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# Morphological and Molecular Characterization of *Alternaria* Species Isolated from Different Plants

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

*Alternaria* belongs to the family of Pleosporaceae, the order of Pleosporales, the class of Dothideomycetes in the phyllum of *Ascomycota*. This fungal genus is characterized by its ability to produce a number of enzymes able to injured many types of crops. The symptoms of Alternaria blight on different agricultural crops such as cabbage, cauliflower, coriander, fenugreek, brinjal, onion, faba bean, dahlia, dracaena, hollyhock, carrot, marigold, tomato and wheat were observed. *Alternaria* produces distinctive "bulls eye" patterned leaf spots in almost all the infected plants. Nineteen *Alternaria* isolates were obtained from fourteen infected plant samples. The observations were recorded from the seven day old culture for colony characteristics on PDA. Based on morphology 10 isolates of *Alternaria alternata*, two isolates *Alternaria tenuissima* and *Alternaria longipes* and one *Alternaria porri*, *Alternaria brassicae*, *Alternaria brassicicola* and *Alternaria* sp. were identified. *Alternaria* species grow rapidly and produced flat, downy to woolly grayish green to black colonies. All the isolates exhibited characteristics dark-coloured multicelled conidia with longitudinal and transverse septa (phaeo-dictyospores) and a beak or tapering apical cells. DNA isolation of all isolates of *Alternaria* was carried out using CTAB method. Isolated DNA was subjected to amplification with ITS1 and ITS4 primers in a thermal cycler. The amplified products ranged from 580-600bp. These amplified products were sequenced and identify the species of different *Alternaria* isolates using BLASTn in NCBI online. All the sequences were published in NCBI public domain. The resulting sequences of all isolates were compared to other sequences in the GenBank as 90-100% identical. Genetic variability was conducted by phylogenetic analysis.

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*Keywords: Alternaria; molecular; morphological; characterization; isolates; conidia; beak length.*

## 1. INTRODUCTION

*Alternaria* belongs to the family of Pleosporaceae the order of Pleosporales the class of Dothideomycetes in the phyllum of *Ascomycota.* It is potentially one of the most ubiquitous, worldwide occurrence opportunistic pathogen affecting many cultivated crops in the field and during postharvest storage of fruit and vegetables. *Alternaria* species attacks all the plant parts and causes severe losses to crops in concern to yield and quality.The genus *Alternaria* was originally described by Nees von Esenbeck [1] with *Alternaria tenuis* as the type. The pathogen produces distinctive "bulls eye" patterned leaf spots. *Alternaria* species grow rapidly producing flat, downy to woolly colonies, covered with grayish, short, aerial hyphae. The surface is grayish white at the beginning which later darkens and becomes greenish black or olive brown with a light border. *Alternaria* forms conidia that arise as protrusions of the protoplast through pores in the conidiophores cell wall. At the onset of conidial development, apex of the conidiophores thickens and a ring-shaped electron-transparent structure is deposited at the apical dome. The classical key taxonomic feature of the genus *Alternaria* is the production of large, multicellular, dark colored (melanized) conidia with longitudinal as well as transverse septa (phaeodictyospores). These conidia are broadest near the base and gradually taper to an elongated beak, providing a club-like appearance. They are produced in single or branched chains on short with erect conidiophores.

Identification of some *Alternaria* species still offers considerable difficulties owing to their high variability and the polymorphism occurring even in pure culture. The conidial features distinguish *Alternaria* from the closely related genera *Ulocladium*, *Stemphylium*, *Embellisia* and *Nimbya*. The difficulty to distinguish between species or even isolates is demonstrated by the presence of nearly one thousand published species designations of *Alternaria* [2]. Over 300 species occurring worldwide have been described [3]. However, errors in the taxonomy of *Alternaria* species have arisen due to the variability of its morphological characters, which are not only affected by intrinsic factors but also by environmental conditions. Hypothetically, the difficulties in taxonomic classification of species within the genus *Alternaria* are partly due to the lack of sexual stages [4]. The molecular taxonomy of the genus *Alternaria* has been recently reviewed. Molecular methods are faster, more sensitive, more stable and less dependent on external factors than morphological methods. The characterization of fungal DNA has been employed to detect and differentiate fungi [5]. Molecular approaches have been developed for the assessment of microbial diversity in complex communities as well [6]. Methods based on DNA analysis can reveal fungal diversity in ecosystems, and offer the potential benefits of highly sensitive and rapid detection [7]. The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal repeat unit has become the primary genetic marker for molecular identification and other species-level pursuits in many groups of fungi [8]. The identification of fungi to species level has been based mostly on the use of variable ribosomal-DNA (rDNA) Internally Transcribed Spacer (ITS) regions. The non-coding ITS region consisting of ITS1, 5.8s rDNA and ITS2, produced a highly sensitive assay as the target sequence for amplification, due to its high copy number in the fungal genome as part of tandemly repeated nuclear rDNA. ITS sequences from well-identified fruiting bodies are estimated to be available for <1% of the hypothesized number of fungal species [9]. These ITS regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the *rRNA* genes. As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions [10,11]. Additionally, the DNA sequences in the ITS region are highly variable and might serve as markers for taxonomically more distant groups. Keeping all these facts and findings, the present investigation started from collection of infected plant species; isolation and screening of the *Alternaria* sp. and identification on the basis of morphological characters. These species identification are further validated through molecular sequence analysis using Internal Transcribed Spacer (ITS) region.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

