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MOLECULAR CHARACTERIZATION OF *Alternaria porri* (Ellis) Cif., THE CAUSAL AGENT OF PURPLE BLOTCH DISEASE OF ONION, WITH THE HELP OF SIMPLE SEQUENCE REPEAT MARKERS

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ABSTRACT

Purple blotch disease of onion, caused by *Alternaria porri* (Ellis) Cif., causes extensive damage to the bulb and seed crops. The present study was aimed to assess the morpho-cultural variations and genetic diversity in 14 isolates *of A. porri* by simple sequence repeat (SSR) markers (selected on the basis of polymorphism pattern). Most isolates showed white fluffy colony growth and yellow to light orange pigmentation with circular, irregular and smooth margin along with clear-cut zonation. During the study period, 36 loci were obtained and the average expected gene diversity was found in the range of 0.0531-0.3707. Shan cluster analysis and un-weighted pair-group method with arithmetic averages (UPGMA) analysis revealed that the isolates 'A.p.B4' and 'A.p.B1' had maximum similarity coefficient (0.9166667), while isolates 'A.p.B12' and 'A.p.B13' had distant similarity coefficient (0.6111111). The SSR primers D.N.alt4 (200-720 bp) and D.N.alt10 (300-600 bp) represented unique bands for all isolates which were used for the identification of isolates. The polymorphic information content (PIC) value for primer D.N.alt-4 and D.N.alt-10 was 0.0, with maximum and minimum PIC value for D.N.alt14 (0.2978) and D.N.alt-5 (0.0495), respectively, thus represented the discriminatory power of a locus. The study revealed that the nine SSR markers tested were helpful in the isolation and molecular characterization of *A. porri* on the basis of polymorphism or genetic diversity.

Keywords: *Alternaria porri,* genetic diversity, onion, purple blotch, SSR markers

INTRODUCTION

Onion (*Allium cepa*) is one of the important bulb crops in India. The estimated onion production in India is 262.92 metric tonnes (MT) ha^{-1} as compared to 260.91 MT in 2019-2020 (NAFED, 2021). Worldwide, India stands 2nd in onion production after China. The major onion producing states in India are Maharashtra, Karnataka, Madhya Pradesh, Gujarat, Bihar, Andhra Pradesh, Rajasthan, Haryana and Telangana. There is a lot of demand for Indian onion in the world and the country exported 15,78,017 MT fresh onion worth 2,826.50 crores rupee (equivalent to 378.49 million USD during the year 2020-21 (APEDA, 2021). Despite being a major vegetable crop, the seed production of onion is not successful in India because of several constraints such as poor soil fertility, unfavorable environmental conditions, lack of resistant varieties, severe attack of insect

pest (thrips, head-border, onion maggot, cut worms, mites, etc.) and diseases (damping off, purple blotch, Stemphylium blight, Colletotrichum blight, downy mildew, smut, etc.). Among the disease pests, purple blotch is the most destructive disease in onion seed production. Seeds are supposed to be an efficient medium for the dispersal of seedborne plant pathogens. The fungi are closely associated with seeds which help in long-term existence and extensive dissemination of pathogens. *Alternaria porri,* the causal agent of the purple blotch of onion, is considered a serious threat to the seed production in onion. In India, onion seed production is mainly carried out in the states of Madhya Pradesh, Maharashtra and Gujrat, while Punjab, Haryana and Rajasthan have abandoned seed production due to the devastations caused by purple blotch. The disease appears on the seed stalk as purple coloured elliptical eye shaped lesions with concentric rings. On disease progression the lesions girdle the stalk and detach the flower from stalk.

A. porri (Ellis) Cif., the causal agent of purple blotch, is one of the most serious onion diseases in India (Tripathi *et al.,* 2009; Islam *et al.,* 2020). Shahanaz *et al.* (2007) have reported that the relative occurrence of *A. porri* causes 50-100% losses to onion crop, and the fungus was isolated from 4% of the seed samples tested. *A. porri* is a seed-borne disease and the phytosanitary legislations have been promulgated in many countries to check its entry into their territories (McDonald, 2004).

