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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2021; 10(1): 625-630

www.thepharmajournal.com
Received: 03-11-2020
Accepted: 06-12-2020

SN Banne
Department of Plant Pathology, College of Agriculture, Latur Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

Sunita J Magar
Department of Plant Pathology, College of Agriculture, Latur Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

Shruti S Kadam
Department of Plant Pathology, College of Agriculture, Latur Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

AP Suryawanshi
Department of Plant Pathology, College of Agriculture, Latur Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

Corresponding Author:
SN Banne
Department of Plant Pathology, College of Agriculture, Latur Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

Cultural and morphological characterization of isolates of *Alternaria alternata* (Fr.) Keissler, causing chrysanthemum leaf blight

SN Banne, Sunita J Magar, Shruti S Kadam and AP Suryawanshi

Abstract
Fungal blights are among the major concern for limiting the cultivation and production of many ornamental and flowering plants. Chrysanthemum is an important cut flower with great export potential. However, it is infected by many pathogens in the protected cultivation. Diseased chrysanthemum samples were collected from chrysanthemum gardens, nurseries and farmers orchards in the Latur district for cultural and morphological characterization of *Alternaria alternata*. The collected isolates of *A. alternata*, from various varieties / hybrids of chrysanthemum were characterized, based on their mycelial growth, growth rate, colony appearance, colony shape and margin, sporulation and concentric zonation and morphological characters (mycelial width, conidia dimensions, beak length and numbers of vertical and horizontal septa). The highest colony growth was found in Ac-1, Ac-3 and ac-4 followed byAc-5. Excellent sporulation was found in isolates such as Ac-1, Ac-3, Ac-5 and Ac-9. Four isolates Ac- 10, Ac-9, Ac-8 and Ac-7 showed large sized mycelial width ranged from 7.15-9.48 µm. Ac-9 and Ac-10 isolates was showed large sized conidia (42.17- 60.29 L X 9.40-11.77 W). Three isolates Ac-5, Ac-9 and Ac-8 showed large sized beak length ranged from 13.52- 15.29 µm, whereas horizontal septation ranged from 1-9 and vertical septation from 1-5.

Keywords: *Alternaria alternata*, blight, cultural characters, morphology

Introduction
Chrysanthemum (*Dendranthema grandiflora* Tzvelev.) is a multi-use flower crop belonging to Asteraceae family and gaining more popularity as a cut flower, loose flower and pot plant. Chrysanthemum is commonly known as Queen of East, produces very attractive flowers of different shape, size and colours. It is an important commercial flower next to rose in the international florist's trade and grown throughout the World (Kher, 1990) [6]. In India, chrysanthemum commercially growing major states are Karnataka, Tamilnadu, Maharashtra, Rajasthan, Madhya Pradesh and Bihar. In Maharashtra, chrysanthemum is grown on an area of 0.39 thousand ha. With the production of 1.65 thousand tonnes loose flowers and 0.05 thousand tonnes cut flowers (Anonymous, 2018) [2]. In Maharashtra, the leading districts in floriculture production are Nasik, Ahmednagar, Thane, Pune, Satara, Sangli and Nagpur. However, Ahmednagar district is specalized as growing district of the Maharashtra (Tupe *et al*., 2017) [17].

There has been a regular demand for chrysanthemum flowers throughout the year in our country. Hence, there is a great potential for the production of chrysanthemum on a commercial scale in India. However, it is difficult to get good quality exportable blooms, higher yields and long lasting post-harvest life of the cultivars under open conditions. The most important factors responsible for the threatened production of chrysanthemum flowers yield is by many diseases, such as, *Alternaria* leaf blight, *Fusarium* wilt, Septorial leaf spot, Ray speck disease, *Pythium* rot, Chrysanthemum rust, Bacterial blight, virus (viriod) and Nematode (Schmidt, 1958; Alfieri, 1968; Strider, 1985; Cook, 2001; Nishi *et al*., 2009 and Luong *et al*., 2010) [14, 1, 16, 3, 11, 9].

Alternaria is a genus of ascomycetous fungi. *Alternaria* species are known as major plant pathogens. There are 299 species in the genus *Alternaria* (Kirk *et al*., 2008, Nowicki-Marcin *et al*., 2012) [7, 12]. Therefore, it was decided to study in detail the morpho-cultural characters of the fungal pathogen.