Fourteen *Alternaria* infected plant samples expressing disease symptoms were collected from adjoining areas of JNKVV campus, Jabalpur as shown in Table 1.

**Table 1. *Alternaria* infected plant samples collected from adjoining area of JNKVV campus, Jabalpur**

| SN | Host Plant | Host plant species | Family | Collection site |
|---|---|---|---|---|
| 1. | Coriander | *Coriandrum sativum* | Apiaceae | Vegetable market, Suhagi |
| 2. | Cabbage | *Brassica oleracea var. capitata* | Brassicaceae | Horticulture field, Maharajpur |
| 3. | Cauliflower | *Brassica oleracea var. botrytis* | Brassicaceae | Vegetable market, Suhagi |
| 4. | Fenugreek | *Trigonella foenum-graecum* | Fabaceae | Vegetable market, Suhagi |
| 5. | Brinjal | *Solanum melongena* | Solanaceae | Horticulture field, Maharajpur |
| 6. | Onion | *Allium cepa* | Amaryllidaceae | Horticulture field, Maharajpur |
| 7. | Dracaena | *Dracaena* sp. | Asparagaceae | Biotechnology centre, JNKVV |
| 8. | Fababean | *Vicia faba* | Fabaceae | Horticulture field, Maharajpur |
| 9. | Hollyhock | *Alcea rosea* | Malvaceae | Garden area of JNKVV |
| 10. | Dahlia | *Dahlia pinnata* | Asteraceae | Garden area of JNKVV |
| 11. | Marigold | *Tagetes erecta* | Asteraceae | Garden area of JNKVV |
| 12. | Carrot | *Daucus carota* sub sp. *Sativus* | Apiaceae | Horticulture field, Maharajpur |
| 13. | Tomato | *Solanum lycopersicum* | Solanaceae | Horticulture field, Maharajpur |
| 14. | Wheat | *Triticum aestivum* | Poaceae | Breeding field |

#### 2.1.1 Media preparation: Potato dextrose agar media (PDA)

Peeled potatoes ~200 g were sliced into small pieces and boiled in 700ml of distilled water up to softening. Extract was filtered through fine strainer and Dextrose (20g) added into it. Volume of the medium adjusted to one liter and 15g agar of was added and sterilized in autoclave at 121°C temperature for 20 min. for making slants, required quantity of medium distributed in test tubes, sealed with nonabsorbent cotton and tilted till get solid.

#### 2.1.2 Isolation of pathogen from host plant

Isolation of *Alternaria* spp. was undertaken from infected plant sample of coriander, cabbage, cauliflower, fenugreek, brinjal, onion, dracaena, fababean, hollyhock, dahlia, marigold, carrot, tomato and wheat. Collected samples were rinsed with sterile water and infected tissues along with adjacent small unaffected tissues were cut into small pieces (2–3 mm length) by using flame-sterilized forceps. Then tissues were further transferred into sterile Petri dishes containing 0.1% mercuric chloride solution for a period of 20 to 30 seconds in laminar air flow and then rinsed three times with sterilized distilled water to ensure freeness from traces of mercuric chloride. Pre-sterilized blotting paper sheet were used to remove excess moisture and then sterilized pieces are aseptically transferred to Petri dishes containing potato dextrose agar media are kept in BOD incubator at 25 ± 2□ temperature with 85 ± 5% relative humidity. After growth of fungal mycelium and sporulation, the fungus was under taken for initial identification. Identification of the selected isolates was again confirmed through slide culture method. Seven days old freshly isolated cultures of *Alternaria* spp. on PDA were used for investigations on various aspects.