Various conventional techniques such as visual seed examination and various incubation methods have extensively been used to detect seedborne fungi but these methods are too laborious, lengthy and less responsive to the low level of seed infection. Currently, PCR based molecular markers are used to identify the pathogens which show high sensitivity and specificity. Of these, random fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites (SSR), internal transcribed spacers (ITS) and single nucleotide polymorphism (SNP) are widely used in plant disease diagnosis and seed health testing, especially to assess the variability and diversity at molecular level (McCartney *et al.,* 2003; Cooke and Lees, 2004; Abbas *et al.,* 2021). Molecular markers are the faster and most accurate method of identification and early detection of plant pathogens. Weir *et al.* (1998) used marker techniques to differentiate between two *Alternaria s*pecies namely *A. solani* and *A. alternata.* SSR markers are considered highly reproducible molecular markers frequently used in population genetic studies of many fungi. Being polymorphic in nature the SSR markers are used to compare the populations based on their allele's frequencies (Ricciardi *et al.,* 2020; Zhong *et al.,* 2021). Ying *et al.* (2011) screened 80 SSR primer pairs against *A. alternata,* the causal organism of *Alternaria* pathotype blotch of apple. Dongo (2005) performed genotypic analysis of *A. brassicicola* using 16 pairs of microsatellite primers. Dinh and Hocking (2006) studied relationships and population genetics amongst 64 isolates of *A. alternata* with the help of 5 microsatellite markers while Benichou *et al.* (2009) used 11 SSR markers to assess allelic variability among 43 isolates of *A. dauci*. Kale *et al.* (2012) studied the efficacy of ISSR markers while examining the high genetic diversity among 20 isolates of *A. alternata.* Singh *et al. (*2014*)* identified and characterized transferable microsatellite loci from *A. brassicicola* to *A. brassicae* so as to develop diagnostic markers. Chethana *et al.* (2018) observed genetic diversity in various isolates of *A. porri*. The accurate and rapid identification of pathogen is important to satisfy the phytosanitary regulations and effective disease management. The present study was aimed to assess PCR based SSR markers for the identification and genetic variability in various isolates of *A. porri.*

MATERIALS AND METHODS

Sample collection

The laboratory experiments on the molecular characterization of *A. porri* were carried out in the Biological Control and Plant Stress Laboratories, of GBPUA&T, Pantnagar (India). Fourteen

isolates of *A. porri*, isolated from the infected onion seeds were collected from farmer's fields of various geographical regions of Uttarakhand (India) used to assess their genetic variability.

Morphological and cultural study of A. porri

The infected onion seeds were collected on the basis of symptoms and microscopic studies. The seed collected from the infected umbel showing symptoms of the disease were extracted and surface sterilized with 0.1% sodium hypochlorite solution for 1-2 min followed by 2-3 successive washing in sterilized distilled water. The sterilized seeds were aseptically placed on solidified malt agar dextrose medium and were incubated at 27±1°C for 7 days. The morphological and cultural characteristics of fourteen isolates of *A. porri* were observed for growth pattern and colony characteristics such as colour of the colony, colony texture, shape, margin, pigmentation and sporulation and compared with authentic literature (Cifferi, 1930; Ellis, 1971).

Molecular characterization of A. porri on the basis of SSR markers

For molecular studies, the genomic DNA of *A. porri* was extracted as per CTAB (cetyl tri-methyl ammonium bromide) method (Lee and Tayler, 1990). For purification, the extracted DNA was treated with RNase and proteinase K (Genei Pvt. Ltd., Bangalore, India). The purification of genomic DNA was checked spectrophotometrically (Bio-Rad SmartSpec 3000 UV/ Visible) at 260/280 nm.

For SSR-PCR amplification, a reaction mixture of 20.0 µL was prepared with the help of a pair of forward and reverse primers (Tautz, 1989). The master mix was prepared by using dNTPs mixture 0.8 µL (10 mM), Taq DNA polymerase 0.3 µL (of 3.0 U μL^{-1}), reaction buffer 2.0 µL $MgCl_2$ 1.2 µL (10x), forward and reverse primers 1 µL each (10 pM μL^{-1} primers) and triple distilled water 12.7 µL. The mixture was homogenized by centrifuging it for 10 sec. Finally, the template DNA 1 µL (of 50 ng μL^{-1}) was added and run for the PCR programme. The nine oligonucleotide SSR primers were designed with the help of NCBI, EST site of Google using website software programme synthesized by Genei Pvt. Ltd., Bangalore (India). The PCR amplification was achieved in M.J. Research Thermocycler (PTC 200).

The optimum reaction conditions were: initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min and annealing at 55°C for 1 min. For next round repeat, the cycle from -2nd step were repeated for 35 times. The steps were followed by extension at 72°C for 1 min and final extension at 72°C for 7 min. Amplicon of PCR product was checked for its purity in 2% agarose gel electrophoresis. Agarose gel (2%) was prepared for SSR markers by using submerged gel electrophoresis unit as per the method of Sambrook *et al.* (1989). It was suspended in proper quantity of 1X TAE buffer and ethidium bromide stain (1.5 µg μL^{-1}) added. The mixture of DNA loading dye (bromo-phenol blue) and amplicon was taken in ratio of 1:6 and loaded in wells with the help of micropipette (200 µL). The electrophoresis unit was performed at 75V for 90 min. After destaining in deionized water, the gel image was viewed in gel documentation system.

