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Morphological and Molecular Characterization of *Alternaria* Isolates Causing Purple Blotch Disease of Onion

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A B S T R A C T

Purple blotch is the most destructive disease commonly prevailing in almost all onion growing pockets except in very cool production areas of the world. The present study was carried out to understand the diversity in *Alternaria* population causing purple blotch disease of onion. Out of fifty six geographical *Alternaria* isolates of Karnataka seventeen were selected as descriptive isolates based on morphological characteristics for understanding the genetic variability. The isolates exhibited Ash, Ashy black, Ashy white, Ashy green and blackish green colour. Sabouraud dextrose agar supported the maximum growth whereas corn meal agar did not support the growth. Asthana Hawker's medium supported the sporulation of the isolates. The isolates showed variability in shape, conidia length, width, beak length and number of septa. Sequencing and blasting of nucleotide sequence of ITS region of ribosomal DNA of isolates revealed 100% matching with five different species of *Alternaria* viz., *A. porri, A. alternata, A. tenuissima, A. palandui and A. brassicicola*. Cluster analysis of ITS rDNA sequences did not provide reliable differentiation between the species and among the isolates. The results put forward that ITS region of ribosomal DNA is inappropriate for taxonomic resolution of *Alternaria* species infecting onion.

Introduction

Onion (*Allium cepa* L.) is a spice and vegetable crop of global importance. Onion is susceptible to numerous foliar, bulb and root pathogens that reduce yield and quality (Cramer, 2000). Excessive rains, humidity, temperature, pests and disease are critical factors of risk to onion cultivation. Irrespective of the varieties, the spectrum of diseases that affect onion remain the same. Purple blotch of onion caused by *A. porri* (Ellis) Ciff. is one of the most serious disease in India (Gupta *et al.,* 1986; Tripathi *et al.,* 2008; Ramjegathesh *et al.,* 2011). The yield losses of bulb and seed crop in India due to this disease under favourable conditions are upto 97% (Gupta and Pathak, 1998; Lakra, 1999).

As a genus, *Alternaria* is a diverse and ubiquitous group of fungi having a high

degree of variability in spore shape and size, pathogenicity and sporulation. To date classification and identification of *Alternaria* species is based primarily on the morphology and development of conidia and conidiophores, to a lesser degree on host plant association and colony morphology (Elliot, 1917; Wiltshire, 1947; Simmons, 1967). Several taxonomists have deliberations on whether morphological characters should be used as the sole criteria for delimiting species, especially regarding small-spored catenulate *Alternaria* species (Rotem, 1994; Kusabaand Tsuge, 1995; Andersen *et al.,* 2001; Peever *et al.,* 2004). These debates are based on the presence of considerable variability in conidium size, shape, and septation within each species as well as within a single *Alternaria* colony (Simmons, 1992). Furthermore conidial characteristics often overlap between closely related species, hindering the establishment of distinct and unambiguous species boundaries. To overcome the limitations of morphological markers, molecular markers (RAPD and RFLP) have been applied in systematic studies of *Alternaria* species.

Recent DNA marker technologies have revolutionized the plant pathogen genomic analysis and have been extensively employed in the identification, early detection and understanding genetic diversity of plant pathogen. Among the different DNA marker techniques, Internal Transcribed Spacer (ITS) regions of fungal DNA (rDNA) are identified as suitable targets for the analysis of fungal communities (White *et al.,* 1990; Peay *et al.,* 2008). The ITS region is the most widely sequenced DNA region in fungi useful for molecular systematics and is of great importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz 2005). Various researchers have reported different species of *Alternaria* causing purple blotch disease of onion in India. Osiru (2008) described the presence of *Alternaria* species is influenced by a number of factors such as climatic conditions, host crops and management conditions. The influence of climatic and management factors on the prevalence of species shows the presence of pathogen diversity. The pathogen population with a high pathogenic diversity poses difficulties in deploying stable resistant varieties, as these succumb to newly evolving pathogenic races or new species of *Alternaria* causing infection in onion crop. Considerable attention has been given for studying the diversity of *Alternaria* all over the world. Although India is the largest producer of onion in the world, limited importance is given to study the pathogen diversity and its geographical distribution despite the magnitude of damage caused by this pathogen incurring huge economic losses in terms of poor quality bulbs and low yields. Thus the present study was conducted to understand the pathogen causing purple blotch disease through morphology and molecular characterization

Materials and Methods

Collection, isolation, identification and maintenance of isolates

In the present investigation a survey was conducted during *Kharif* 2011 in eleven districts of Karnataka (Bangalore, Kolar, Tumkur, Raichur, Bijapur, Bidar, Gulbarga, Dharwad, Gadag, Belgaum and Shimoga) for the collection of purple blotch disease infected samples of onion field (Table 1). Onion leaves showing typical symptoms of purple blotch disease were collected and the pathogen was isolated within 24h following standard tissue isolation. The cultures were purified by single spore isolation using water agar and potato dextrose agar. The purified culture were maintained on PDA slants at 5°C under refrigeration and renewed once in 30days.