#### 2.1.3 Single spore isolation for pure culture of *Alternaria* spp.

The cultures were purified by single spore technique. A dilute spore suspension was poured on plain agar in Petri dishes and the spores were allowed to settle down on the agar surface. The amount of suspension was so adjusted as to form a very thin layer over the surface of the agar. Spores, which settled quite apart from each other were selected under the microscope, marked and encircled on the back of the bottom of the Petri dish with a glass marking pencil. They were lifted along with agar and transferred to Petri dishes already poured with potato dextrose agar medium. After the proper growth of the transferred single spore, regular sub-culturing was done to maintain and check further contamination. After purification, the culture was multiplied and maintained at 2% Potato dextrose agar slant in refrigerator at 4□ for further studies. The cultures were multiplied and renewed after every two months.

All stock cultures were maintained on PDA slants and sub cultured. The slants were incubated at 25°C for 7 days and then stored at 4°C.

#### 2.1.4 Microscopic observation of the fungal isolates

The observations from the seven day old culture were recorded for colony characters on PDA. The morphological features like colour, branching and septation of the hyphae, conidiophores and conidia of the pathogens were studied under compound microscope. Conidia (100 per isolate) were examined under 10x magnification of microscope in laboratory. A slide culture technique was also used to observe the morphology of the fungi [12].

#### 2.1.5 Inoculation and incubation of broth culture

In each conical flask 100 ml potato dextrose broth was poured, sealed with aluminum foil and autoclaved at 121□ tempreture for 20 min. The sterilized PDB were then allowed to cool down upto room temperature. Small pieces of culture were cut from Petridish culture by using a sterilized cork borer and one piece was kept into each of the conical flasks by sterilized inoculation needle under laminar air flow. The inoculated flasks were cotton plucked and incubated in BOD incubator at 25 ± 2└ with 80 ± 5% RH for desired growth of the fungus.

### 2.2 Harvesting of mycelium

After seven days of incubation, fully grown mycelium mat was harvested. Drain out of medium from conical flask, then mycelium was washed with sterile water 3-4 times to removal traces of medium and spores from mycelium mat. Invert conical flask on watch glass drained out excess water from mycelium. Pre-sterilized blotting paper sheet was used to remove moisture from fungal mycelium. Dried mycelium is wrapped into aluminum foil and kept it into -20ºC deep freezer for overnight.

#### 2.2.1 DNA extraction of fungal isolate

Isolation of fungal DNA was carried out by protocol described by Gontia-Mishra et al. [13], with minor modification. Fresh fungal mycelium ~200 mg was carefully grind in liquid nitrogen and crushed with a mortar pestle to a fine powder and 800 µl of extraction buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 2.5 M NaCl, 3.5% CTAB, 150 µl of 20 mg/ml proteinase K) was added. The mixture was vortexed at high speed on a homogenizer (Spinix, Tarsons, India) for 5 min. The samples were placed in a water bath at 65ºC for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was mixed. The samples were again centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform-iso-amyl-alcohol (24:1) was mixed. Samples were again centrifuged. Supernatant was collected and equal volume of ice-cold isopropanol was added. Samples were incubated at -20ºC for 1 to 2 h. The samples were centrifuged for 15 min at 13,000 rpm to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800 µl of 70% ethanol. DNA pellet was air-dried and dissolved in 200 µl TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). Added 5 µl RNaseA (20 mg/ml) to DNA samples, mixed and incubated at 37ºC for 1h. DNA was recovered and air-dried. DNA was reconstituted in TE buffer for further use for PCR amplification.

#### 2.2.2 PCR amplification using ITS primers

The ITS region was amplified by PCR with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCRs were carried out in a 20 µl reaction volume that contained the DNA templates (2 µl of purified DNA), 18 µl Master Mix, contains of 2 µl 10x PCR buffer, 2.4 µl of $MgCl_2$, 0.4 µl of dNTPs, 2 µl of primers ITS1 and ITS4, 0.2 µl of *Taq*DNA polymerase and rest of the volume nuclease free water.

Amplification was performed in Agilent Master Cycler Gradient. The optimal annealing temperature was 55□ for amplification of DNA using ITS1/ITS4 primers. The PCR amplification conditions included: initial denaturation at 94°C for 5 min and 35 cycles: Denaturation at 94°C for 30 sec, annealing at 55-57°C for 60 sec followed by extension step at 72°C for 90 sec and a final extension at 72°C for 7 min. The resulting PCR products were visualized on 1.5% agarose gel containing ethidium bromide at 80 volts. After electrophoresis, the DNA bands were visualized by illumination under ultraviolet (UV) light.

