA - $\text{NaNO}_3$ )	$22.88\pm 3.47$
M2 (CDA - $\text{KH}_2\text{PO}_4$ )	$17.62\pm 2.14$
M3 (CDA - $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ )	$5.00\pm 0.00$
M4 (CDA - KCl)	$10.13\pm 0.63$
M5 (CDA - $\text{FeSO}_4$ )	$5.00\pm 0.00$
M6 (CDA) (control)	$24.38\pm 3.15$
LSD (0.01)	4.31

Our results showed higher mycelial growth (55.18 mm) and sporulation ( $10.60\times 10^4$  spores  $\text{ml}^{-1}$ ) at  $25^\circ\text{C}$  indicating that this temperature was most favourable for the growth as well as sporulation of *A. brassicicola*. However, significant reduction in mycelial growth and sporulation was observed at  $35^\circ\text{C}$ . Though,  $15^\circ\text{C}$  also favoured moderate growth and sporulation of the fungi but it was significantly lower than that recorded at  $25^\circ\text{C}$ . Similar observations have been reported in *Alternaria brassicicola* isolated from cabbage (*Brassica oleracea* var. *capitata* L.) cultivated on PDA (Tu, 2019). The results are in agreement with Degenhardt et al. (1982) who reported that *Alternaria brassicicola* isolated from cabbage required more than  $15^\circ\text{C}$  temperature and more than 95% relative humidity for best spore germination. Neergaard (1945) had also





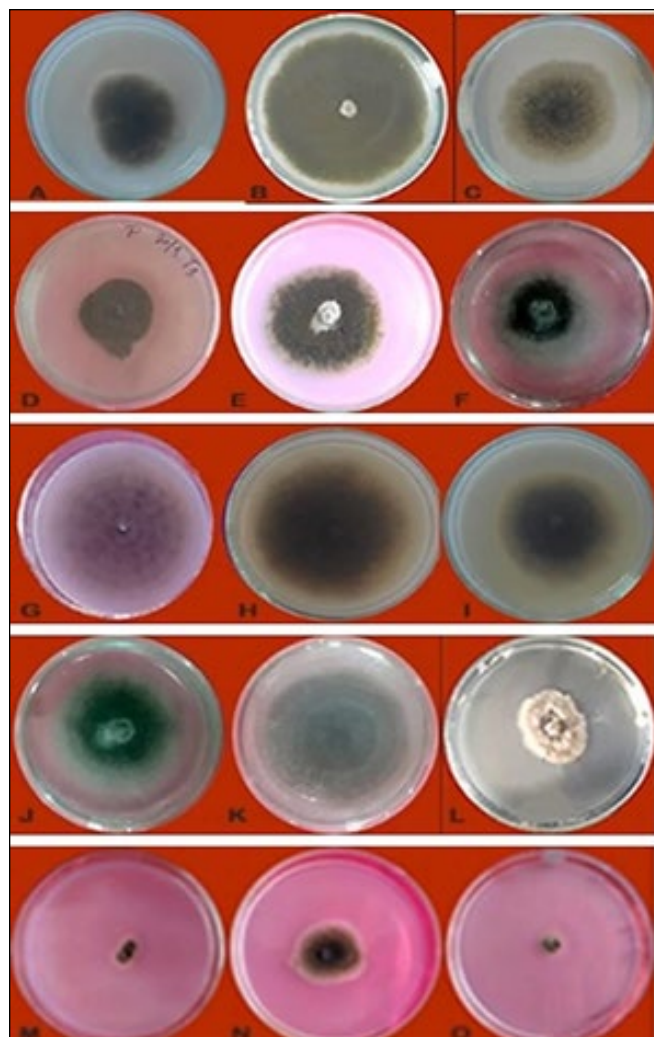


Figure 2: Growth of *Alternaria brassicicola* on Potato Dextrose Agar (A, B, C); Richard's Agar (D, E, F); Czapek Dox Agar (G, H, I); Asthana & Hawker's Agar (J, K, L) and Minimal media (M, N, O) at 15°C, 25°C and 35°C respectively

observed good growth and sporulation of various species of *Alternaria* at different temperatures between 23–28°C. Similar findings have been reported by other workers for *Alternaria* on different hosts like cabbage, rapeseed-mustard and Indian mustard (Humpherson-Jones and Phelps, 1989; Sinha et al., 1992; Shrestha et al., 2005). The range of temperature (from 15 to 35°C) at which the isolate of *Alternaria brassicicola* from *B. juncea* could grow and sporulate indicates a high level of phenotypic flexibility that allows it to survive and proliferate over a wide range of environmental conditions and in a larger geographical area.

Sporulation capacity of a pathogen plays a major role in disease epidemics by contributing to spread of disease and establishment of successful infection (Agrios, 2005; Gupta and Chandel, 2023). Temperature is known to invariably affect the number of spores produced by fungi on the culture medium (Agrios, 2005; Rahman et al., 2012). A positive correlation

( $r=0.60$ ) of sporulation capacity with radial growth has been found at 15°C indicating that a reduction in mycelia growth will reduce the number of inoculums as well as the disease development under relatively cooler conditions. On the contrary, it was negatively correlated ( $r=-0.61$ ) at 35°C, indicating that although higher temperature restricted the radial growth but improved sporulation in *A. brassicicola*. Similar results were also reported in *Alternaria alternata* (Damialis et al., 2015). It may be suggested that a higher temperature would correspond to slower mycelium growth but higher spore production in *A. brassicicola* that could be considered as a compensatory mechanism in the pathogen to overcome the reduced growth for better survivability under warmer conditions. Such compensatory mechanism was not observed at relatively lower temperature of 15°C.

