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Variability of *Alternaria brassicicola* Causing Leaf Blight of Cabbage in Major Cabbage Growing Districts of Assam, India

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ABSTRACT

The research was conducted at Assam Agricultural University, Jorhat, Assam, India during September, 2021–May, 2022 to study the variation of *Alternaria brassiciola* infecting cabbage. Fifteen isolates of *Alternaria brassicicola* which infected cabbage were collected from six different districts in Assam and their variability was investigated. Within the *in vitro* setting, it was observed that all isolates exhibited significant variation in terms of conidial length, breadth, and septal count. The isolates displayed a range of average conidial length and breadth spanning from 20.45µm to 29.74µm and 6.56µm to 9.89µm, respectively. Additionally, the isolates exhibited a variation in transverse septa with counts ranging from 4 to 6 and longitudinal septa, ranging from 0.13 to 0.41. Regarding the colony colour, four distinct variations, ranging from dark grey-green to dark brown-green and from mild olive green to greyish olive green were observed. These colonies featured edges in shades of white, greyish, brownish, or green edges and their shapes varied between round and irregular, accompanied by either a fluffy or compressed growth pattern. The diameter of the radial growth, observed on the fifteenth day after inoculation, varied from 57.3 mm to 84.2 mm. In RAPD analysis it was evident that the Jorhat isolates (AbJor_2) and the Barpeta isolate, (AbBa_1) exhibited the highest genetic similarity, as indicated by the lowest genetic distance with the coefficient of similarity 0.484. Conversely, the Jorhat isolates (AbJor_3) and Barpeta isolate (AbBa_1) shared the least genetic similarity, with the coefficient of similarity 0.021.

KEYWORDS: Cultural, morphological, molecular, RAPD, variability

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1. INTRODUCTION

Cabbage (*Brassica oleracea* L. var. capitata) is one of the most common winter vegetables in India which belongs to the Brassicaceae family. After China, India is regarded as the world's second-largest producer of cabbage (Chadha, 2003). West Bengal, Orissa, Gujrat, MP, Assam, and Bihar are the top six cabbage-producing states of India (Anonymous, 2022). In India, cabbage was grown in an area of 398.5 thousand hectares during the year 2017–18 with a production of 9037300 t and productivity of 22.7 t ha^{-1}. Assam produced 640130 t of cabbage in 2017–18 in an area of 33.24 thousand ha with a productivity of 19.26 t ha^{-1} (Anonymous, 2018), which is much less than the national average of 22.7 t ha^{-1}. The reason for low production of the crop may be due to the attack of number of insect pests and diseases during different stages of crop growth. The major diseases of cabbage faced by the farmers of Assam are leaf spot and blight (*Alternaria brassicae* and *A. brassicicola*), damping off (*Pythium debaryanum*), downy mildew (*Perenospora parasitica*), wire stem (*Rhizoctonia solani*), black leg (*Phoma lingam*), *Sclerotinia* rot (*Sclerotinia sclerotiorum*) and black rot (*Xanthomonas campestris* pv. *campestris*) (Sharma et al., 2018). Among the diseases, Alternaria leaf spot and blight is the major obstacle for farmers and may cause up to 80% loss in yield (Hossain and Mian, 2004). The fungus is a widespread and profoundly destructive plant pathogen capable of infecting every part of the plant. In severe instances; its presence invariably results in complete defoliation (Peralta et al., 2005). The disease is more prevalent in regions with higher levels of humidity, rainfall, and temperature. Yield losses due to Alternaria infection in cabbage have been reported to be 10–70% in India (Singh et al., 2017). Hence, one of the primary contributors to the lower crop yield is the prevalence of Alternaria leaf spot and blight disease, recognized as the most prevalent and harmful disease affecting cabbage and Brassicas (Meah et al., 2002).