#### 2.2.3 DNA sequencing and phylogenetic tree construction

DNA sequencing of PCR products was done by the dideoxynucleotide chain termination method

[14] with outsourcing of Chromous Biotech Pvt. Ltd., Bangalore. The sequence homology search was performed using the BLAST program through the internet server at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/). Sequences and accession numbers for compared isolates were retrieved from the GenBank database. Sequence pair distances among related and different fungal isolate were scored with the ClustalW program and phylogenetic tree analysis was performed with the MEGA-7 [15]. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal) [16].

## 3. RESULTS

### 3.1 Isolation and Morphological Characterization of *Alternaria* Isolates from Infected Samples

The present investigations were carried out to study morphological and molecular identification of *Alternaria* species from different plants. Experiments were conducted to study the cultural, morphological characters and molecular characterization of *Alternaria* spp. by using ITS primers. The symptoms of *Alternaria* blight on different crops *viz.,* brinjal, tomato, cabbage, cauliflower, coriander, fenugreek, onion, fababean, dahlia, dracaena, hollyhock, wheat, marigold and carrot were studied during (2017-18). The diseases have appeared on plant parts including leaves, fruits and flower. The characteristic symptoms appeared on different plantswere small, circular, brown necrotic spots all over the foliage, lower leaves, the spots gradually enlarged in size and later became irregular in shape, or remained circular with concentric rings or zones (Fig. 1).

The pathogen was successfully isolated from 14 different plant samples and pure culture was maintained on potato dextrose agar. Nineteen isolates of *Alternaria* spp. were obtained from 14 infected plant samples. The developing fungi was sub-cultured and maintained in pure form for further studies. The morphological characters of all isolates were included in Table 2. Similarly colony and spore morphology were presented in Figs. 2 and 3.

#### 3.1.1 Molecular identification of different *Alternaria* isolates

Total 19 PCR products of different isolates were sequenced and seq. length ranges from 508-528 bp. The fragment sizes of nineteen accessions are mention in Table 3.

#### 3.1.2 Similarity based species identification through BLAST tool

Each sequence was subjected to online BLASTn in NCBI (www.ncbi.nlm.nih.gov/blast/) for analysis to identify the species of different *Alternaria* isolates. The searches resolved the different sequences up to genus level. However, due to several hits at the same parameters of max score, total score, query cover, E value and identity percent, one of the specie was not identified unambiguously. The closest matches were used to confirm the exact taxonomic group of each isolate. All isolates were determined to have identical sequences in the ITS region. The ITS region and morphological characterization were used to identify *Alternaria* spp. The resulting sequences of all isolates were compared to other sequences in the GenBank and were 90-100% identical as shown in Table 3.

