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RESEARCH ARTICLE

Diversity Assessment Among *Alternaria solani* Isolates Causing Early Blight of Tomato in India

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Abstract Early blight (EB), caused by the fungus, *Alternaria solani,* is one of the most destructive diseases of tomatoes and other solanaceous crops; particularly in warm and humid climate. This study was targeted to explore the genetic and pathogenic diversity of *A. solani* from major tomato producing states of India. Thirty-three isolates were chosen for this study. These isolates exhibited considerable intra as well as inter-state variation. The phylogenetic tree generated with the ISSR sequences confirmed this result. Aggressiveness of the isolates towards susceptible tomato genotype was assessed in vitro, using detached leaf method. Considerable amount of variability in virulence was observed among the isolates. Specific activity of polygalacturonase and pectin methyl esterase was also estimated, to observe the relation among these, with the virulence of isolates. The information generated in the present study provides initial data on the population variability of the EB pathogen. It could be a valuable aid for tomato breeding strategies, aimed at obtaining cultivars with resilient resistance. This will provide a basis for planning disease protection strategies for sustainable agriculture which is required for producing crop plants which harmonize with the environment.

Keywords *Alternaria solani* · Molecular variation · Hydrolytic enzymes · Polygalacturonase · Pectin methyl esterase · ISSR

Significance statement Diversity assessment among *A. solani* isolates from different states of India will provide valuable aid for resistance breeding strategies and basis for planning disease protection for sustainable agriculture.

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Introduction

In developing countries like India, a major concern is the fact that agricultural land is being continuously limited. Decrease in area under crop with different biotic and abiotic stresses had affected productivity which has resulted in threat to food security. These stress conditions in agricultural ecosystems can occur at variable intensities. Continuous exposure to these stress factors; either biotic or abiotic, affects the growth, development and production of the plant. EB, caused by *Alternaria solani,* is one of the most destructive diseases in solanaceous vegetable crops (tomato, potato and eggplant etc.). It affects the aerial parts i.e. fruits, stem and leaves of tomatoes and stem, foliage and tubers of potatoes. Warm and humid (25–29 °C, 90–100%) environmental conditions are conducive to infection. Germination of *Alternaria* conidia requires free moisture and an optimum temperature of 28–30 °C. Theaverage time for its germination has been notice is approximately 40 min (apsnet.org). EB is distributed worldwide and predominantly occurs wherever tomato and potato aregrown. The losses due to early blight disease in solanaceous crops may be up to 80% [1]. It has already been established that variation in populations of plant pathogens directly affects disease management, especially the strategies related to the deployment of resistant cultivars and fungicide usage [2]. The present research is

focused on the diversity present in identified *A. solani* isolates from different states of India. The authors hope to generate information which will be useful for integrated disease management (IDM) programs against EB and for developing new control strategies of the disease.

The interaction between the pathogen and its host possesses diversity that is mainly dependent on the geographical and environmental conditions prevailing in the area where the crop is grown. When a plant is attacked by a pathogen, the plant and the pathogen try to overcome each other by producing some biochemicals. The first line of barrier to colonize the pathogenin plant is cell wall of host plant. Secretion of cell wall degrading enzymes by the pathogen is the first step towards its establishment in the plant. Pectin is one of the major and most complex components of plant cell wall. Plant pathogenic fungi are known to produce a range of cell wall degrading enzymes that macerate plant cell walls; including pectolytic enzymes such as pectin methylesterase (PME), polygalacturonase (PG) and pectate lyase (PNL and PL, respectively) that may have important roles in the infectivity in the progress of disease symptoms [3, 4]. The plant cell contains cell wall and pectin in it. Hence, the importance of such enzymes in pathogenicity is supported by the ability of purified enzymes to reproduce disease symptoms [5] and by the correlation of the pectolytic enzyme level with the degree of the symptoms shown by the plant [6]. This has been taken in account here for assessing the pathogenicity of different isolates of *Alternaria,* as they are the necrotroph. Along with the above mentioned cell wall degrading enzymes, many DNA based markers have been utilized to investigate the genetic diversity among plant pathogens. Studies of genetic variations in the population of plant pathogens in the last decade has increased because of availability of molecular markers. These techniques consist of random amplified polymorphic DNA [7, 8], microsatellites, restriction fragment length polymorphism (RFLP) [9], amplified fragment length polymorphism (AFLP) [10], and inter simple sequence repeats (ISSR) [11]. Other markers, which are being used for this purpose, are internal transcribed regions [12], rRNA coding regions of nuclear DNA [13] and mitochondrial DNA [14].