The study of variability plays a crucial role in documenting shifts within populations and individuals, as an indicator of the presence of distinct pathotypes (Nagesh et al., 2021). Although *A. brassicola* reproduces asexually, the isolates of this pathogen causing leaf blight disease in cabbage show significant variability in morphology, culture and genetic composition. This high level of variability suggests the existence of different pathotypes within the population (Kumar et al., 2016; Prakash et al., 2022). The intensity of Alternaria blight on cabbage displays variations not only among crops in a particular region but across different seasons and geographical locations. *A. brassicola* adapts to changing environments and develops fungicide resistance through variation caused by heterokaryosis or mutation. However, high variation increases disease and infection risk, challenging cultivar resistance stability and making it difficult to control the disease with resistant cultivars (Adhikari et al., 2017). The existence of morphological variability within the isolates of other *Alternaria* species has been reported by earlier workers (Kaur et al., 2007; Mohsin et al., 2016; Roopa et al., 2016). Variability in the morphological and cultural characteristics of *A. brassicicola* isolates from different regions of India has been reported by Deep et al. (2014). The RAPD-PCR approach has been used by several researchers to examine the molecular variability in *Alternaria* species, and they have demonstrated the variety within the species (Swati et al., 2014; Aneja et al., 2016). Therefore, the present investigation was attempted to explore the understanding of these causal organisms with respect to cultural, morphological and molecular variability among the isolates of *A. brassicicola*, from the major cabbage growing districts of Assam, India.

2. MATERIALS AND METHODS

The research was conducted at Assam Agricultural University, Jorhat, Assam, India during September, 2021–May, 2022. The infected cabbage leaves with characteristic symptoms were collected from six different districts, including Dhubri, Kokrajhar, Barpeta, Nalbari, Nagaon, and Jorhat shown in Table 1.

2.1. Isolation, cultural and morphological characterisation of the isolates

The fungal cultures from the diseased samples were then isolated on a PDA medium as per the method described by Singh et al. (2009). Following that, these isolates were purified using the single-spore method (Tousson and Nelson, 1976). Utilising the nine-day-old cultures of each isolate, the morphological variability of 15 isolates of *Alternaria brassicicola* was examined. Each isolate's conidial morphology, including the size of the spore body including breadth, length, and the total number of transverse and longitudinal septa, was noted. A phase contrast binocular microscope attached to a computer was used to acquire pictures of the conidial structure of each isolate. Each isolate's cultural characteristics, such as colony colour and texture on PDA media, were directly observed from the culture developed on Petri plates. Additionally, the growth behaviour was evaluated, including morphological characteristics and form (circular/irregular). The Royal Horticultural Society, London (R.H.S) colour chart was used to determine the colour of the culture. On the fifteenth day following the inoculation of the culture on the Petri plates, the radial growth measurement was obtained.

2.2. DNA isolation and PCR amplification

By using the CTAB method, the whole genomic DNA

Table 1: Geographical locations for collection of Alternaria leaf blight of cabbage from different districts of Assam

Sl. No.	Isolates	Sample collected area		
		District	Area	Geographical location
1.	Ab-Dhu_1	Dhubri	Golakganj	26.10°N latitude and 89.83°E longitude
2.	Ab-Dhu_2		Bilasipara	26.21°N latitude and 90.22°E longitude
3.	Ab-Kok_1	Kokrajhar	Bashbari	26.07°N latitude and 90.39°E longitude
4.	Ab-Kok_2		Kazigaon	26.20°N latitude and 90.00°E longitude
5.	Ab-Kok_3		Gossaigaon	26.45°N latitude and 89.97°E longitude
6.	AbBa_1	Barpeta	Sorbhog	26.48°N latitude and 90.87°E longitude
7.	AbBa_2		Mandia	26.26°N latitude and 90.95°E longitude
8.	AbNal_1		Nadla	26.43°N latitude and 91.31°E longitude
9.	AbNal_2	Nalbari	Mukalmua	26.27°N latitude and 91.35°E longitude
10.	Ab-Nag_1	Nagaon	Rupahi	26.38°N latitude and 92.67°E longitude
11.	Ab-Nag_2		Panigaon Choiali	26.35°N latitude and 92.70°E longitude
12.	Ab-Nag_3		Raidongia	26.39°N latitude and 92.60°E longitude
13.	AbJor_1	Jorhat	Barbheta (AAU Orchard)	26.72°N latitude and 94.20°E longitude
14.	AbJor_2		Teok	26.83°N latitude and 94.43°E longitude
15.	AbJor_3		Alengmora	26.81°N latitude and 94.12°E longitude