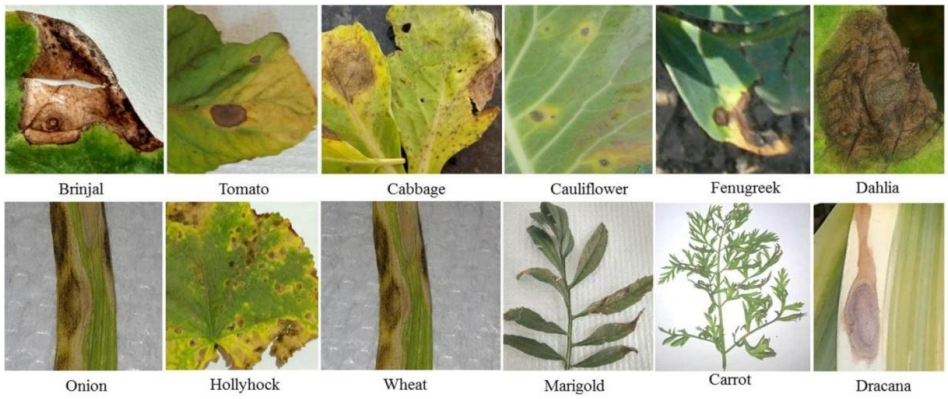


**Fig. 1. Symptoms of Alterneria Blight on different plants**

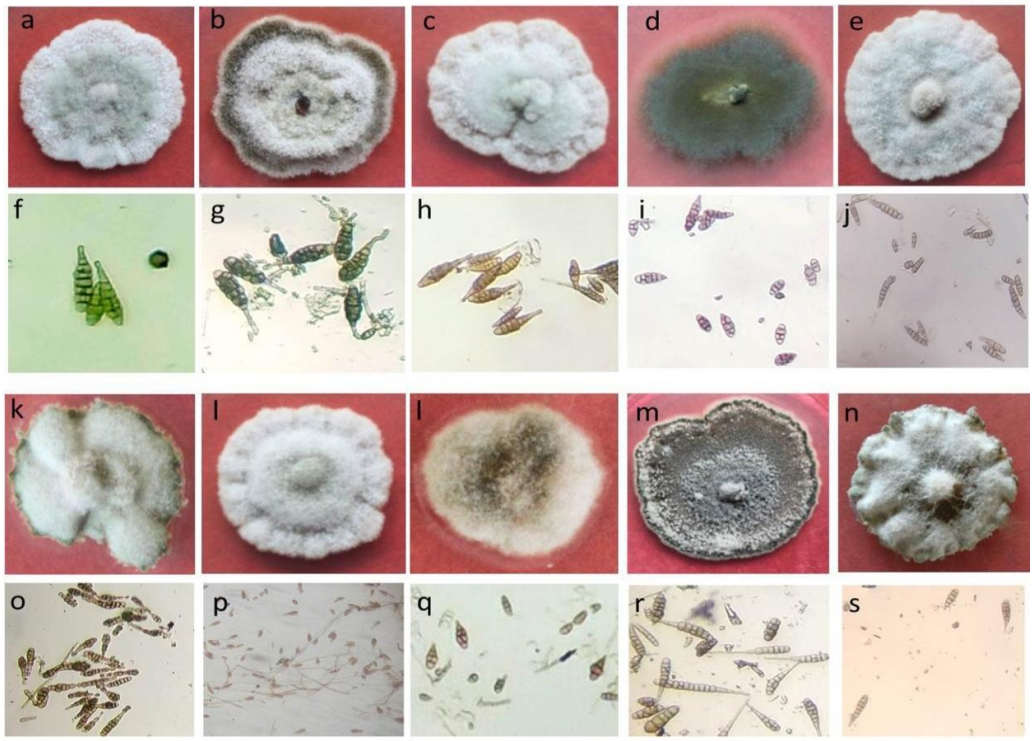


**Fig. 2. Morphology of colony and conidia of isolate number 1-10**

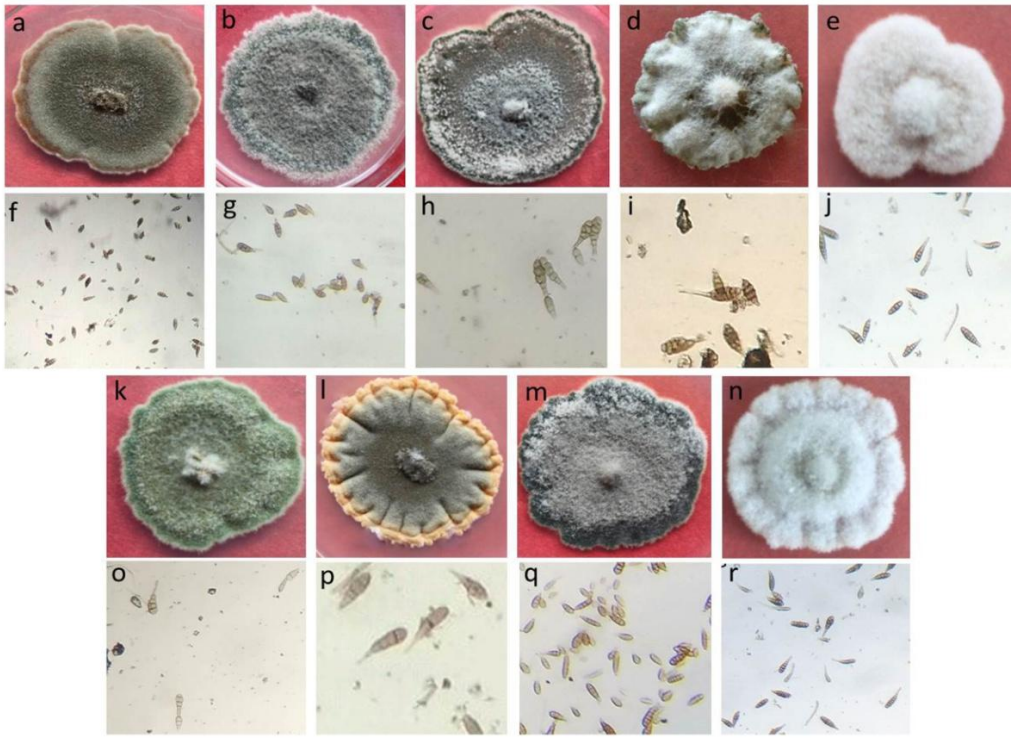


**Fig. 3. Morphology of colony and conidia of isolate number 11-19**

**Table 2. Morphological characteristics of *Alternaria* species**

| SN | Host name | Colony characters in PDA | Size of conidia (µm) | | Septation | Organisms |
|---|---|---|---|---|---|---|
| | | | Horizontal | Vertical | | |
| 1 | Brinjal | Felty to wolly, fluffy mycelial growth and irregular margins. | 35.7 × 5.23 | 3–8 | 0-2 | *Alternariaarborescens* |
| 2 | Brinjal | Grayish green to sage green, fluffy mycelial growth and irregular margins. | 45.16 × 12.42 | 1-3 | 2-5 | *Alternariaalternata* |
| 3 | Tomato | Greenish gray colony, fluffy mycelial growth and irregular margins. | 47.16 × 13.49 | 1-3 | 13.49 | *Alternariaalternata* |
| 4 | Tomato | Greenish gray to dark olivaceous gray, fluffy mycelial growth and irregular margins. | 44 × 12 | 3-8 | 1-2 | *Alternarialongipes* |
| 5 | Cabbage | Smoky grayish, fluffy mycelial growth and irregular margins | 67.12 × 12.26 | 1-5 | 1- 6 | *Alternariabrassicicola* |
| 6 | Cauliflower | Dark Olive gray to grayish green, fluffy mycelia growth and irregular margins. | 89 × 29 | 11-15 | 6-14 | *Alternariaalternata* |
| 7 | Coriander | Dark olive to iron gray, fluffy mycelial growth and irregular margins. | 47.56 × 8.95 | 3-7 | 1-4 | *Alternaria sp.* |
| 8 | Fenugreek | Greenish gray, fluffy mycelial growth and irregular margins. | 47.10 × 13.47 | 2-7 | 1- 3 | *Alternariaalternata* |