Materials and Methods

Collection of diseased sample

Leaves exhibiting typical symptoms of chrysanthemum blight (*Alternaria alternata*) disease were collected in the paper bags from chrysanthemum gardens, nurseries and farmers orchards in the Latur district.

Isolation and maintenance of fungal pathogen

Naturally leaf blight diseased specimen of chrysanthemum (Cv. White probiotic) were collected from the local floriculture nursery for isolation of fungal pathogen. The infected plant parts were cut into smaller pieces with a sterile scalpel and disinfected with 2 % aqueous solution of sodium hypochlorite (NaOCl) solution for 2 min., with three subsequent washings in sterilized distilled water. Then, cut samples were dried by sterilized blotting paper. The isolation of blight causing, fungal pathogen was made by applying hyphal-tip technique on potato dextrose agar and was incubated at 27 ± 2 °C temperature. The growth of fungi noticed after seven days of incubation, profuse mycelial growth free from any contaminant was obtained. Thirty to thirty five days old potted seedlings / plants of chrysanthemum (Cv. White probiotic) were selected for proving the pathogenicity of the fungal pathogen. The pathogenicity of test fungus was proved in artificial epiphytotics condition with high relative humidity. The pathogenicity of isolated cultures was proved by spraying spore and mycelial suspension (approx. 2×10^6 spore/ml). The reisolation was carried out from artificially infected leaves in the same way as described earlier.

Cultural variability

Ten isolates (Ac-1 to Ac-10) of *A. alternata* obtained from eight varieties and two hybrids of chrysanthemum were grown on autoclaved and cooled PDA in sterilized glass Petri plates (90 mm dia.) were subjected to study for their cultural variability. The experiment was planned in CRD and the ten test isolates were replicated thrice.

Observations on cultural/colony characteristics such as, colour, appearance, shape, zonation and margin of the colony, growth speed/24 hr were recorded after a week of incubation. Sporulation was recorded at 12 days of incubation. For counting sporulation, at 12 days of incubation, sporulating culture of the test isolates in Petri plates was flooded with 10 ml distilled water and was gently scrapped with Camel hair brush to obtain spore suspension. Temporary mount on glass slide of the spore suspension was prepared, mounted under Research microscope and counted the spores under five random microscopic fields (400 X) and averaged and sporulation frequency was categorized as below (Kumar and Choudhary, 2006) [8].

Grade	Sporulation	No. of spores/ microscopic field
-	Absent	Nil
+	Poor	1-10
++	Fair	11-30
+++	Good	31-50
++++	Excellent	More than 50

Morphological variability

For studying morphological characters, temporary mounts in Lactophenol cotton blue stain on glass slides of the sporulated cultures of 10 isolates were prepared and covered with glass slide. The morphological characteristics such as shape and size (length and breadth in µm) of conidia, septation of conidia, length of beak and colour and size of conidiophores were recorded, under five different microscopic fields (400 X), by using Image -J launcher software 1.50i.

Results and Discussion

Isolation and characterization of pathogenic fungi

The isolated fungi from the blighted chrysanthemum leaves were compared with reisolated fungal pathogen. Based on typical symptoms of *Alternaria* blight (naturally and artificially diseased chrysanthemum plants), pathogenicity test, morpho-cultural characteristics and microscopic observations, the test pathogen was identified as *Alternaria alternata* (Fries.) Keissler., the cause of chrysanthemum *Alternaria* blight. The pathogenicity studies by spraying spore and mycelial suspension showed that, the fungus infects the gerbera foliage causing typical symptoms of disease. The symptoms such as: small dark spots, oval to irregularly shape with brown concentric ring at the center of leaf, within a week of inoculation.

Cultural variability among the *A. alternata* isolates

The results obtained on cultural characteristics such as mycelial growth, growth rate, colony appearance, colony shape and margin, sporulation and concentric zonation etc. are presented in Table 1 and 2 and Fig. 1.

Colony growth

The results (Table 2) showed that, among the isolates, mycelial growth was varied from 58.10 mm (Ac-10) to 90.00 mm (Ac -1, Ac -3 and Ac - 4). However, it was significantly highest in Ac -1 Ac -3 and Ac -4 (each 90.00 mm), followed by Ac -5 (88.85 mm) and Ac -8 (88.33 mm) which were on par, Ac -9 (81.00 mm), Ac -6 (70.50 mm), Ac -2 (69.60 mm), Ac -7 (60.50 mm) and Ac -10 (58.10 mm), respectively.