Mean followed by the same letter in the superscript(s) are statistically at par; NS- Nonsignificant

was extracted (Doyle and Doyle, 1990). Ten RAPD PCR primers were used to effectively amplify the DNA (OPD1: ACCGCGAAGG, OPD2: GGACCCAACC, OPD3: GTCGCCGTCA, OPD4: TCTGGTGAGG, OPD5: TGAGCGGACA, OPD6: ACCTGAACGG, OPD7: TTGGCACGGG, OPD8: GTGTGTCCCA, OPD9: CTCTGGAGAC, OPD10: GGTCTACACC). In a 15 µl PCR mix, which included double distilled water (8 µl), 10X PCR buffer (1.5 µl), MgCl2 (1 µl), DNTP (1.5 µl), Primer (1.5 µl), Taq DNA polymerase (0.5 µl), and Target DNA (1 µl), the PCR reaction was carried out (Sharma et al., 2013). After that, the PCR processes were carried out in a gene Amp 9700 thermal cycler. The thermocycler was used to do the amplification, which started with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, primer annealing for 1 min at 32°C, primer elongation for 2 min at 72°C, and an extended elongation for 10 min at 72°C. The technique was used to determine which RAPD primers would be best for analysing the molecular differences between the isolates.

2.3. Phylogenetic analysis

The amplification reactions were examined using electrophoresis on 1.2% agarose gel in a TBE buffer. The electrophoretic gel was operated at 75V until the dye had moved a third of the gel's length. A UV transilluminator was used to see the migrated DNA, and the result was a picture that was saved to a computer. Gel pictures were manually scored, and a present/absent matrix was kept. The similarity index was calculated using this data and the NTSYS PC 2.02i programme (Rolf, 1997). UPGMA created a dendrogram from the distance matrix (Sneath and Sokal, 1973).

3. RESULTS AND DISCUSSION

3.1. Morphological variation

The morphological parameters of the fifteen *A. brassicicola* test isolates varied in terms of length, breadth, and septation. The average conidial length was found to be 20.45 µm in Nalbari isolate (AbNal_2) and 29.74 µm in Jorhat isolate (AbJor_2) with a range of 15.5 µm to 32.9 µm. The average conidial breadth was found to be in the range of 5.2 µm to 11.2 µm with the highest breadth (9.89 µm) in Dhubri isolate (AbDhu_2) and the lowest (6.56µm) in Kokrajhar isolate (AbKok_1). The average no of transverse septa was the lowest (4.0) in Nalbari isolate (AbNal_1) and the highest (6.0) in Dhubri (AbDhu_2) and Jorhat (AbJor_1, AbJor_2) isolates with a range from 3-8. The average number of longitudinal septa varied from 0.13 to 0.41 (range 0-2). All the conidia were found without a true beak (Table 2 and Figure 1). Maximum mycelial growth was found in AbNal_2 (84.2 mm) isolate, followed by AbJor_3 (83.3 mm), AbNal_1 (83.2 mm), AbJor_2 (83.0 mm), AbBa_1 (82.3 mm), AbNag_1 (82.2 mm), AbKok_1 (81.3 mm), AbNag_3 (79.0 mm), AbKok_2 (76.7 mm), AbBa_2 (73.7 mm), AbDhu_2 (69.5 mm), AbDhu_1 (67.2 mm), AbKok_3 (66.3 mm), AbJor_1 (65.0 mm)and AbNag_2 (57.3 mm). Several researchers have also documented morphological variability among the *Alternaria* spp. isolates (Singh et al., 2007; Goyal et al., 2011). *Alternaria brassicicola* conidia are muriform and lack a beak (Chand and Chandra, 2014). The Conidia were 13–120 µm long and 6–16 µm

Table 2: Morphological variability among the isolates of *Alternaria brassicicola*