| 9 | Onion | Olive gray, fluffy Mycelium with zonation and smooth margins. | 87 × 20.13 | 3-14 | 0-8 | *Alternariaporri* |
| 10 | Fababean | Greenish brown colony, fluffy mycelial growth and irregular margins. | 42.12 × 11.45 | 2- 6 | 1- 3 | *Alternariaalternata* |
| 11 | Dahlia | Brownish green colony, fluffy mycelial growth | 43.12 × 11.9 | 1-3 | 2-10 | *Alternariaalternata* |
| 12 | Dracaena | Pea to sage green colony, fluffy mycelial growth and irregular margins. | 46.15 × 16.87 | 1-3 | 2-5 | *Alternariaalternata* |
| 13 | Hollyhock | Dark Greenish colony, fluffy mycelial growth and irregular margins. | 48.19 × 12.22 | 2- 7 | 1- 3 | *Alternariaalternata* |
| 14 | Wheat | Greenish gray colony, fluffy mycelial growth and irregular margins. | 47.14 × 13.46 | 1-3 | 2-8 | *Alternariaalternata* |
| 15 | Marigold | Sage green to pea green, fluffy mycelial growth and irregular margins. | 47.56 × 12.45 | 1-3 | 2-10 | *Alternariaalternata* |
| 16 | Marigold | Olive buff to dark grayish green colony, fluffy mycelial growth with Zonation and smooth margins. | 45.12 × 9.32 | 2-11 | 0-10 | *Alternariatenuissima* |
| 17 | Carrot | Greenish gray to olivaceous black, fluffy mycelial growth with zonation and irregular margins. | 62.7 × 9.4 | 3-6 | 0-2 | *Alternariatenuissima* |
| 18 | Carrot | Dark greenish colony, fluffy mycelial growth and irregular margins. | 47.13 × 13.54 | 1-3 | 2-10 | *Alternariaalternata* |
| 19 | Carrot | Pale black to olivaceous brown, fluffy mycelial growth and irregular margins. | 18-20 × 10 | 3-5 | 0-2 | *Alternarialongipes* |