Cultural and Morphological characterization

Cultural characteristics of all the isolates were studied on PDA medium. A culture disc of 5mm diameter was cut using cork borer from the peripheral region of the seven day old culture. The disc was placed at the center of the PDA plate and incubated at 27±1°C for 15 days. For each isolate four replications were maintained. Colony diameter was measured on the 7th day after inoculation. Observations on cultural characters *viz.,* colony growth, colour of mycelium, growth pattern, zonation, sectors, margin growth and colour of colony on reverse side of the culture plate were recorded.

Observations on sporulation characters were recorded after 15 days of inoculation. To assess the conidia count in each of the isolate conidial suspension was prepared by adding 10 ml of sterile distilled water to each of the culture plates. The conidial suspension was serially diluted and conidial concentration (conidia/ml) was determined using a Neubauer Heamocytometer.

Variability among the cultures isolated was studied on synthetic (Sabourauds Dextrose Agar, Richards Agar and Asthana and Hawker's Agar) and non-synthetic media (Oat Meal Agar, Corn Meal Agar, Bean Extract Agar, Host Extract Agar, Malt Extract Agar, Potato Carrot Agar and Potato Dextrose Agar). Three replicates were maintained for each media. The composition and preparation of the following media were obtained from "Ainsworth and Bisby's Dictionary of the fungi" (Ainsworth, 1961) and "Plant Pathological methods, fungi and bacteria" (Tuite, 1969) excepting host leaf extract agar medium.

Conidial characterization

The cultures obtained from single spore isolation were grown on PDA medium at 27±1°C for ten days. The culture was observed for sporulation upto 15 days after inoculation. The cultures which were not sporulated were inoculated on the onion leaves or inflorescence stalk by detached leaf method and observed for sporulation. The conidial characteristics *viz.,* colour, shape, size, number of transverse and longitudinal septa and length of the beak were recorded in fifty conidia selected randomly from different microscopic fields of Carl Zeiss bright field microscope under 20X magnification. The average of fifty observations was taken for each parameter using the formula given below.

$$\text{Average/mean} = \frac{\text{Sum of all the observations}}{\text{total number of observation taken}}$$

Molecular characterization

Extraction of genomic DNA, amplification and phylogenetic analysis

DNA extraction was done from the mycelia obtained from 7 day old still potato dextrose broth cultures of *Alternaria* isolates incubated at 27±1°C. Mycelium was harvested from the liquid broth by filtration through Whatman No.1 filter paper, blot dried and subsequently dried to a fine powder in liquid nitrogen. The mycelial biomass was quick frozen in liquid nitrogen and ground into fine powder.

DNA was extracted from the frozen mycelial powder employing a slightly modified method of Raeder and Broda (1985).by incubating 37°C for 10 min after the phenol: chloroform: isoamyl alcohol (25:24:1) precipitation. This was followed by precipitation with 0.54volumes of isoamyl alcohol.

The ITS region of rDNA of the fungal isolates were amplified by PCR with universal primer pairs (White *et al.,* 1990). PCR reaction (50 µl) were performed with primer ITS1(Gardes

and Burns, 1993) and ITS4 (White *et al.,* 1990) in an Eppendorf master cycler by 40 cycles of denaturation at 94°C for 30s, annealing at 53°C for 40 s and extension at 72°C for 45s with an initial denaturation of 5 min at 94°C and final extension of 10min at 72°C after cycling. Amplified PCR products were separated in 1% agarose gel in Tris-Borate –EDTA (TBE) buffer Ethidium bromide was added to the agarose gel at 0.5 µg/ml.

The gel is visualized by trans-illuminator in Gel doc unit with image capture system and photographed. Primers and all the reagents used in this study were procured from Fermentas life sciences, Bangalore. The amplified ITS region of rDNA products with primer pair(ITS1 and ITS4) using QIA quick PCR Purification Kit (Qiagen Inc., Mississauga, Ontario) yielded 560 bp. The purified product (20ng) was sent for sequencing. Sequencing of the purified product of PCR was done using the BigDye Terminator Cycle Sequencing system (Applied Bio systems) USA and analysed with ABI 3100 analyzer capillary machines. Sequencing of the PCR product was performed in both directions. Nucleotide homology searches were performed with the nucleotide program BLAST (http://ncbi.nlm.nih.gov/) confirm the identity of the pathogen.