Isolate	Conidial length (µm)		Conidial breadth (µm)		Septation			
	Average	Range	Average	Range	Transverse		Longitudinal	
					Average	Range	Average	Range
AbDhu_1	21.70^{d}	17.2-25.3	7.63^{e}	6.8-8.7	5.00^{b}	4–7	0.22	0-1
AbDhu_2	26.49^{b}	23.8-30.4	9.89^{a}	6.9-10.7	6.00^{a}	5–8	0.24	0-1
AbKok_1	24.06^{c}	19.6-29.5	6.56^{f}	5.5-7.8	5.67^{a}	3–7	0.26	0-1
AbKok_2	21.36^{d}	16.6-24.1	6.76^{f}	5.2-8.1	4.67^{bc}	5–8	0.41	0-2
AbKok_3	25.52^{bc}	20.8-27.6	9.53^{a}	7.5-11.2	5.67^{a}	4–6	0.30	0-2
AbBa_1	21.49^{d}	17.1-25.4	8.67^{bc}	5.6-10.7	4.33^{cd}	5–7	0.30	0-2
AbBa_2	21.31^{d}	15.5-25.3	7.96^{cde}	6.2-9.3	5.00^{b}	4–6	0.21	0-1
AbNal_1	21.05^{d}	15.6-24.7	7.96^{cde}	6.2-8.9	4.00^{d}	4–7	0.25	0-2
AbNal_2	20.45^{d}	16.6-23.3	7.79^{de}	5.8-9.8	5.67^{a}	4–7	0.20	0-1
AbNag_1	21.56^{d}	16.7-24.4	8.52^{bcd}	6.8-10.1	4.33^{cd}	3–8	0.35	0-2
AbNag_2	28.70^{a}	25.0-32.9	8.39^{bcde}	6.8-9.6	5.00^{b}	4–7	0.23	0-1
AbNag_3	25.48^{bc}	19.8-31.8	9.14^{ab}	7.1-10.1	5.00^{b}	3–6	0.13	0-1
AbJor_1	24.11^{c}	21.9-28.2	8.51^{bcd}	6.1-10.4	6.00^{a}	3–7	0.24	0-1
AbJor_2	29.74^{a}	26.1-32.7	8.70^{bc}	6.1-10.5	6.00^{a}	5–8	0.20	0-1
AbJor_3	28.70^{a}	26.2-30.7	7.97^{cde}	7.6-9.7	5.67^{a}	3–6	0.18	0-1
SEd±	0.72	-	0.40	-	0.32	-	NS	-
CD (p=0.05)	1.54	-	0.83	-	0.66	-	NS	-

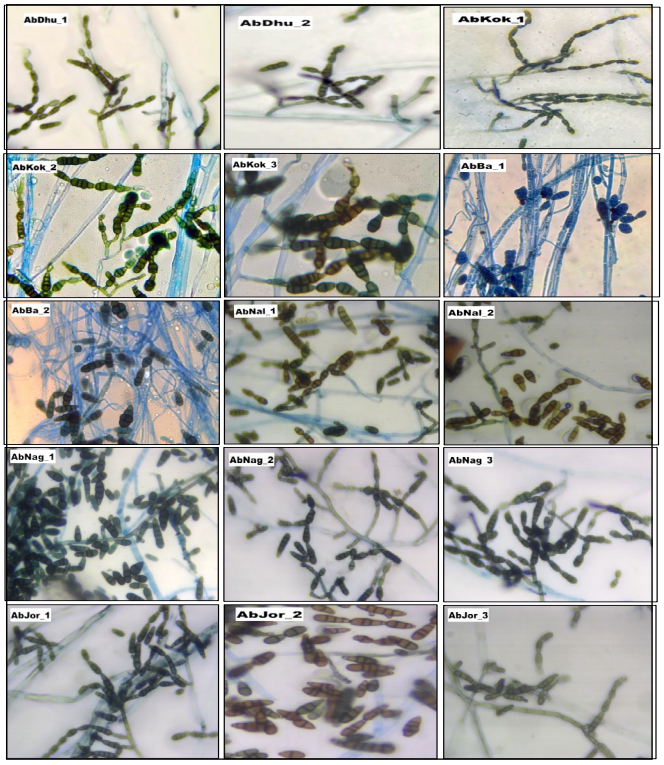

Figure 1: Morphology of conidia along with conidiophore of fifteen isolates of *Alternaria brassicicola*

wide, with a cross and longitudinal septum. Meena et al. (2010) also described that *Alternaria brassicicola* produces conidia with no discernible beaks and has 5 to 8 transverse and 0 to 4 longitudinal septation.

3.2. Cultural variation

Variations in the cultural characteristics and colony colour were also observed in the test isolates. The colours varied from greyish olive green (AbNag_2, AbJor_1, AbJor_3, AbNal_1, AbDhu_1), dark grey-green (AbKok_1, AbKok_3, AbNag_1), moderate olive green (AbDhu_2, AbKok_2) and dark brown green (AbBa_1, AbBa_2, AbNal_2, AbNag_3, AbJor_2) and mostly the colonies had fluffy and compressed growth with slight variations and regular to irregular margins with a whitish, greyish, brownish and greenish rim among the isolates (Table 3, Figure 2). The results revealed that all fifteen isolates of *A. brassicicola* exhibited great variability with respect to mycelial growth, colony color, colony diameter and colony zonation. Deep et al. (2014) examined thirty-two *A. brassicicola* isolates for colony colour and radial development on PDA and reported that the colonies ranged in colour from olive green to dark olivaceous black. Similarly, Kumar et al. (2020) reported cultural differences among 16 isolates of *A. brassicicola* that cause dark patches of mustard. They found a range of colony colours, including olivaceous grey,