**Table 3. Identification of Alternaria isolates associates with different plants based on ITS sequence**

| Host | fragment size (bp) | Accession number | Related species | % Identity |
|---|---|---|---|---|
| Brinjal | 528 | MH356755 | *Alternaria arborescens* KU645989 | 90 |
| Brinjal | 522 | MH356762 | *Alternaria alternata* MG991256 | 100 |
| Tomato | 527 | MH356763 | *Alternaria alternata* MG025876 | 90 |
| Tomato | 525 | MH356770 | *Alternaria longipes* KJ722535 | 100 |
| Cabbage | 522 | MH356771 | *Alternaria brassicae* KF542552 | 100 |
| Cauliflower | 522 | MH356772 | *Alternaria brassicicola* KU204772 | 100 |
| Coriander | 526 | MH356773 | *Alternaria sp.* KT723004 | 100 |
| Fenugreek | 525 | MH356764 | *Alternaria alternata* KT192223 | 100 |
| Onion | 508 | MH356766 | *Alternaria porri* KY419560 | 100 |
| Fababean | 528 | MH356756 | *Alternaria alternata* MF927539 | 100 |
| Dahlia | 527 | MH356757 | *Alternariaalternata*KX783404 | 100 |
| Dracaena | 508 | MH356758 | *Alternaria alternata* MF422133 | 100 |
| Hollyhock | 525 | MH356759 | *Alternaria alternata* MG991256 | 90 |
| Wheat | 522 | MH356765 | *Alternaria alternata* MG596645 | 100 |
| Marigold | 522 | MH356761 | *Alternaria alternata* MF927539 | 100 |
| Marigold | 522 | MH356768 | *Alternaria tenuissima* KX783377 | 100 |
| Carrot | 525 | MH356767 | *Alternaria tenuissima* MG132062 | 100 |
| Carrot | 522 | MH356760 | *Alternaria alternata* MG025876 | 90 |
| Carrot | 522 | MH356769 | *Alternaria longipes* KJ722535 | 100 |

## 3.2 Phylogenetic Analysis

A database search for closely related fungal species were aligned using the ClustalW Program. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.03939477 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic tree was divided into major and minor group. Minor group has only *Alternaria* sp. and major group again subdivided into three groups as A, B and C. The subgroup 'A' represents total 12 isolates of *Alternaria alternata* and *Alternaria tenuissima* were closely related. The subgroup 'B' represents total three isolates *Alternaria porri*, *Alternaria brassicicola* and *Alternaria brassicae* whereas *Alternaria brassicicola* and *Alternaria brassicae* were shows 100% similarity. The subgroup 'C' represents total three isolates; two of *Alternaria longipes* and one of *Alternaria arborescens* (Fig.4).

### 3.2.1 Pairwise distances amongst sequences

The overall average pairwise distances calculated as 0.006. The pairwise distance amongst sequences ranges 0-0.035. The minimum pairwise distance calculated as 0.00 and maximum value 0.035 found between two pairs of first one *Alternaria longipes* and *Alternaria* sp. and another pair of *Alternaria arborescens* with *Alternaria* sp.

### 3.2.2 Estimation of divergence between sequences

The estimation of evolutionary divergence between all the sequences done to study the evolutionary changes in isolates. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 508 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [15]. The results showed that the range of evolutionary divergence between all the sequences was 0-0.035. Minimum divergence (0) found between *Alternaria brassicicola* and *Alternaria brassicae*. The maximum value (0.035) found between two combinations of *Alternaria longipes* and *Alternaria arborescens* with *Alternaria* sp. most diverse isolates based on molecular study.

## 4. DISCUSSION

All the ten isolates of *Alternaria alternate* isolated produced grayish to green colony. All the isolates produced light brown conidia with varied in shape

from obclavate to mostly ellipsoidal, muriform having tapered apex. Conidia and beak length was measured about 42.12-48.19 x 11.9-17.37 µm and 18.7µm to 23.81 µm. The character agreed with the original descriptions given by Keissler [17].

Morphological variability like colour of colony, margin of colony, colony growth and sporulation on PDA was studied among ten isolates of *Alternaria alternate* collected from different plant sources. The isolates found to have distinct morphological variability. Ramjegathesh and Ebenezar [18] also studied the morphology of ten isolates of *A. alternata* causing leaf blight disease of onion in Tamil Nadu and found conidia were muriform and light brown colour, length of their conidia was varied from 30.99 to 42.47 µm. The width of the conidia was varied from 11.90 to 17.37 µm. Generally, all the isolates produced both beaked and unbeaked conidia. The number of cells in each conidium varied from 2 to 9. All the isolates took 13-16 days for sporulation.