Data analysis

A multiple-sequence alignment was performed with similar reference sequences of other *Alternaria* isolates available in the NCBI database using Molecular Evolutionary Genetic Analysis (MEGA 4) software and a BLAST similarity test was performed. The regions of the ambiguity and positions that were not available for all the sequences compared were omitted, Phylogenetic analysis were constructed from the aligned sequences using MEGA.

Results and Discussion

Fifty six geographical isolates of *Alternaria* spp. collected from eleven districts of Karnataka showed variation in morphological and cultural characteristics. Out of these isolates seventeen isolates were selected as descriptive isolates for understanding the genetic variability of the pathogen by morphological and molecular criteria. On potato dextrose medium the isolates showed significant variation in cultural characters *viz.,* colony colour, growth pattern, margin and colony colour on the reverse side of the plate. The isolates were characterized by regular, irregular, circular, smooth and rough colonies and did not produce any diffusible pigments which resulted in change of colour of the medium. However zonation or sectoring was absent in all the isolates. Based on colony colour isolates were classified into five groups *viz.,* ash, ashy black, ashy green, ashy white and blackish green (Table 1). The results of colony colour revealed variability among the isolates which are in accordance with the reports of Pryor and Michailides (2002) who categorized isolates of *Alternaria* infecting pistachio into four groups. Similarly Prasad *et al.,* 2009 grouped the *A. helianthi* isolates into four groups. Chowdappa *et al.,* (2012) working on diversity analysis of *A. porri* described the colour of the colonies on PDA medium were either greyish orange or brownish orange in color having cottony texture showing dark orange colour on the reverse side of the plate. Several workers have noticed the diversity in colony color, margin and topography among the isolates of *A. solani* (Kaul and Saxena, 1988; Perez and Martinez, 1996 and Babu *et al.,* 2000).

All the isolates exhibited substantial variation in the mycelial growth rate on potato dextrose agar. Based on colony diameter/ growth rate, the isolates were classified into seven groups. Among the isolates the fastest colony growth was recorded in the isolate OLHi1-3 whereas

the isolate OLPk recorded the slowest colony growth (Table 2). Based on sporulation habit isolates were classified into three groups.

Extending studies on cultural variability, the *Alternaria* isolates were characterized on different synthetic and non-synthetic media. The isolates showed varied growth with varying margin type, colony colour and colour of the colony reverse. Likewise Goyal *et al.,* (2011) observed extensive variation in mycelial growth and sporulation among the isolates of *A. brassicae*. Supporting variation in mycelial growth rate Chowdappa *et al.,* 2012a classified the isolates of *A. porri* into fast, intermediate and slow growing groups. Observations on variation in cultural characteristics of *Alternaria* isolates on different media are in line with Ramjegathesh and Ebenezar (2012). Among the media evaluated Sabourauds dextrose agar, potato dextrose agar and oat meal agar has supported maximum growth of the fungus (Table 3). Naik *et al.,* (2010) reported Sabourauds dextrose agar as the best medium supporting the growth of *A. solani*. Mohan (1996) and Karthikeyan (1999) reported potato dextrose agar as the best medium for the growth of *A. palandui* causing leaf blight of onion. Results on oat meal agar medium supporting the growth of *A. palandui* and *A. alternata* are in line with the findings of Kannan (1992). *Alternaria* isolates showing slow growth in corn meal agar, potato carrot agar and Richard's agar may be due to the availability of poor quality sugars present in these medium (Karlatti, 1983; Prasad, 2002 and Savitha, 2004).

Variation in sporulation was observed among the isolates on different culture media. All the isolates showed maximum sporulation on Asthana Hawker's medium followed by potato dextrose agar. Meena *et al.,* 2012 have reported good sporulation of all the isolates of *A. brassicae* in Asthana and Hawker's medium.

In the present study, spore size and shape of conidia were used for identifying the fungus (Fig. 1). The average conidial length of the isolates ranged between 17.90 to 76.15µm however, longest conidial length was recorded in OLNau (76.15 µm) (Table 4) followed by OLA1 (70.99µm) and shortest in OLTor (17.90µm). The average conidial width among different isolates varied from 6.24 to 25.38 µm, and was observed to be highest in OLA1 (25.38µm) followed by OLNau (23.82 µm) and lowest in OLTor (6.24µm). All the isolates produced beaked conidia. The beak length varied from 3.31 to 55.48µm. The isolate OLA1 had the longest beak (55.48µm) followed by OLShi (14.40µm), while it was short in OLTor (3.65µm), OLPk (3.88µm) and OLHbg (3.31µm).