Table 3: Cultural variability among the isolates of *Alternaria brassicicola*

Isolates	Cultural characteristics			
	Colour	Growth pattern	Margin	Radial growth after 15 days (mm)
AbDhu_1	Greyish olive green	Fluffy	Circular with whitish rim	67.2^{fg}
AbDhu_2	Moderate olive green	Fluffy	Circular with greyish rim	69.5^{ef}
AbKok_1	Dark grey green	Fluffy	Circular with whitish rim	81.3^{ab}
AbKok_2	Moderate olive green	Compressed	Circular with whitish rim	76.7^{cd}
AbKok_3	Dark grey green	Compressed	Irregular with whitish rim	66.3^{fg}
AbBa_1	Dark brown green	Fluffy	Irregular with whitish rim	82.3^{ab}
AbBa_2	Dark brown green	Fluffy	Irregular with brownish rim	73.7^{de}
AbNal_1	Greyish olive green	Fluffy	Circular with whitish rim	83.2^{ab}
AbNal_2	Dark brown green	Compressed	Circular with whitish rim	84.2^{a}
AbNag_1	Dark grey green	Compressed	Irregular with whitish rim	82.2^{ab}
AbNag_2	Greyish olive green	Fluffy	Irregular olivaceous green rim	57.3^{h}
AbNag_3	Dark brown green	Fluffy	Circular with whitish rim	79.0^{bc}
AbJor_1	Greyish olive green	Fluffy	Irregular with whitish rim	65.0^{g}
AbJor_2	Dark brown green	Compressed	Circular with brownish rim	83.0^{ab}
AbJor_3	Greyish olive green	Compressed	Circular greyish rim	83.3^{ab}
SEd±				0.212
CD (p=0.05)				0.435

Mean followed by the same letter in the superscript(s) are statistically at par

brown, greyish brown, light olive green, chocolate brown, greenish straw, grey, whitish grey, dark brown, light brown, and olivaceous green, as well as the presence and absence of zonation, with round and irregular margins.

3.3. Molecular variation

The RAPD analysis revealed genetic variability in all fifteen test isolates of *A. brassicicola*. Out of the 10 primers tested, the primers viz., OPD1, OPD2, OPD5 and OPD6 revealed amplifications above 45 (Table 4 and Figure 3). The simple matching coefficient values were generated using Jaccard's similarity coefficient where the lowest genetic distance or highest similarity was found in Jorhat isolate

Table 4: PCR- amplicon of 10 different Primers

Primer	Number of PCR amplification fragments generated			Percentage of polymorphic loci (PPB)	Total No. of bands amplified
	Total No. of bands	Polymorphic bands	Monomorphic bands		
OPD1	8	8	0	100.00	81
OPD2	7	7	0	100.00	71
OPD 3	5	5	0	100.00	34
OPD 4	4	4	0	100.00	22
OPD 5	6	6	0	100.00	56
OPD 6	7	7	0	100.00	48
OPD 7	5	5	0	100.00	30
OPD 8	6	6	0	100.00	38
OPD 9	5	5	0	100.00	25
OPD 10	6	6	0	100.00	36