Determination of genetic diversity using inter simple sequence repeats (ISSR) is widely used in genotyping the *Alternaria* species as well as other fungal genera [15, 16]. This marker analysis is based on PCR-technique. ISSRs are amplified by PCR, using microsatellite core sequences as primers, with a few selective nucleotides as anchors, into the non-repeat adjacent regions (16–18 bp). Approximately 10–60 fragments from multiple loci are generated simultaneously, depending on the size of genome, separated by gel electrophoresis and scored as the presence or absence of fragments of a particular size. ISSR primers produce dominant molecular markers. ISSR fingerprinting shows higher levels of polymorphisms, when compared to some of the other PCR-based techniques. Its strength is that it does not require the sequence information for use. Due to the fact that this marker analysis is reliable and reproducible, it is preferred over other dominant markers.

Material and Methods

Fungal isolates

Totally, 33 isolates of *A. solani* (Table 1) were taken for the present study. Out of 33 isolates of *A. solani*, 28 were procured from National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan (Uttar Pradesh), 02 from Indian Agricultural Research Institute, New Delhi and and one each was collected from Kanpur, Varanasi, and Kalyani-West Bengal. Maintenance of the isolates and production of mycelium were performed as per instruction of the institutes, NBAIM and IARI.

Cultural Variation

The cultures were grown on the potato dextrose agar (PDA) plates at 25 ± 2 °C (12 h light/12 h dark) in a culture room. The isolates were characterized on the basis of colony color and radial growth after 5 and 10 days of inoculation. The experiment was performed in three replicates.

Detached Leaf Assay for Pathogenicity

The CO-3 variety of tomato, susceptible to *A. solani* [17] was grown in pots containing soil-compost mixture (2:1) in a greenhouse (25–28 °C). For detached leaf assay, healthy tomato leaves (5 weeks old plants) were surface sterilized using 2% of NaOCl for 10–15 s. Sterilized leaves were then rinsed thrice using sterile distilled water. Two (2) mm agar discs were placed on the adaxial side of leaves with the mycelium side facing down. Three leaves were inoculated per isolate and incubated at 25 ± 2 °C (12 h light/12 h dark) for 7 days. Disease severity was determined using the rating scale: 0 = no symptoms, 1 = necrosis around the lesion, 2 = necrosis covering ½ to ¾ of leaf, 3 = whole leaf necrosis, 4 = petiole necrosis (petiole detached from stem) and 5 = necrosis on plant stem [18]. The experiment was performed in triplicates.

Hydrolytic Enzyme Activities

Activities of cell wall degrading enzymes such as pectin methyl esterase (PME) and polygalacturonase (PG) were