The isolates i.e. OLMb, OLNau, OLLin, OLSik, OLAnn, OLShi, OLHi1-3and OLHi1-4 produced medium beak while the isolates OLWg, OLD1, OLTor, OLNg, OLHbg, OLPk, OLCb and OLSo produced small beak. The average number of transverse septa, varied from 3 to 8, highest being observed in OLA1 and lowest is in OLTor. The number of longitudinal septa varied from 1 to 3, highest was noticed in OLA1 and most of the isolates showed at least one longitudinal septa. The spores of isolates OLA1 and OLNau have been identified as bigger spores with 7-8 septa in conidum.

The isolates i.e. OLMb, OLLin, OLD1, OLShi, OLAnn, OLHi1-3, OLHi1-4 and OlCb produced medium spore with 5-6 septa. The isolates OlWg, OLTor, OLSik, OlNg, OLHbg, OlPk and OlSo produced small spores with 3-4 septa in conidium. The characteristics of the *Alternaria* isolates in question were compared with *Alternaria* species already reported on the host. The variation in conidial size was significant among the isolates of *Alternaria* infecting onion.

Table.1 Cultural characteristics of *Alternaria* isolates infecting onion crop

Sl. No.	Isolates	Geographical origin	Colony colour	Growth pattern	Zonation	Sectors	Margin	Colony reverse
1	OL Mb	Muddebihal	Ashy black	Adherent, circular	Absent	Absent	White, flat, thick, smooth	Black
2	OL Nau	Naubad	Ash, grey	Adherent, raised, not circular	Present (2)	Absent	White, thick, rough	Blakish brown
3	OL Lin	Lingasuguru	Ash white	Circular, raised,	Absent	Absent	White, smooth, thick, raised	Brown
4	OL Wg	Wagdal	Ashy green	Adherent, circular,	Absent	Absent	Light white, raised, thick	Black
5	OLD1	Dharwad	Ashy white	Circular, fluffy	Absent	Absent	Smooth, thick, Light white raised	Brown
6	OL Sik	Sikandrapur	Ashy white	Adherent, Circular	Absent	Present (3)	Greenish white, smooth, thick	Whitish green
7	OL Ann	Annigeri	Ashy white	Adherent, circular,	Absent	Absent	Smooth Thin, raised Light green with whitish tinge	Brown
8	OLA1	Aralur	Ash	Adherent, circular,	Absent	Absent	Smooth Thin, raised Light green with whitish tinge	Black
9	OLHi1-3	Hirehalli	Ashy green	Adherent, circular	Absent	Absent	Smooth, thin, white and flat	Black
10	OLHbg	Hirebagewadi	Ahy green	Circular, Adhernt	Absent	Absent	Circular	Ashy green
11	OL Shi	Shirahatti	Ashy black	Adherent, circular	Absent	Absent	Rough Thick, raised, white	Black
12	OL Tor	Torvi	Ashy	Adherent, circular,	Absent	Absent	Thick, smooth, ashy white	Brown
13	OLHi1-4	Hirehalli	Ashy green	Raised, circular,	Absent	Absent	Rough, thin, white and raised	Brown
14	OL Pk	Pamankallur	Blackish green	Raised, irregular	Absent	Absent	Smooth, raised, thick, white	Black
15	OLCb	Chickkaballapur	Ashy white	Adherent, irregular	Absent	Absent	Smooth Thin, raised whitish tinge, circular	Brown
16	OL Ng	Nargund	Blackish green	Adherent, circular	Absent	Absent	Thick, ash adhered, flat White	Blackish green
17	OL So	Sorab	Ashy green	Adherent,circular	Absent	Absent	Smooth Thin, white raised	Blackish green

Table.2 Colony diameter and sporulation behaviour of *Alternaria* isolates of onion