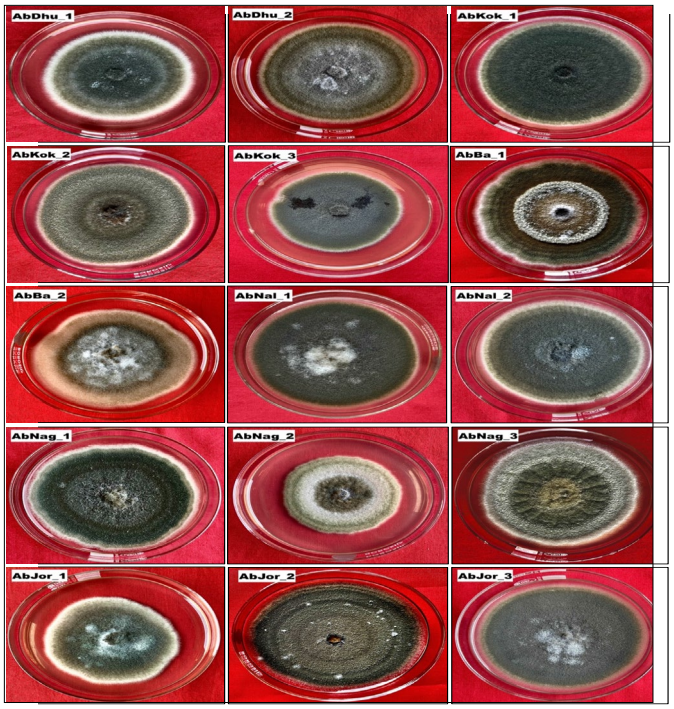

Figure 2: Cultural variation of *Alternaria brassicicola* isolates after 15 days of radial growth

(AbJor_2) and Barpeta isolate (AbBa_1) with a coefficient of similarity (0.484), followed by 0.459 coefficient of similarity between Kokrajhar isolate (AbKok_3) and (AbKok_1) and between Jorhat (AbJor_1) and Kokrajhar (AbKok_2) with 0.435 similarities. The least genetic similarity (0.021) was found between Jorhat isolate (AbJor_3) and Barpeta isolate (AbBa_1) and also between Jorhat isolate (AbJor_3) and (AbJor_2) with 0.023 similarities (Table 5). The dendrogram generated with the similarity data was grouped into five clusters, Cluster I consist of 2 isolates (AbDhu_1 and AbDhu_2), Cluster II consist of 5 isolates (AbKok_2, AbJor_1, AbBa_2, AbNal_2 and AbNag_1), Cluster III consist of 2 isolates (AbBa_1 and AbJor_2), Cluster IV consists of 5 isolates (AbKok_1, AbKok_3, AbNal_1, AbNag_3 and AbJor_3) and Cluster V consists of a single isolate AbNag_2 as an outlier (Figure 4). Selvamani, (2014) investigated the genetic variants of forty isolates using 26 RAPD markers, and found that all of the isolates had a significant degree of variation. With a 0.67 similarity value, the isolates were divided into six separate clusters. Gherbawy, (2005) found significant genetic variation across Alternaria isolates, even within the same species group.

Table 5: Similarity matrix for *A. brassicicola* from different districts

	AbDhu_1	AbDhu_2	AbKok_1	AbKok_2	AbKok_3	Ab Ba_1
AbDhu_1	1					
AbDhu_2	0.34	1				
AbKok_1	0.364	0.24	1			
AbKok_2	0.356	0.303	0.382	1		
AbKok_3	0.204	0.15	0.459	0.279	1	
AbBa_1	0.203	0.209	0.132	0.313	0.1	1
AbBa_2	0.304	0.25	0.36	0.415	0.3	0.261
AbNal_1	0.196	0.2	0.316	0.233	0.417	0.111
AbNal_2	0.333	0.235	0.246	0.397	0.122	0.298
AbNag_1	0.273	0.213	0.275	0.289	0.139	0.375
AbNag_2	0.182	0.13	0.277	0.183	0.128	0.087
AbNag_3	0.232	0.12	0.333	0.222	0.371	0.149
AbJor_1	0.27	0.264	0.316	0.435	0.229	0.354
AbJor_2	0.151	0.171	0.192	0.25	0.212	0.484
AbJor_3	0.306	0.159	0.311	0.297	0.303	0.021

Table 5: Continue...

	Ab Ba_2	AbNal_1	AbNal_2	AbNag_1	AbNag_2	AbNag_3	AbJor_1	AbJor_2	AbJor_3
AbBa_2	1								
AbNal_1	0.231	1							
AbNal_2	0.327	0.109	1						

Table 5: Continue...

	Ab Ba_2	AbNal_1	AbNal_2	AbNag_1	AbNag_2	AbNag_3	AbJor_1	AbJor_2	AbJor_3
AbNag_1	0.286	0.125	0.375	1					
AbNag_2	0.234	0.081	0.298	0.196	1				
AbNag_3	0.265	0.294	0.102	0.157	0.174	1			
AbJor_1	0.352	0.222	0.288	0.373	0.121	0.327	1		
AbJor_2	0.317	0.161	0.239	0.275	0.15	0.219	0.245	1	
AbJor_3	0.163	0.258	0.154	0.059	0.139	0.233	0.231	0.023	1