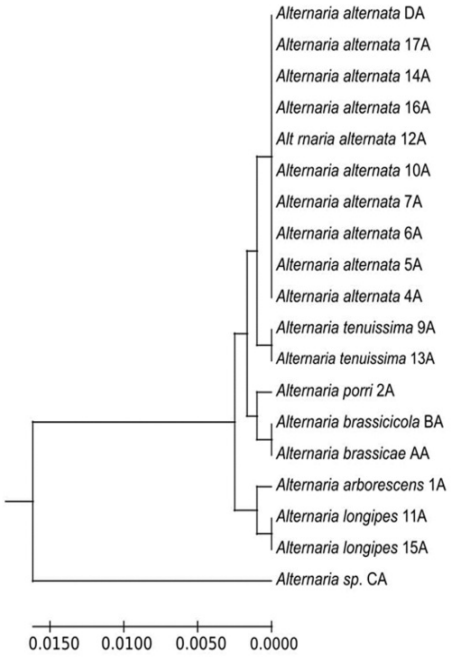


**Fig. 4. Dendrogram representing phylogenetic affiliation of different isolates**

DNA sequencing has greatly enhanced the ability to accurate and reproducibly identifies the fungi. In the present finding *Alternaria brassicola* and *A. brassicae* successfully identified on cole crop by using ITS1-ITS4 primer set. Similar experiment was conducted by Mishra et al. [19] on morphological, biochemical and molecular characterization of *Alternaria brassicicola* and *A. brassicae* using a primer set, ABS28F and ABS28R as an internal diagnostic marker to differentiate and analyze *A. brassicicola* from *A. brassicae* and other fungi associated with leaves, stem and siliquae of rapeseed-mustard. The ITS marker could not distinguish the other *Alternaria* species analyzed and more markers, such as the mitochondrial small subunit (mt SSU) or the glyceraldehydes-3-phosphatedehydrogenase (gpd) gene used by Pryor and Bigelow [20], are needed. In the present study one of the species was not identified by ITS region unambiguously. *Alternaria* sp. isolated from onion was identified as *Alternaria porri* by primer set ITS1-ITS4. Likewise, molecular variability of *A. porri* by using seven RAPD primers and two ITS primers ITS1 and ITS4 in polymerase chain reaction (PCR) were also carried out by Mohsin et al. [21]. Three isolates of *Alternaria* spp. were found from carrot and identified as *Alternaria tenuissima*, *Alternaria alternata* and *Alternaria longipes* using ITS1and ITS4 primers. However, Tulek and Dolar [22] reported *A. radicina*, *A. alternata*, *A. tenuissima* and *A. dauci* using ITS1 and ITS4 primer in carrot. *Alternaria alternata* and *A. longipes* isolated from tomato leaves were identified by using ITS1 and ITS4 primers set. Earlier same experiments were conducted in tomato and identified *Alternaria alternata* using ITS1 and ITS4 primers by Xie et al. [23].

In UPGMA phylogeny analysis, the isolates were grouped into two clusters. Minor group has only one *Alternaria* sp. and major group again subdivided into three sub-groups as A, B and C. The sub-group 'A' represents total 12 isolates of *A. alternate* and *A. tenuissima*. The subgroup 'B' represents total three isolates viz., *A. porri*, *A. brassicicola* and *A. brassicae*. The subgroup 'C' represents two isolates of *A. longipes* and one isolate of *A. arborescens*. Similar clustering patterns have also been reported in *Alternaria* isolates by Pryor and Gilbertson [24] and Xie et al. [23].

In this study, the ten *Alternaria alternata* isolates MH356765, MH356764, MH356756, MH35657, MH356758, MH356759, MH356760, MH356761, MH356762 and MH356763 exhibited the maximum similarity with published ITS sequences of KT192223, MF927539, KX783404, MF422133, MG991256, MF927539. Out of nineteen sequenced isolates two accession

MH356767 and MH356768 showing similarity with *A. Tenuissima* i.e. MG132062 and KX783377, two accession MH356769 and MH356770 matching with *A. longipes* i.e. KJ722535, KJ722535 and single accession MH356771 showing similarity with *A. brassicae* i.e. KF542552; MH356772 similar with *A. brassicicola* i.e. KU204772; MH356766 similar with *A. porri* KY419560; MH356755 similar with *A. arborescens* i.e. KU645989 and accession MH356773 similar with *Alternaria* sp. i.e. KT723004 were published in public domain NCBI.

## 5. CONCLUSIONS

On the basis of morphological characters eight different species were identified *viz., Alternaria alternate, A. arborescens, A. porri, A. longipes, A. tenuissima, A. brssicicola, A. brassicae and Alternaria* sp. Out of nineteen isolates ten were *Alternaria alternata*, although they were different in their colony and conidial characters. For molecular characterization and identification ITS regions was used and the BLASTn analysis revealed sequence similarity of 99-100% with the sequences in database of NCBI GenBank for all the isolates. Phylogenetic analysis revealed minimum divergence between *A. brassicicola* and *A. brassicae* and the maximum divergence between two isolates of *A. longipes* and *A. arborescens* with *Alternaria* sp.

The species detection specific to *Alternaria* and development of molecular tools is an upcoming challenge for the plant pathologist. Study will help for sustainable plant disease management.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors**.**

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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