The tested culture media differentially influenced the growth and sporulation of *A. brassicicola* isolated from Indian mustard. PDA was recorded as the most suitable for *in vitro* growth of *A. brassicicola* at 25°C. Minimal media on the other hand appeared to be less suitable for rapid mycelial growth and spore production of *A. brassicicola* at all the tested temperatures supposedly due to lack a sole carbon source. Media containing high carbon, nitrogen, phosphate, potassium, magnesium, sulphur elements including sugars are required for the growth of fungi at pH range of 5 to 6, and a temperature range from 15 to 37°C (Basu et al., 2015; Nasreen et al., 2017). The importance of various organic media for mycelial growth and spore production have been recognized by several workers for different groups of fungi (Kim et al., 2005; Zhao et al., 2010; Pradeep et al., 2013). PDA is one of the most commonly used culture media because of its simple formulation and its ability to support mycelial growth of a wide range of fungi (Xu et al., 1984; Maheshwari et al., 1999; Khadega and AlHussaini, 2015). Several reports have indicated PDA as a good medium for the growth and sporulation of *Alternaria brassicicola* and other species like *A. brassicae*, *A. solani*, *A. porri* and *A. alternata*. (Bonde, 1929; Neergard, 1945; Pawar and Patel, 1957; Arshour and El-Kadi, 1959; Sitarama and Mehta, 1982; Rotem, 1994; Sharma et al., 2013; Deep et al., 2014). Richard's agar (RA) was also reported to be a suitable media for *Alternaria alternata* and *A. porri* (Arshour and El-Kadi, 1959; Sitarama and Mehta, 1982). Moderate growth & profuse sporulation of *A. ricini* has been reported on RA by Pawar and Patel (1957). We recorded that Czapek dox agar supported maximum mycelia growth at 35°C, after 7 days of incubation which was 74% more than mycelia growth recorded on PDA. At 15°C, however, the test fungi revealed heavy conidial production ( $14.47 \times 10^4 \text{ ml}^{-1}$ ) after ten days of incubation on Richard's agar (RA). This is likely due the presence of distinct source of cations and anions in Czapek dox agar (CDA) and Richard's agar (RA). In our study, *A. brassicicola* failed to grow in the absence of  $\text{FeSO}_4$  and  $\text{MgSO}_4$  at 35°C. Sulphur is known to support *in vitro* fungal growth and sporulation in *A. brassicae* and *A. alternata*

(Hasija, 1969; Vishwanath, 1987; Kumar et al., 2016). Singh and Tandon (1971) also observed poor growth of *Alternaria tenuis* isolates on culture medium devoid of a sulphur source. Magnesium sulphate additive has been reported to best support the growth of *A. triticina* (Kumar and Rao, 1979). Concurrent observations have also been reported in field trials by Vishwanath (1987) wherein soil application of sulphur @ 50 kg ha<sup>-1</sup> resulted in 40% higher *Alternaria* blight disease severity on pods of toria over the check (with no sulphur application). However, further increase in sulphur application negatively correlated with the disease development. Richie et al. (2007) observed upregulation in autophagy in *Aspergillus fumigatus* in response to starvation stress, resulting in the generation of a pool of metal ions. Autophagy contributes to the elaboration of appressorium for virulence in necrotrophic fungi (Veneault et al., 2006). Certain metal ions may act as enzyme production inducers in fungi (Mahmoud et al., 2013). Reports suggest stimulation of glucose 6-phosphate dehydrogenase enzyme in *Alternaria alternata* by Mg<sup>2+</sup> levels within the physiological range (Jiang and Niehaus, 1986). Levels of magnesium (Mg) can either complement or antagonize the uptake of other minerals and result in varied disease responses depending on environmental conditions. There are several examples of disease control through nutrient manipulation which can be achieved by either modifying the nutrient availability or modifying the nutrient uptake (Huber and Graham, 1999; Huber and Jones, 2012). In pathogens like *Fusarium oxysporum*, that are favoured by warmer, acidic soils, application of NO<sub>3</sub><sup>-</sup> reduces the availability of manganese (Mn) and iron (Fe) causing increase in pH and resulting in the reduction of the pathogen (Dordas, 2008; Gupta et al., 2017).

#### 4. Conclusion

Significant effect of incubation temperature and growth medium on the cultural characteristics of *A. brassicicola* have been observed in this study. The fungus is cosmopolitan in distribution in the presence of specific elements for growth and reproduction. Manipulating the plant-pathogen-environment interaction over time can therefore aid in disease management. Therefore, manipulating some nutrients like iron and magnesium has provided new innovative antifungal strategies against this pathogen by targeting potential weaknesses in fungal metabolism for sustainable management.

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