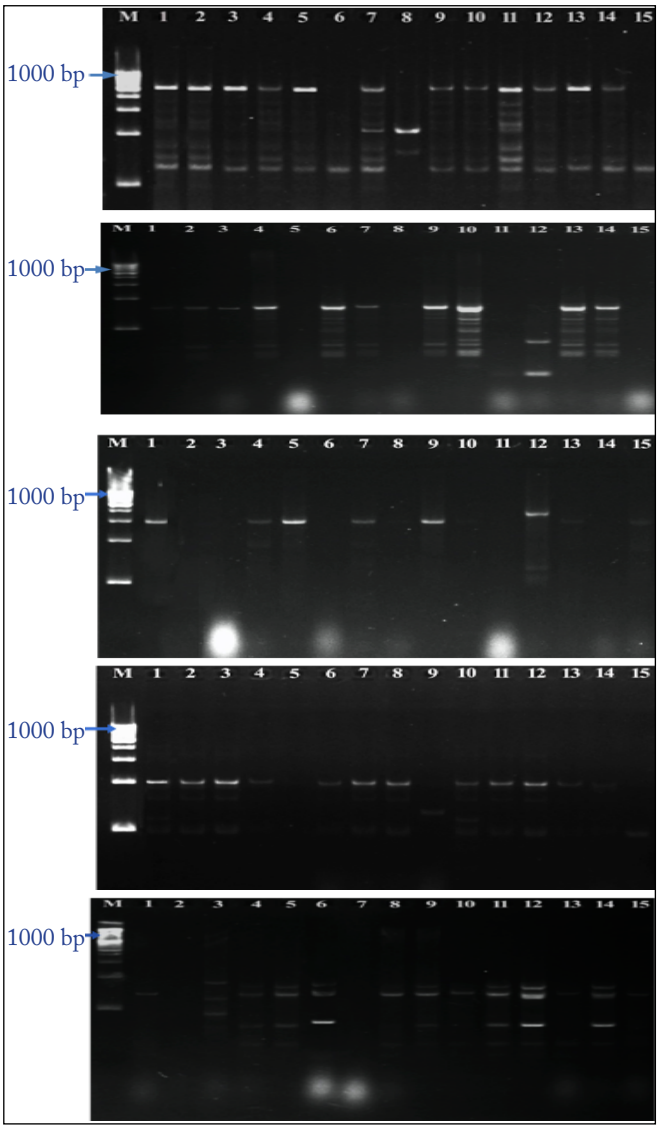

Figure 3: RAPD banding profile of primer OPD: 1-5 in 15 isolates of *Alternaria brassicicola*

Other species of Alternaria infecting crucifers have shown similar results (Meena et al., 2012; Sharma et al., 2013). Many other researchers used RAPD genetic markers to describe variation within an *Alternaria* species (Sharma and Tewari, 1995, 1998; Kumar et al., 2008).

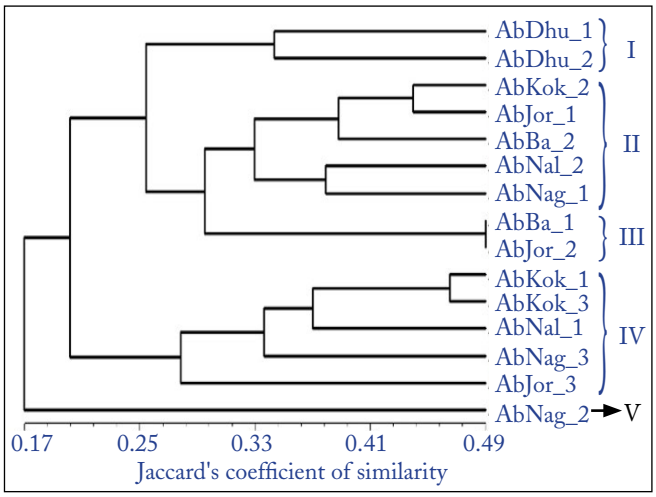

Figure 4: UPGMA based dendogram of 15 isolates of *Alternaria brassicicola* derived from using 10 RAPD Primers

4. CONCLUSION

The conidial length, breadth, and number of both transverse and longitudinal septa that varied greatly across all of the isolates. *In vitro*, there was significant cultural variation in terms of mycelial growth rate, morphological characteristics, colony form and colour along with compressed and fluffy morphological characteristics. Molecular variation among the isolates was also found when RAPD molecular analysis was performed on the fifteen isolates. There is a great need for research into developing resistant varieties of cabbage against huge variability within the same species of *Alternaria*.

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