Isolates	Colony diameter (mm) after 7 days of inoculation	Colony growth rate (mm/day)	Sporulation	Conidia/ml
OL Mb	65	9.29	+++	$6x10^5$/ml
OL Nau	62	8.86	+	$0.5x10^5$/ml
OL Li	50	7.14	+	$0.3x10^5$/ml
OL Wg	45	6.43	++	$3x10^5$/ml
OLD1	67	9.57	++	$3x10^5$/ml
OL Sik	66	9.43	+++	$7x10^5$/ml
OL Ann	50	7.14	++	$2.5x10^5$/ml
OLA1	68	9.71	+++	$5x10^5$/ml
OLHi1-3	65	9.29	++	$3.5x10^5$/ml
OL Hbg	60	8.57	+++	$8x10^5$/ml
OL Shi	57	8.14	++	$2.0x10^5$/ml
OL Tor	65	9.29	+	$0.5x10^4$/ml
OLHi1-4	70	10.00	++	$2.5x10^5$/ml
OL Pk	35	5.00	+	$0.5x10^5$/ml
OL Cb	50	7.14	++	$0.5x10^5$/ml
OL Ng	60	8.57	+++	$6x10^5$/ml
OL So	58	8.29	++	$4.5x10^5$/ml

Table.3 Effect of different media on the mycelial growth of Alternaria isolates

Isolates	**Colony diameter on different media(mm)**										**Mean**
Media	SDA	RA	AHA	OMA	CMA	BEA	HEA	MEA	PCA	PDA	
OL Mb	70.00	70.00	55.00	85.00	37.50	74.00	65.00	68.00	68.00	80.00	67.25
OL Nau	73.50	52.00	55.00	77.50	60.00	80.00	72.00	77.50	60.00	72.00	67.95
OL Lin	78.50	85.00	80.00	85.00	52.00	80.00	72.5	70.00	70.00	80.00	75.30
OL Wg	74.30	66.00	82.00	70.00	40.00	76.00	75.00	50.00	70.00	61.00	66.43
OLD1	77.00	63.00	57.50	81.50	42.50	83.50	75.00	53.00	56.50	80.00	66.95
OL Sik	85.00	40.00	80.00	68.00	42.00	85.00	80.00	63.00	60.00	46.00	64.90
OL Ann	85.00	80.00	85.00	85.00	40.00	85.0	80.00	55.00	70.00	85.00	75.00
OLA1	79.50	75.00	70.00	85.00	45.00	70.00	70.00	64.00	58.00	85.00	70.15
OLHi1-3	80.00	80.00	70.00	80.00	65.00	22.00	62.00	60.00	50.00	80.00	64.90
OL Hbg	80.00	50.00	72.00	85.00	50.00	65.00	75.00	84.00	70.00	85.00	71.60
OL Shi	70.00	60.00	62.00	85.00	65.00	75.00	68.00	70.00	57.00	80.00	69.20
OL Tor	80.00	58.00	72.50	68.50	40.00	82.00	75.00	56.00	57.50	81.00	67.05
OL Hi1-4	85.00	85.00	80.00	50.00	60.00	80.00	75.00	83.00	65.00	80.50	74.35
OL Pk	78.5	75.00	70.00	80.00	52.00	60.00	72.5	45.00	50.00	80.00	66.30
OLCb	80.00	65.00	75.00	78.00	55.00	63.00	70.00	75.00	45.00	79.00	68.50
OL Ng	85.00	55.00	85.00	88.00	40.00	80.00	80.00	85.00	77.50	85.00	76.05
OL So	80.00	70.00	72.00	85.00	55.00	75.00	80.00	85.00	65.00	80.50	74.75
Avg mean	78.90	66.41	71.94	78.62	49.47	72.68	73.35	67.26	61.74	77.65	