Colony colour

On the basis of colony colour, the test isolates were categorized into six groups. Group I consisted of four isolates with white colony (Ac -6, Ac -7, Ac -9 and Ac -10), Group II contained of two isolates with creamy white colony (Ac -5 and Ac -8), Group III of consisted one isolate with off white colony (Ac -4), Group IV of consisted one isolate with grayish black colony (Ac -2), Group V of consisted one isolate with olivaceous black (Ac -3) and Group VI consisted of one isolate with black colony (Ac -1).

Colony appearance

Colony appearance of the test isolates was cottony (Ac-1, Ac-3, Ac-8 and Ac-9), fluffy (Ac-4, Ac-7 and Ac-10) and feathery (Ac-2, Ac-5 and Ac-6), respectively.

Growth rate

Based on growth rate, the test isolates were grouped as fast growing (Ac -1, Ac -3, Ac -4, Ac -5 and Ac -8), moderately growing (Ac -6 and Ac - 9) and slow growing (Ac -2, Ac -7 and Ac -10).

Colony shape and margin

Shape of the colony was circular with smooth margin in five isolates (Ac 1, Ac -3, Ac - 4, Ac -8 and Ac -9) and irregular with rough margin in rest of the five isolates (Ac -2, Ac -5, Ac -6, Ac -7 and Ac -10).

Sporulation

The sporulation induced by the test isolates varied from fair (++) to excellent (++++). However, it was excellent (++++) in four isolates, such as, Ac -1, Ac -3, Ac -5 and Ac -9; good (+++) in three isolates such as Ac -4, Ac -6, and Ac -8 and fair (++) in three isolates such as Ac -2, Ac -7 and Ac -10, respectively.

Table 1: Cultural variability among the isolates of *A. alternata*, from various cultivars / hybrids of chrysanthemum

Characters	Isolates									
	Ac -1	**Ac -2**	**Ac -3**	**Ac -4**	**Ac -5**	**Ac -6**	**Ac -7**	**Ac -8**	**Ac -9**	**Ac -10**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Colony Dia. (mm)	90.00	69.60	90.00	90.00	88.85	70.50	60.50	88.33	81.00	58.10
Colour	Black	Grayish black	Olivaceous black	Off white	Creamy white	White	White	Creamy white	White	White
Appearance	Cottony	Feathery	Cottony	Fluppy	Feathery	Feathery	Fluppy	Cottony	Cottony	Fluppy
Growth rate	Fast	Slow	Fast	Fast	Fast	Moderate	Slow	Fast	Moderate	Slow
Shape	Circular	Irregular	Circular	Circular	Irregular	Irregular	Irregular	Circular	Circular	Irregular
Margin	Smooth	rough	Smooth	Smooth	rough	Rough	Rough	smooth	Smooth	rough
Sporulation	++++	++	++++	+++	++++	+++	++	+++	++++	++
Zonation	Present	Absent	Present	Present	Absent	Absent	Present	Absent	Present	Absent

*Sporulation: ++++ = Excellent, +++ = Good, ++ = Fair, + = Poor, Dia: Diameter. Colony/mycelial growth: SE±: 0.52, CD (P = 0.01): 1.55