Table 1 List of *Alternaria solani* isolates and corresponding institutes

S. no.	Isolate name	Procured/collected from	State from which collected
1.	Kanpur	Collected self	Uttar Pradesh
2.	0118	NBAIM, Mau	Rajasthan
3.	2655	NBAIM, Mau	Rajasthan
4.	2659	NBAIM, Mau	Jammu
5.	2663	NBAIM, Mau	Jammu
6.	2664	NBAIM, Mau	Kashmir
7.	2669	NBAIM, Mau	Himachal
8.	2657	NBAIM, Mau	Uttar Pradesh
9.	2672	NBAIM, Mau	Karnataka
10.	0113	NBAIM, Mau	Andhra Pradesh
11.	WB	Collected Self	West Bengal
12.	5350	IARI	Dharwad, Karnataka
13.	UPT-7	IIVR, Varanasi	Uttar Pradesh
14.	4632	IARI	New Delhi
15.	2671	NBAIM, Mau	Karnataka
16.	0114	NBAIM, Mau	Karnataka
17.	2662	NBAIM, Mau	Jammu
18.	1982	NBAIM, Mau	Karnataka
19.	0112	NBAIM, Mau	Karnataka
20.	2666	NBAIM, Mau	Himachal Pradesh
21.	0117	NBAIM, Mau	Haryana
22.	2667	NBAIM, Mau	Himachal Pradesh
23.	2658	NBAIM, Mau	Varanasi, Uttar Pradesh
24.	2678	NBAIM, Mau	Karnataka
25.	2668	NBAIM, Mau	Himachal Pradesh
26.	2665	NBAIM, Mau	Himachal Pradesh
27.	2656	NBAIM, Mau	Varanasi, Uttar Pradesh
28.	0110	NBAIM, Mau	Manikala, Uttar Pradesh
29.	2408	NBAIM, Mau	Shimla, Himachal
30.	0111	NBAIM, Mau	New Delhi
31.	0139	NBAIM, Mau	Chhattishgarh
32.	2674	NBAIM, Mau	Banglore, Karnataka
33.	0116	NBAIM, Mau	Kasargod, Kerala

determined spectrometrically. Fungal strains grown in induction medium containing pectin as a substrate were incubated for 10 days at 25 ± 2 °C. After incubation, the contents were filtered and the filtrate was used as an enzyme extract.

Polygalacturonase (PG) Assay

To 0.8 ml of enzyme extract, 2 ml of assay buffer (2 mg/ml pectin in 0.1 M citrate buffer, pH 5.0) was added and the contents were incubated at 37 °C for 1 h. Five and half ml of phenol sulphuric acid reagent (PSA; 0.5 ml 80% phenol in 5 ml sulphuric acid) was added to 1 ml reaction mixture and absorbance of the resulting solution was measured at 480 nm. The activity of the enzyme was calculated by using a standard calibration curve obtained using galacturonic acid as reducing sugar. One unit corresponded to 1 µM of reducing sugar liberated from substrate in 1 h at 37 °C [19].

Pectin Methyl Esterase (PME) Assay

Enzyme extract (0.5 ml) was mixed with 7 ml assay buffer (10 mg/ml pectin in 0.02 M Tris–HCl buffer, pH 8.0). Initial pH of the solution was maintained at 8.0 by using 0.05 M NaOH. The reaction mixture was incubated for 1 h at 37 °C in a water bath and the initial pH was noted. The pH was again adjusted to 8.0 by titrating against 0.02 N NaOH containing 5 mM sodium azide. The volume that was required to bring back the pH was noted. The amount of enzyme that was utilized was calculated as 1 µM/h NaOH [20]. Calculations for the activity were done according to the formula given by Balaban et al. [20]. Both the biochemical assays were carried out in triplicates and repeated twice. Induction medium with no fungal inoculation served as the control.

Analysis of Genetic Variation Through ISSR Markers

Fungal Strains and Genomic DNA Isolation

For genomic DNA isolation, isolates were subcultured in 20 ml Potato dextrose broth (PDB) medium and incubated at 25 ± 2 °C for 10 days. The fungal material was removed from the broth and modified Doyle and Doyle [21] method was used for DNA isolation. Genomic DNA quality was checked with electrophoresis in 0.8% agarose gel and the quantity was measured with a nanodrop spectrophotometer at a wavelength of 260 nm (Nanovue, GE).

Development of ISSR Fingerprinting Method

The ISSR primers were custom synthesized from IDT, India. Initially, 25 primers were screened using five *A. solani* DNA samples. Finally, primers which produced reproducible and consistent profiles (11 ISSR) were selected for profiling all DNA samples (Table 2).