Table.4 Variation in conidial morphology of *Alternaria* isolates of onion

Sl. No.	Onion isolates	Colour	Surface	Shape	Beak	No of septa (Transverse Longitudinal)		Conidia		Beak	
								Length	width	Length	Thickness
						T	L	Mean (µm)		(µm)	
1	OL Mb	Dark brown	Smooth	Obclavate	Medium	5-6	1	30.51	10.55	11.71	**3.89**
2	OL Nau	Chocolate brown	Smooth	Obclavate	Medium	7	1	76.15	23.82	10.53	**8.65**
3	OL Lin	Golden brown	Smooth	Obclavate	Medium	6	2	45.10	13.70	10.80	**3.81**
4	OL Wg	Dark brown	smooth	Ovate	Short	4	1	27.95	10.39	6.21	**3.23**
5	OLD1	Golden brown	Smooth	Obclavate	Short	6	1	36.04	13.24	8.27	**3.85**
6	OL Sik	Golden brown	Smooth	Obclavate	Medium	6	1	57.48	19.47	13.32	**9.15**
7	OL Ann	Dark brown	Smooth	Obclavate	Medium	6	1	46.53	13.44	11.32	**4.5**
8	OLA1	Golden brown	Smooth	Obpyriform	Long	7-8	3	70.99	25.38	55.48	**7.51**
9	OL Hi1-3	Light brown	Smooth	Obclavate	Medium	5-6	1	28.49	10.50	10.06	**3.81**
10	OL Hbg	Light brown	Smooth	Obclavate	Short	4	0-1	24.21	9.86	3.31	**3.18**
11	OL Shi	Golden brown	Smooth	Obpyriform	Medium	5	1-2	29	9.74	14.4	**4.68**
12	OL Tor	Brown	Rough	Ovate	Short and blunt	3	2	17.9	6.24	3.65	**3.60**
13	OL Hi1-4	Light brown	Smooth	Obclavate	Medium	6	1	26.89	9.50	11.06	**4.51**
14	OL Pk	Golden brown	Smooth	Ovate	Short	4	2	35.98	11.04	3.88	**3.84**
15	OL Cb	Light brown	Smooth	Obclavate	Medium	5	1	29.28	7.81	10	**4.82**
16	OL Ng	Light brown	Smooth	Obclavate	Short	4	1	22.85	7.25	4.76	**4.41**
17	**OL So**	**Golden brown**	**Smooth**	**Obpyriform**	**Medium**	**4**	**1**	**34.07**	**9.69**	**7.16**	**4.55**

Table.5 Details of the species identification of *Alternaria* isolates based on ITS rDNA

Sl. No.	Isolates code	Place of collection	No. bases	Gene bank Accession number	Species identified
1	OL Mb	Muddebihal, Vijayapura	509	JX294489	*A. porri*
2	OLNau	Naubad, Bidar	467	JX294490	*A.alternata*
3	OL Lin	Lingasagur, Raichur	485	KC357742	*A.tenuissima*
4	OL Wg	Wagdal, Kalaburgi	455	JX666590	*A.porri*
5	OLD1	Dharwad, Dharwad	507	JX666601	*A.palundi*
6	OL Sik	Sikandrapur, Bidar	505	JX666589	*A.tenuissima*
7	OL Anni	Annigere, Gadag	505	KC357739	*A.alternata*
8	OLA1	Aralur, Kolar	508	JX666599	*A.porri*
9	OL Hi1-3	Hirehalli, Tumakuru	498	JX666597	*A.alternata*
10	OL Hbg	Hirebagewadi, Belagavi	509	JX666591	*A.brassicicola*
11	OL Shi	Shirahatti, Dharwad	505	JX666592	*A.porri*
12	OL Tor	Torvi, Kalaburgi	505	JX666593	*A.porri*
13	OL Hi1-4	Hirehalli, Tumakuru	509	JX666600	*A.alternata*
14	OL Pk	Pamankallur, Raichur	501	JX294491	*A.alternata*
15	OL Cb	Chickkaballapur	508	KC357741	*Alternaria* spp.
16	OL Ng	Nargund, Dharwad	509	JX666594	*A.tenuissima*
17	OL So	Soraba, Shivamogga	505	KC357740	*A.alternata*

Fig.1 Conidial morphology of *Alternaria* isolates causing purple blotch of onion