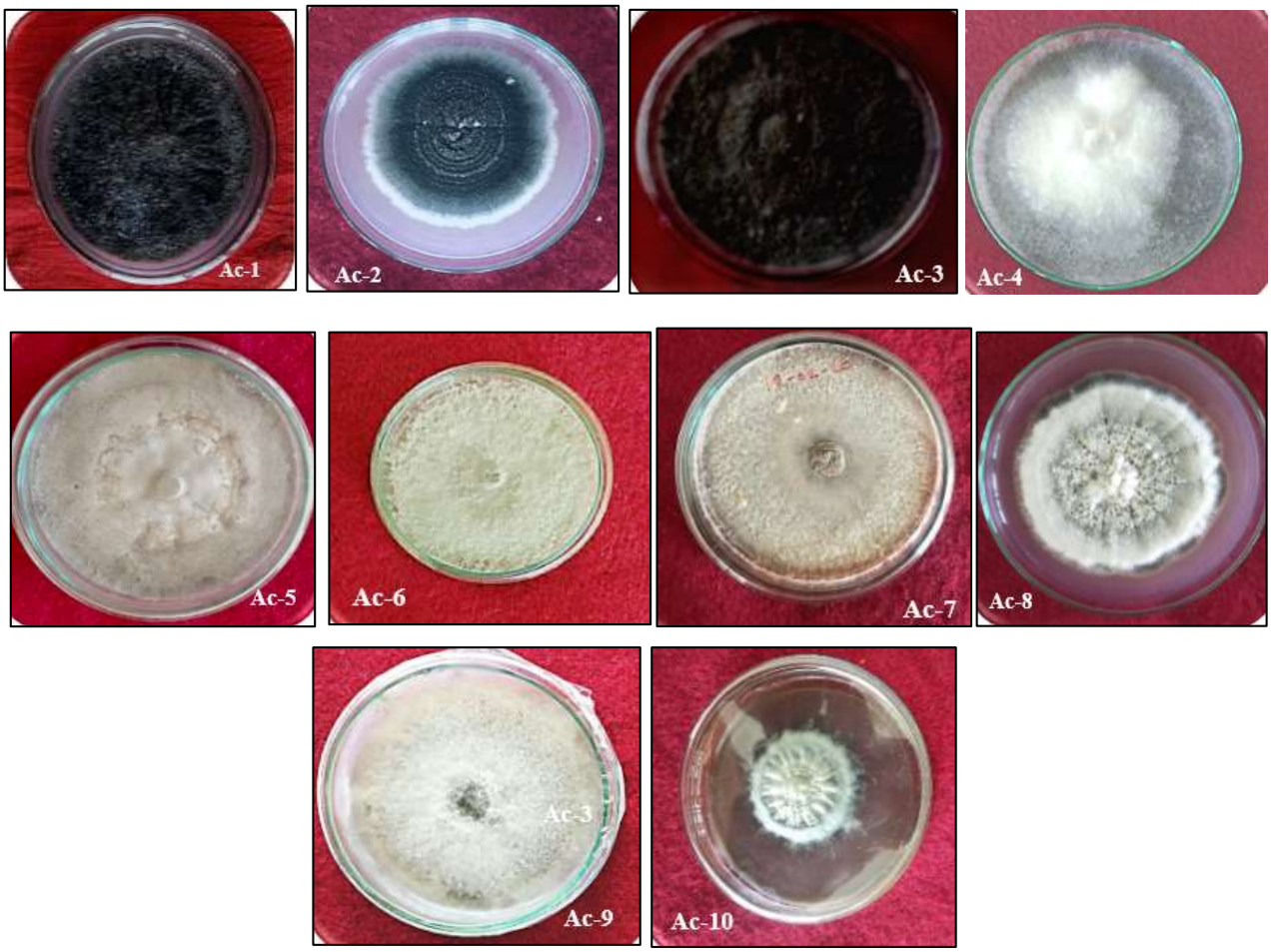

Plate I: Cultural variability among various isolates of *A. alternata*, from chrysanthemum varieties / hybrids

Zonation

The zonation was present in about five test isolates such as Ac -1, Ac -3, Ac -4, Ac -7 and Ac -9; while, it was absent in rest of the five test isolates such as Ac -2, Ac -5, Ac -6, Ac -8 and Ac -10, respectively.