ISSR-PCR amplification was carried out in 25 µl reaction mixture in 200 µl PCR plates. Each reaction mixture contained 50 ng genomic DNA, 200 µM of each dNTPs, 0.5 unit of Taq polymerase, 1 X Taq polymerase buffer solution and 0.2 µM of primer. Amplifications were performed in a thermal cycler (Bio Rad, USA) programmed for an initial denaturation of 4 min at 94 °C, 45 cycles of denaturation at 94 °C for 1 min, annealing temperature

Table 2 ISSR primers used for study of genetic diversity among *A. solani* isolates

S. no.	Name of primer	Sequence (3′–5′)	Annealing temperature (°C)	Total no. of bands	PIC
1.	ISSR 801	ATA TAT ATA TAT ATA TT	45	NA	NA
2.	ISSR 807	AGA GAG AGA GAG AGA GT	45	NA	NA
3.	ISSR 808	AGA GAG AGA GAG AGA GC	45	7	0.457
4.	ISSR 809	AGA GAG AGA GAG AGA GG	45	7	0.497
5.	ISSR 810	GAG AGA GAG AGA GAG AT	45	6	0.422
6.	ISSR 811	GAG AGA GAG AGA GAG AC	45	NA	NA
7.	ISSR 812	GAG AGA GAG AGA GAG AA	48	NA	NA
8.	ISSR 814	CTC TCT CTC TCT CTC TA	48	NA	NA
9.	ISSR 815	CTC TCT CTC TCT CTC TG	45	NA	NA
10.	ISSR 816	CAC ACA CAC ACA CAC AT	48	4	0.498
11.	ISSR 817	CAC ACA CAC ACA CAC AA	48	NA	NA
12.	ISSR 818	CAC ACA CAC ACA CAC AG	48	4	0498
13.	ISSR 819	GTG TGT GTG TGT GTG TA	48	3	0.485
14.	ISSR 820	GTG TGT GTG TGT GTG TC	45	NA	NA
15.	ISSR 821	GTG TGT GTG TGT GTG TT	45	NA	NA
16.	ISSR 835	AGA GAG AGA GAG AGA GYC	45	5	0.490
17.	ISSR 864	ATG ATG ATG ATG ATG ATG	48	7	0450
18.	ISSR 824	TCT CTC TCT CTC TCT CG	45	1	NA
19.	ISSR 836	AGA GAG AGA GAG AGA GYA	45	6	0.494
20.	ISSR 840	GAG AGA GAG AGA GAG AYT	45	6	0.387
21.	ISSR 843	CTC TCT CTC TCT CTC TRA	45	NA	NA
22.	ISSR 857	ACA CAC ACA CAC ACA CYG	50	NA	NA
23.	ISSR 859	TGT GTG TGT GTG TGT GRC	50	NA	NA
24.	ISSR 873	GAC AGA CAG ACA GAC A	45	NA	NA
25.	ISSR 880	GGA GAG GAG AGG AGA	45	NA	NA

specific for each primer for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. The PCR products were separated on 2% agarose gel in 1X TAE buffer at 65–70 V for 3–4 h. DNA fragments were visualized under UV light and documented using a Gel Documentation System (BioDoc-It 220 Imaging System, UK). All PCR amplifications were performed at least twice for each isolate to assure reproducibility.

The ISSR data for genotyping was assembled into a binary matrix by scoring unambiguous polymorphic bands manually. Presence of a band was denoted as "1", and absence was marked as "0". The dendrogram was constructed using the neighbour-joining (NJ) method through the software program Darwin 6.0 (Perrier & Jacquemoud-Collet 2006, https://darwin.cirad.fr/darwin).

Results and Discussion

Radial Growth and Color

Radial growth observed for isolates presented in Table 3 was significantly not different for most of the isolates, with the lowest radial growth for 00113 (at 5th day after inoculation) and the highest radial growth for 00111 (at 10th day after inoculation). However, considerable variation was observed among the isolates in terms of colour (Table 3, Fig. 1).

Pathogenicity

Inoculation of detached tomato (susceptible line CO-3) leaves with *A. solani* isolates resulted in clearly defined necrosis for most of the isolates. Observations were noted on the 7th day after inoculation (Fig. 2). Eight isolates could not cause any necrotic lesion on the leaves and were categorized as nonpathogenic isolates. Sixteen isolates caused necrosis in the leaves around the lesion and they were rated as 1. Nine isolates were given the rating 2, as the necrosis caused by these covered ½ or ¾ area on the leaves surrounding the lesion. The results of the pathogenicity tests have been summarized in Table 3 and Fig. 2.