For absence or presence of bands, the DNA fingerprints were scored in the form of binary matrix. Jaccard's coefficient was obtained for 14 isolates of *A. porri* with the help of NTSYS-pc (version 2.11 W; Exeter Biological Software, Setauket, NY, USA (Rohlf, 1993). Jaccard's coefficient was calculated by SIMQUAL program.

$$\text{Jaccard's coefficient} = NAB / (NAB + NA + NB)$$

Where, NA and NB = number of bands in sample A and B; NAB = number of bands shared in the samples.

The dendrograms was constructed by means of un-weighted pair-group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973). Software programme Seqid was used for the calculation of molecular weight of each band. The average expected gene diversity was calculated by means of the bands scored in gel. It was assessed by using molecular marker technology in studies on plant genetic diversity (*http://www.ipgri.cgiar.org/publications*).

The estimation of discriminatory power of a locus polymorphic information content (PIC) that provides an estimate of discriminatory power of a locus or loci, taking into account not only for

the number of alleles that are expressed, but also relative frequencies of those alleles was estimated using the formula:

$$PIC = 1 - 1/n \sum_{i=1}^{n} Pij$$

Where; Pij is the frequency of j^{th} allele in the i^{th} primer.

RESULTS AND DISCUSSION

Morpho-cultural characterization

The 14 isolates of *A. porri*, collected from different geographical regions of Uttarakhand (India) were assessed for genetic variability on the basis of morphological and molecular criteria. All the isolates showed significant variation in cultural characters such as growth pattern, colony colour, margin, pigmentation, and zonation on malt extract agar medium (Table 1). Most of the isolates showed white fluffy growth of mycelia with pink grayish colour. The colonies were circular or irregular with smooth margin. Most of the colonies of *A. porri* like those of isolates A.p.B1, A.p.B2 A.p.B4, A.p.B6, A.p.B7, A.p.B8, A.p.B9, A.p.B10 and A.p.B14 showed yellow or light orange colour pigmentation in the beginning which later became dark orange to brownish and then purple. The isolates A.p.B3 A.p.B5 A.p.B11 and A.p.B13 produced light yellow colour of pigmentation. A distinct zonation was observed in isolates A.p.B3, A.p.B5 and A.p.B12 while isolates A.p.B1, A.p.B8, A.p.B10, A.p.B11 and A.p.B13 showed slight zonation. In isolates A.p.B2 A.p.B4 A.p.B6 A.p.B7 A.p.B9 and A.p.B14 the zonations were absent. Our results are in confirmatory with Shahnaz *et al*. (2013) who reported that the mycelial characteristics of *A. porri* varied from smooth to fluffy and whitish to dark olivaceous with presence of zonation in most of the isolates.

Table 1: Some colony characteristics of *Alternaria porri* isolates, collected from Uttarakhand (India)

S. No.	Isolates	Source	Colour	Colony texture	Shape	Margin	Pigmentation	Zonation
1	A.p.B1	Pantnagar	Creamish white	Cottony	Circular	Smooth	Light yellow to orange	Slight
2	A.p.B2	Haldwani	Peach	Compact	Circular	Smooth	Orange to brownish orange	Absent
3	A.p.B3	Gaulapar	Grayish	Condensed	Circular	Smooth	Pale	Distinct
4	A.p.B4	Halduchaur	Pinkish white	Cottony	Irregular	Webby	Slight yellow to orange	Absent
5	A.p.B5	Ramnagar	Grayish white at periphery	Condensed	Circular	Smooth	Pale	Distinct
6	A.p.B6	Ranikhet	Pinkish white	Cottony	Irregular	Slightly webby	Orange to yellowish	Absent
7	A.p.B7	Almora	Pinkish White	Cottony	Irregular	Slightly webby	Yellowish to orange	Absent
8	A.p.B8	Jeolikot	Pinkish gray	Condensed, flattened	Circular	Webby	orange to dark brownish	Slight
9	A.p.B9	Champawat	Peach to grayish	Condensed, bulky	Circular	Slightly webby	Dark brownish	Absent
10	A.p.B10	Jashpur	Pinkish brown	Condensed	Irregular	Smooth	Brownish orange	Slight
11	A.p.B11	Khatima	Grayish black	Condensed	Circular	Webby	Pale	Slight
12	A.p.B12	Haridwar	Greyish green	Condensed	Circular	Smooth	Light yellow	Distinct
13	A.p.B13	Dehradun	Greyish white	Floccose	Circular	Smooth	Pale	Present
14	A.p.B14	Ranibagh	Peach	Condensed	Irregular	Webby	Dark orange	Absent