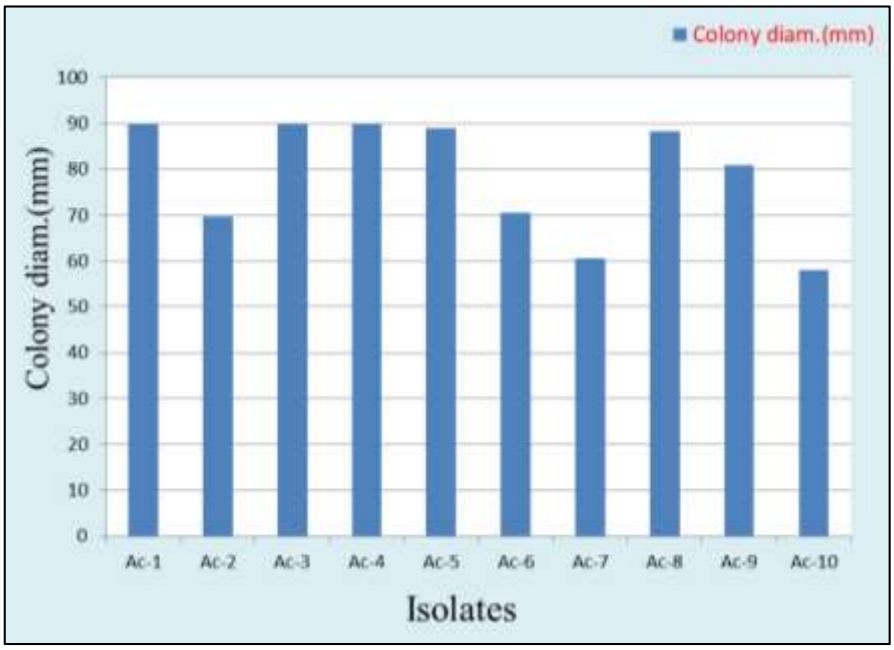

Fig 1: Variability in colony diameter among the isolates of *A. alternate*

All the 10 test isolates were further categorized into seven groups based on colony growth, colour, margin, appearance, growth rate, sporulation and zonation. The results obtained are presented in Table 2.

Table 2: Grouping of *A. alternata* test isolates based on cultural variability

Groups	Cultural Parameters	Isolates / No.
1. Colony growth (mm)		
Group-I	Maximum (> 80 mm)	Ac-1, Ac-3,Ac-4,Ac-5, Ac-8 and Ac-9 (6)
Group-II	Medium (70 -80 mm)	Ac-6 (1)
Group-III	Minimum (< 70 mm)	Ac-2, Ac-7 and Ac-10 (3)
2. Colony colour		
Group-I	White colony	Ac -6, Ac -7, Ac -9 and Ac -10 (4)
Group-II	Creamy white	Ac -5 and Ac -8 (2)
Group-III	Off white colony	Ac -4 (1)
Group-IV	Grayish black	Ac -2 (1)
Group-V	Olivaceous black	Ac -3 (1)
Group-VI	Black	Ac -1 (1)
3. Colony appearance		
Group-I	Cottony	Ac-1, Ac-3, Ac-8 and Ac-9 (4)
Group-II	Fluffy	Ac-4, Ac-7 and Ac-10 (3)
Group-III	Feathery	Ac-2, Ac-5 and Ac-6 (3)
4. Growth rate		
Group-I	Fast	Ac -1, Ac -3, Ac -4, Ac -5 and Ac -8 (5)
Group-II	Moderate	Ac -6 and Ac - 9 (2)
Group-III	Slow	Ac -2, Ac -7 and Ac -10 (3)
5. Colony shape and margin		
Group-I	Circular and smooth	Ac -1, Ac -3, Ac - 4, Ac -8 and Ac -9 (5)
Group-II	Irregular and rough	Ac -2, Ac -5, Ac -6, Ac -7and Ac -10 (5)
6. Sporulation		
Group- I	Excellent	Ac -1, Ac -3, Ac -5 and Ac -9 (4)
Group-II	Good	Ac -4, Ac -6 and Ac -8 (3)
Group-III	Fair	Ac -2, Ac -7 and Ac-10 (3)
7. Zonation		
Group-I	Present	Ac -1, Ac -3, Ac -4, Ac -7 and Ac -9 (5)
Group-II	Absent	Ac -2, Ac -5, Ac -6, Ac -8 and Ac -10 (5)

Morphological variability

Results (PLATE II and Table 3 and 4) revealed that, all the ten test isolates of *A. alternata* exhibited a wide range of morphological variability such as mycelial width, conidial dimensions, beak length and numbers of vertical and horizontal septa.

Mycelial width

On the basis of mycelial width, the test isolates were categorized into three groups *viz*., large, medium and small sized (Table 3 and 4). Four isolates (Ac -10, Ac -9, Ac -8 and Ac -7) were large sized, of which mycelial width ranged from 7.15-9.48µm. However, it was highest in Ac -10 (9.48 µm), followed by Ac -9 (8.25 µm), Ac -8 (7.20µm) and Ac -7

(7.15µm).Three isolates (Ac -1, Ac -3 and Ac -5) showed medium sized mycelial width in the range of 5.25-6.20 µm. However, it was maximum in Ac -3 (6.20 µm), followed by Ac -5 (5.78 µm) and Ac -1 (5.25 µm). Rest of the three isolates (Ac -4, Ac -6 and Ac -2) showed small sized mycelial width in the range of 3.48-4.55µm. However, it was maximum in Ac -4 (4.55 µm), followed by Ac -6 (4.25 µm) and Ac -2 (3.48 µm), respectively.