Table 3 Growth and virulence characteristics of *A. solani* isolates

S. no.	Name of *A. solani* isolate	State from which collected		Color	Pathogenicity reaction (detached leaf method CO-3 variety, [18])
1.	Kanpur	Uttar Pradesh	6.37	Grey with b&w pigments	1
2.	0118	Rajasthan	5.90	Grey with black periphery	0
3.	2655	Rajasthan	4.27	Greenish grey	2
4.	2659	Jammu	5.40	Grey with cream pigment	1
5.	2663	Jammu	5.33	Grey	2
6.	2664	Kashmir	4.27	Grey with white pigment	0
7.	2669	Himachal	6.73	Grey with cream pigment	2
8.	2657	Uttar Pradesh	4.50	Black at periphery and white at center	0
9.	2672	Karnataka	7.33	Black	0
10.	0113	Andhra Pradesh	4.17	White	0
11.	WB	West Bengal	4.90	Grey	2
12.	5350	Dharwad, Karnataka	4.07	Blackish grey	1
13.	UPT-7	Uttar Pradesh	7.70	Creamish with yellow pigments	1
14.	4632	New Delhi	6.10	Whitish grey	1
15.	2671	Karnataka	6.27	Dark grey	1
16.	0114	Karnataka	6.17	Grey with white pigment	1
17.	2662	Jammu	5.57	Brown with cream outside	1
18.	1982	Karnataka	4.07	Grey	1
19.	0112	Karnataka	6.80	Black with grey tinge	2
20.	2666	Himachal Pradesh	2.70	Green with cream pigments	2
21.	0117	Haryana	4.40	Greenish black with white at periphery	2
22.	2667	Himachal Pradesh	5.83	Greenish black	0
23.	2658	Varanasi, Uttar Pradesh	7.37	Greyish with black touch	1
24.	2678	Karnataka	6.43	Dark grey	1
25.	2668	Himachal Pradesh	7.17	Brownish	2
26.	2665	Himachal Pradesh	6.10	Brown with greenish touch	1
27.	2656	Varanasi, Uttar Pradesh	6.10	Greenish black with white pigmentation	1
28.	0110	Manikala U.P.	6.47	Black	2
29.	2408	Shimla, H.P.	6.97	Brownish black	0
30.	0111	New Delhi	7.63	Black grey pigments	1
31.	0139	Chhattishgarh	6.47	Black with brown pigments	0
32.	2674	Banglore, Karnataka	3.93	Greenish black with white at periphery	1
33.	0116	Kasargod, Kerala	7.13	Brownish black	1

Biochemical Studies

Severe damage to plant tissue is a measure of fungal action for pathogenicity which results in disease production in the host [22]. The first line of defense in plants is the cell wall and it is composed of pectin (constituent of middle lamella and primary cell wall) and other compounds. Fungus has the ability to produce polygalacturonase (PG) and pectin methyl esterase (PME) enzymes that hydrolyze pectin [23]. It has been observed that pectic enzymes are sufficient to cause cell death and create huge losses to the plant [24]. Figure 3 shows the value of specific activity of polygalacturonase, which ranges from 0.012 (for isolate 2655) to 0.111 uM/ml (for isolate 2666). All the isolates with their respective PG activity values are shown in Table 4 and Fig. 3.

Another enzyme, pectin degrading pectin methylesterase (PME) was estimated for its specific activity in these isolates and it ranged from 0.63 (for 2665) to 3.54 Units/ml (for 0139). It is secreted in combination with pectin-degrading enzyme, polygalacturonase, and together, decays the plant cell wall to establish infection and absorb nutrients from the host [25]. The specific activity of PME is represented in Table 4 and the bar graph (Fig. 4).