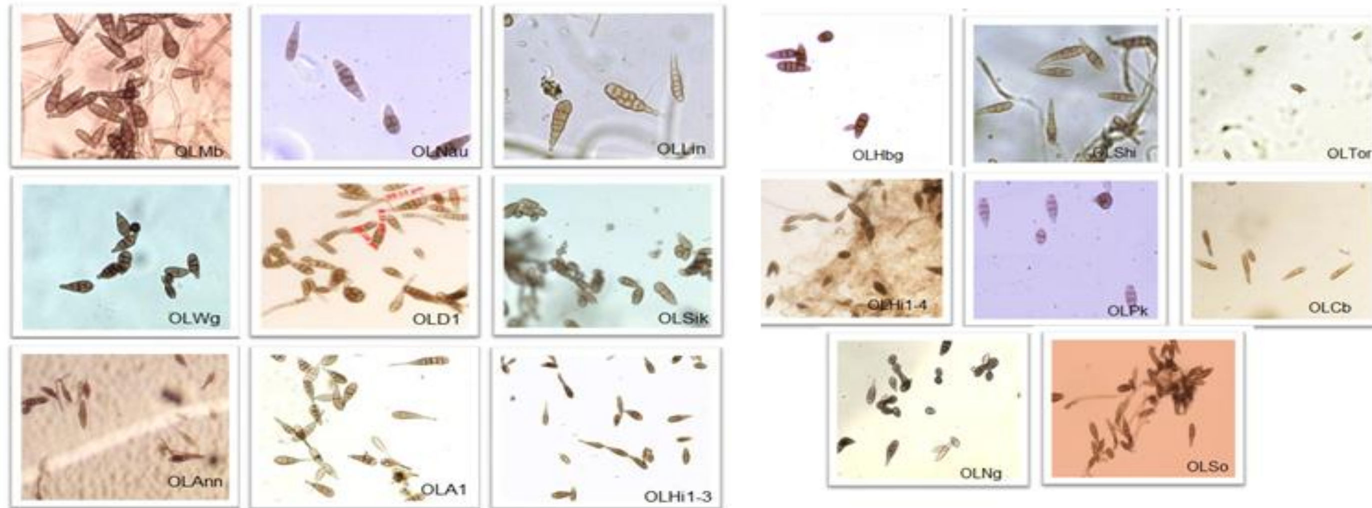
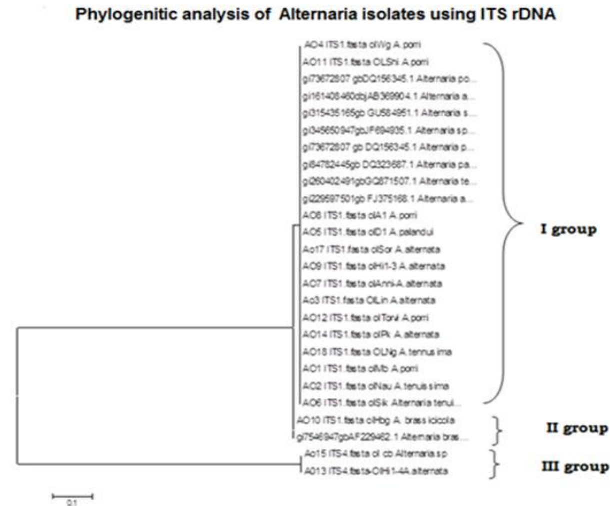


Fig.2 Phylogenitic analysis of *Alternaria* isolates of onion



All the isolates produced conidia of smooth surface, golden brown to dark brown and obclavate shaped conidia measuring about 17.9-76.15 µm× 7.25-25.32 µm and beak length of 5.65-13.32 µm × 2.51-9.15 µm with 3–8 transverse septa and 1-3 longitudinal septa. In the present investigation, all the isolates showed variation in cultural characteristics which is not adequate to claim the existence of races of *Alternaria* species. Discussing the cultural variability Rotem, 1994 admitted many *Alternaria* species differ in cultural characteristics and acknowledged that if these characteristics suffice for identification of races then the number of races may be almost as big as the number of isolates being tested.

The conidial characters of the isolates are in accordance with those described by Ellis, 1971. However, Nolla (1927) reported the dimension of conidia as 105-220 µm -17.5-26 µm in *A. porri* (Nolla, 1927), 10.26-77.52 µm × 4.56-14.82 µm in *A. alternata* (Neergaard, 1945; Abubakar and Ado, 2009), 22-95µm × 8–19 µm in *A. tenuissima* (Wiltshire, 1933), 10.5-77 × 3.5-14µm in *A. palandui* (Ayyangar, 1928), 38-146 × 10-73 µm in *A. cepulae* (Ponnappa, 1974), 18-130 × 8-30 µm in *A. brassicola* (Ellis, 1971). The conidial dimensions and colour of *Alternaria* isolates of onion collected from different localities of Karnataka were within the range of morphological explanation of the earlier recognized species. The conidial dimension of the isolates matched with *A. alternata, A. tenuissima, A. palandui, A. cepulae* and *A. brassisicola.* Similar results on variation in conidial size in the isolates of *A. solani* were reported by Rotem (1994), Varma *et al.,* (2007). The results on morphological variability of isolate are in agreement with earlier workers (Meena *et al.,* 2005; Kaur *et al.,* 2007; Singh *et al.,* 2007) who observed variability in different geographical isolates within *Alternaria* species.

Molecular characterization

Molecular characterization of geographical isolates was carried out based on the sequence of ITS region of rDNA. Sequencing and blasting of ITS rDNA of isolates revealed matching of five different species of *Alternaria viz., A. porri, A. alternata, A. tenuissima, A. palandui* and *A. brassicicola*. Cluster analysis of ITS rDNA sequences did not provide reliable differentiation between the species and among the isolates thus suggested the region of ribosomal DNA gene is inappropriate for taxonomic resolution of *Alternaria* species infecting onion. Correlation was not observed among the group of isolates based on conidial morphology, cultural characteristics, pathogenicity and nucleotide sequence of ITS region of rDNA.