Table 3: Morphological variability among the isolates of *A.alternata*

Sr. No.	Isolates	Av. mycelial width (µm)	Av. size of conidia (L X W µm)	Av. beak length (µm)	Number of septa (Range)	
					H	V
1	Ac -1	5.25	29.43 X 5.45	8.54	1-5	1-2
2	Ac -2	3.48	30.28 X 6.52	9.18	2-6	1-4
3	Ac -3	6.20	37.22 X 8.74	11.25	1-4	1-3
4	Ac -4	4.55	38.21 X 7.25	8.84	3-5	1-3
5	Ac -5	5.78	30.48 X 6.88	15.29	2-5	1-2
6	Ac -6	4.25	29.98 X 6.07	9.03	3-5	1-3
7	Ac -7	7.15	32.39 X 9.82	12.18	2-6	1-2
8	Ac -8	7.20	32.23 X 8.23	13.52	2-5	1-5
9	Ac -9	8.25	60.29 X 11.77	15.18	3-9	1-3
10	Ac -10	9.48	42.17 X 9.40	11.63	4-6	2-4

Conidial size

Based on length and breadth of the conidia, the test isolates were grouped as large, medium and small sized conidia. Two isolates (Ac -9 and Ac -10) exhibited large sized conidia (42.17 -60.29 L X 9.40-11.77 W), however, highest average conidial size (length X width) was recorded in Ac -9 (60.29 X 11.77 µm^2), followed by Ac -10(42.17 X 9.40 µm^2). Four isolates (Ac -4, Ac -3, Ac -7 and Ac -8) exhibited medium sized conidia (32.23 -38.21 L X 7.25- 9.82 W µm^2), however, it was maximum in Ac -4 (38.21 X 7.25 µm^2), followed by Ac -3 (37.22 X 8.74 µm2), Ac -7 (32.39 X 9.82 µm^2) and Ac -8 (32.23 X 8.23 µm^2). Rest of the four isolates (Ac -1, Ac -2, Ac -5 and Ac -6) exhibited small sized conidia (29.43 -30.48 L X 5.45-6.88 W µm^2).