SSR based molecular variation among A. porri isolates

To assess the genetic variability among the isolates of *A. porri*, nine SSR primers were chosen on the basis of polymorphism pattern. Overall, 36 bands (locus) were found for 14 isolates. SSR primers D.N.alt4 (200-720 bp) and D.N.alt10 (300-600 bp) showed distinctive bands for 14 isolates which were used in the identification of isolates. The polymorphic information content (PIC) value was lowest (0.0) in primer D.N.alt-4 and D.N.alt10, and highest (0.2978) in D.N.alt14 primer, while it was 0.1587 on an average for 9 primers (Table 2). The average expected gene diversity (Hi), assessed on the basis of banding pattern of each primer, ranged from 0.0531 (D.N.alt5) to 0.3707 (D.N.alt14). There was a great genetic diversity among various isolates of *A. porri*.

Shan cluster analysis and UPGMA methods were applied for the construction of dendrogram (Fig. 1). SSR dendrogram revealed that the isolates A.p.B4 and A.p.B1 had highest similarity coefficient (0.9166667) with a maximum bootstrap value of 92%. It showed very close genetic relationship in these two isolates. The high degree of similarity coefficient (0.8888889) was exhibited among the isolates A.p.B7 and A.p.B6, A.p.B11 and A.p.B10, and A.p.B3 and A.p.B5; whereas distant similarity was observed in isolates A.p.B12 and A.p.B13 with minimum bootstrap values of 72 and 74%, respectively.

The nine SSR primers made on an average four bands in each primer while other isolates gave monomorphic amplification with a common band of 200, 380, 400 and 720 bp with primers D.N.alt.4; 500, 800 and 1000 bp with primer D.N.alt.5; 300 and 400 bp with primer D.N.alt.-9; 300,

Table 2: Information about the SSR markers used in assessing the genetic diversity of the isolates of *Alternaria porri*

S. No.	Primer code	Primer sequence (5'to3')	Amplified product range (bp)	Total bands	Mono bands	Poly bands	% Poly-morp-hism	Unique bands (bp)	Major-allel freq-uency	Average expected gene diversity (hi)	PIC
1	D.N.alt1-F	GTTTGGAATGACTGTGTGTGCT (-22)	170-560	6	1	5	83.3	120	0.7619	0.3231	0.2572
	R	AGATCGTGAGTATGCCAACCTT (22)									
2	D.N.alt4-F	GTAACCACAAACCAAATGCGT (21)	200-720	4	4	0	0.0	200, 380, 400, 720	1.0000	0	0
	R	CAATGCAGCCATGACAAAATAC (22)									
3	D.N.alt5-F	ACGAGACAGCATATCACGCTTA (22)	250-1000	5	3	2	40.0	500,800 1000	0.9714	0.0531	0.0495
	R	GAGCTTCGAGACCTTTGACACT (22)									
4	D.N.alt8-F	GTGGAATGGTGTCAAGCAGTTA (22)	180-500	5	1	4	80.0	180	0.7857	0.2837	0.2255
	R	AGATCGTGAGTATGCCAACCTT (22)									
5	D.N.alt9-F	AGATCGTGAGTATGCCAACCTT (22)	240-400	3	2	1	33.3	300,400	0.9524	0.0816	0.0716
	R	GTGGAATGGTGTCAAGCAGTTA (22)									
6	D.N.alt10-F	ATGGTGTTCCTTTGGCTGTAGT (-22)	300-600	3	3	0	0.0	300,400,600	1.0000	0	0
	R	TCTTACAATGCGGTAATTGGTC (22)									
7	D.N.alt12-F	TCTAACGACGGCTACTTGTTGA (22)	390-800	3	0	3	100.0		0.7619	0.3197	0.2579
	R	ACCGACTTGATTTGGATAGACG (22)									
8	D.N.alt13-F	TCTAACGACGGCTACTTGTTGA (22)	200-700	4	1	3	75.0	700	0.8036	0.2832	0.2285
	R	ACCGACTTGATTTGGATAGACG (22)									
9	D.N.alt14-F	TCGGTTTAGGAGTATATGGCGT (22)	170-400	3	0	3	100.0		0.7381	0.3707	0.2978
	R	ATTGATGAACTGACGGACTGTG (22)									
		Total		36	15	21					
		Average		4	1.66	2.33	56.84		0.8591	0.1964	0.1587