It is evident from the ITS rDNA sequences that *A. alternata* and *A. porri* are the most prevalent species infecting onion in Karnataka. The ITS rDNA sequences of the isolates were submitted to the gene bank and the accession numbers obtained are listed in Table 5. Multiple sequence alignment of the isolates and of the matching species is given in Figure 2. Pryor and Michailides (2002) used ITS rDNA for the identification of *Alternaria* Isolates associated with *Alternaria* blight of pistachio. Correspondingly Chowdappa *et al.,* (2012) analysed genetic variability in the isolates of *A. porri* on the basis of ITS sequences of rDNA. Similarly, Fernandez and Vargas (2007) identified 34 selected morphotypes among the isolates of *Alternaria* spp. Occurring on onion foliage belonging to *A. destruens*, *A. tenuissima*, *A. palaundi* and *A. porri* species group, based on internal transcribed spacer (ITS) region. The sequence data of the total size of the ITS1 and ITS2 regions, of the isolates including the 5.8SrDNA gene, of the isolates studied ranged from 455 to 509. The results inferred

that, the sequence of ITS region varied among the *Alternaria* isolates. The results of the present study are in agreement with the previous reports which showed that the ITS region vary among the species within a genus or among populations (Gardes *et al.,* 1991).

Phylogenetic analysis

Phylogenetic analysis of ITS rDNA sequences executed by neighbour-joining method grouped the isolates into three clades (Fig. 2). The first clade consists of 14 isolates *viz.,* OLWg, OLD1, OLSo, OLHi1-3, OLAnni, OLLin, OLSik, OLNau, OLPk, OLNg, OLMb, OLShi, OLA1 and OLTor. Only one isolate OLHbg was found under second clade. The third clade consists of two isolates OLCb and OLHi1-4. The *Alternaria* isolates clustered in clade I included five reference isolates from GenBank; AB087220 (*A. porri*), AB369904 (*A. alternata*), DQ323687 (*A. palandui*), GQ871507 (*A. tenuissima*) and JF694935 (*Alternaria* spp.). Clade II included only one reference isolate from the gene bank with Accession no AY154707 (*A. brassicicola*). Clade III included two reference isolates from GenBank FJ375168 (*A. alternata*) and GU584951 (*Alternaria* sp). The species *A. alternata*, *A. palandui*, *A. porri* and *A. tenuissima* were placed in monophyletic clade, whereas the species *A. brassicicola* and *Alternaria* sp. were placed separately in two clades. The clustering of isolates based on geographical origin was not evident. Clustering of isolates based on sequence of ITS rDNA did not provide reliable differentiation between the species and also among the isolates**.** Considering the improper differentiation between the species it is suggested that ITS region is inappropriate for taxonomic resolution of *Alternaria* species infecting onion.

Cluster analysis of the isolates is in agreement with Fernandez and Vargas (2007) in deriving phylogenetic relationship among the *Alternaria* isolates of onion based on ITS sequences of rDNA. They suggested that rDNA were not sufficient to differentiate *Alternaria* species infecting onion. Kadam *et al.,* (2009) targeted the internal transcribed spacer (ITS) sequences within the ribosomal DNA (rDNA) region to delineate genetic variability among eight *Alternaria* species causing diseases in other crops and inferred that divergent sequences within the ITS regions can be employed to design species-specific PCR primer for use in molecular diagnostics.

The Alternaria isolates of onion were studied to understand their morphological and molecular diversity. The isolates exhibited variation among the isolates in colour and texture of the colony. Sabouraud dextrose agar supported the maximum growth and corn meal agar did not support the growth. Asthana Hawker's medium supported the sporulation. On PDA medium, the isolates showed significant difference pertaining to mycelial growth and sporulation. Variation in conidia shape, conidia length, width, beak length and number of septa was prominent among the isolates. Sequencing and blasting of nucleotide sequence of ITS region of ribosomal DNA of isolates revealed 100% matching with five different species of Alternaria viz*., A. porri, A. alternata, A. tenuissima, A. zpalandui*and *A. brassicicola*. Cluster analysis of ITS rDNA sequences did not provide reliable differentiation between the species and among the isolates. The results put forward that ITS region of ribosomal DNA is inappropriate for taxonomic resolution of *Alternaria* species infecting onion.

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