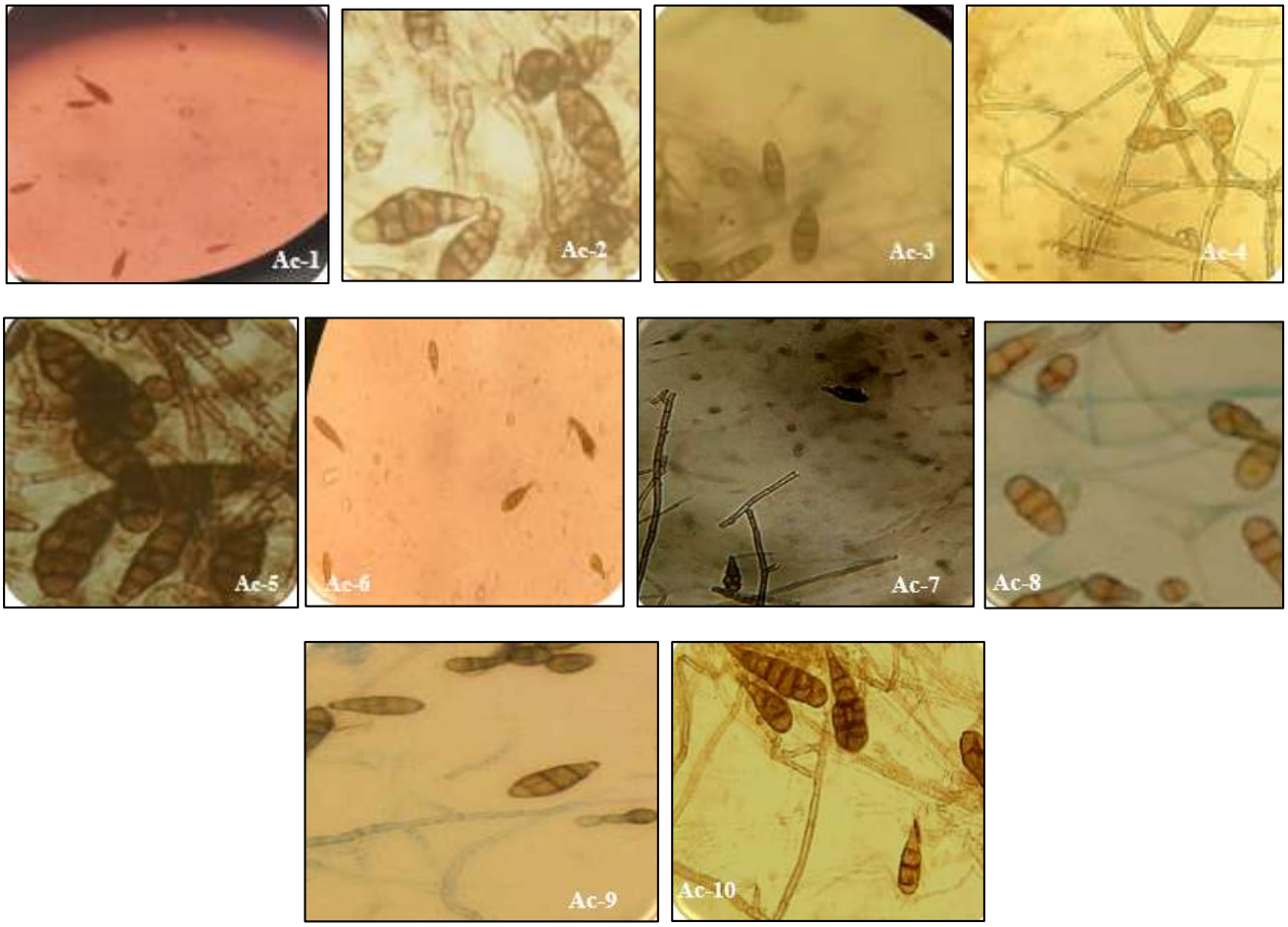

Plate II: Morphological variability among various isolates of *A. alternata*, from chrysanthemum varieties / hybrids

Beak length

Three isolates (Ac -5, Ac -9 and Ac -8) showed large sized beak length (13.52-15.29 µm). However, it was highest in Ac -5 (15.29 µm), followed by Ac -9 (15.18 µm) and Ac -8 (13.52 µm), Three isolates (Ac -7, Ac -10 and Ac -3) showed medium sized beak length (11.25-12.18 µm), however, it was highest in Ac -7 (12.18 µm), followed by Ac -10 (11.63 µm)

and Ac -3 (11.25 µm). Rest of the four isolates (Ac -2, Ac -6, Ac -4 and Ac -1) exhibited small sized beak length (8.54-9.18 µm).

Septation

In all of the test isolates, horizontal septation ranged from 1-9 and vertical septation from 1-5.

Table 4: Grouping of *A. alternata* test isolates based on morphological variability

Sr. No.	Conidia	Size (µm)	No. of isolates
I. Mycelial width			
1	Large	7.15-9.48	04
2	Medium	5.25-6.20	03
3	Small	3.48-4.55	03
II. Conidia size (LX W µm)			
1	Large	42.17 -60.29 X 9.40-11.77	02
2	Medium	32.23 -38.21 X 7.25-9.82	04
3	Small	29.43 -30.48 X 5.45-6.88	04
III. Beak Length			
1	Long	13.52-15.29	03
2	Medium	11.25-12.18	03
3	Short	8.54-9.18	04

Thus, these results indicated morpho-cultural variability among *A. alternata*, causing chrysanthemum blight. Morpho-cultural variability within and among various species of *Alternaria* (*A. alternata, A. tagetica, A.tenuissima, A. helianthi, A. sesami, A. solani*) has earlier been reported by several workers (Gohel, 2004; Mangala *et al*., 2006; Patel *et al*., 2010; Shamala and Janardhana, 2015 and Devi *et al*., 2016) [5, 10, 13, 15, 4].

Acknowledgment

We are thankful to the Head of the Department of Plant Pathology, College of Agriculture, Latur for providing the laboratory facilities.

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