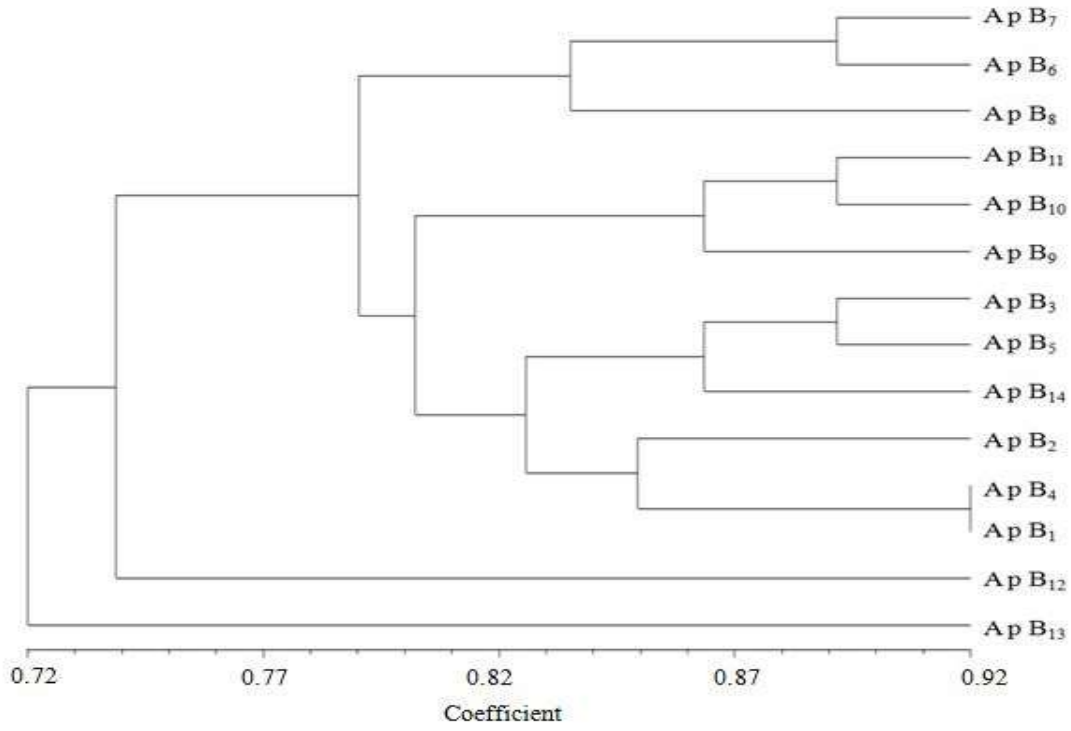

Fig. 1: UPGMA dendogram showing clustering of 14 isolates of *A. porri* based on 9 SSR markers

400 and 600 bp with primer D.N.alt10, 120 bp with primer D.N.alt1, 180 bp with primer D.Nalt.8 and 700 bp with primer D.N.alt13 which may be useful in the identification of *Alternaria porri* isolates. The primers D.N.alt4 and D.N.alt10 could not differentiate isolates while a number of primers yielded polymorphic bands within the isolates of *A. porri*. It was observed that SSR primers D.N.alt.5, D.N.alt.4, D.N.alt-10 and D.N.alt.-9 expressed high degree of similarity. On the other hand SSR primer D.N.alt.1, D.N.alt.-14, D.N.alt.8, D.N.alt13 and D.N.alt-12 illustrated high degree of variability. Our findings are in line with Dongo (2005), Dinh and Hocking (2006) and Benichou *et al.* (2009). Kale *et al*. (2012) reported that out of the 87 amplified loci, 98% loci were polymorphic which reflected high genetic variation among the 20 isolates *A. alternata*. Chethana *et al.* (2018) revealed 100% matching with in 5 different species of *Alternaria* viz., *A. porri, A. alternata, A. tenuissima, A. palandui* and *A. brassicicola* by means of sequencing and blast analysis of nucleotide sequence of ITS region of ribosomal DNA of isolates. Singh *et al.* (2014) identified 8,457 microsatellites from transcript sequences of *A. brassicicola* observed that the most frequent repeat was tri-nucleotide (74.03%), whereas penta-nucleotide (1.14%) was the least frequent.

Conclusion: The morphological and molecular diversity studies among various isolates of *A. porri* revealed variation in colour and texture of colony. This work highlighted that SSR markers are helpful for quick identification and genetic variability among the various isolates of *A. porri*. The findings would be helpful in developing purple blotch resistant varieties of onion and thus enhance onion seed production

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