Fig. 1 Cultural diversity among the different isolates of *A. solani*

Genetic Diversity Study Using ISSR Primers

Totally, twenty-five primers were selected for the present study and among these, only 11 primers gave amplification in all the isolates. Of these, eleven tested primers with total 56 alleles were amplified by PCR. Each of these primers generated 1–7 scorable bands, ranging from 200 to 1500 bp.

The dendrogram (Fig. 5) generated by DARWIN clearly showed two distinct groups of the isolates. The largest group, "Group 1", comprised of nineteen isolates and "Group II" consisted of 13 isolates. However, a single isolate, 2665, could not be clustered into the above mentioned main clusters. Both the groups have isolates from diverse geographical distribution and with different virulence ability. Group 1 is divided into two subgroups that have 11 and 8 isolates. Five isolates in the subgroup showed no pathogenicity, while four showed high virulence. Two isolates were moderately virulent. This subgroup has four members from the hill collection i.e. 2664, 2663, 2669 and 2659. Two isolates in this SG are from Rajasthan, two from Karnataka and three belong to Uttar Pradesh, Andhra Pradesh and West Bengal, respectively.

The second subgroup comprised of eight isolates with moderate virulence, except 0112, that showed more virulence than other isolates in this group. Four isolates in this SG II have been isolated from Karnataka, two from Uttar Pradesh, one from New Delhi and one from Jammu.

The second group consisted of thirteen isolates among which four isolates showed more virulence and six revealed comparatively low virulence. Three isolates did not show any virulence in the present study. This group consisted of more diverse isolates; four from Himachal Pradesh, three from Uttar Pradesh, two from Karnataka,

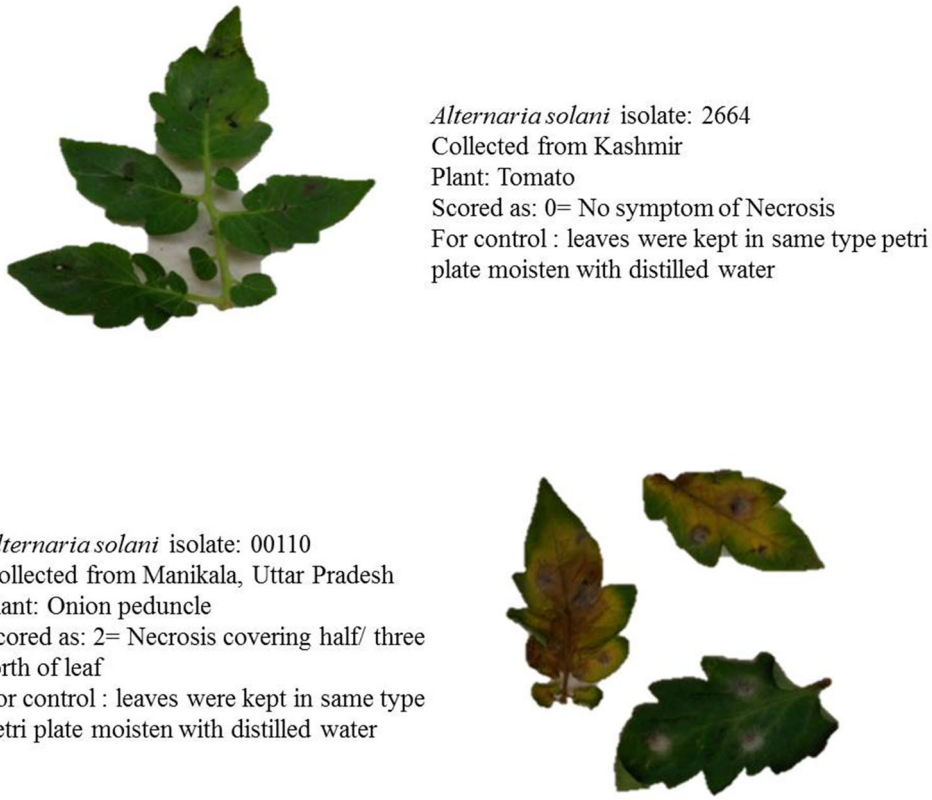

Fig. 2 Scoring for virulence as 0 and 2 [18]

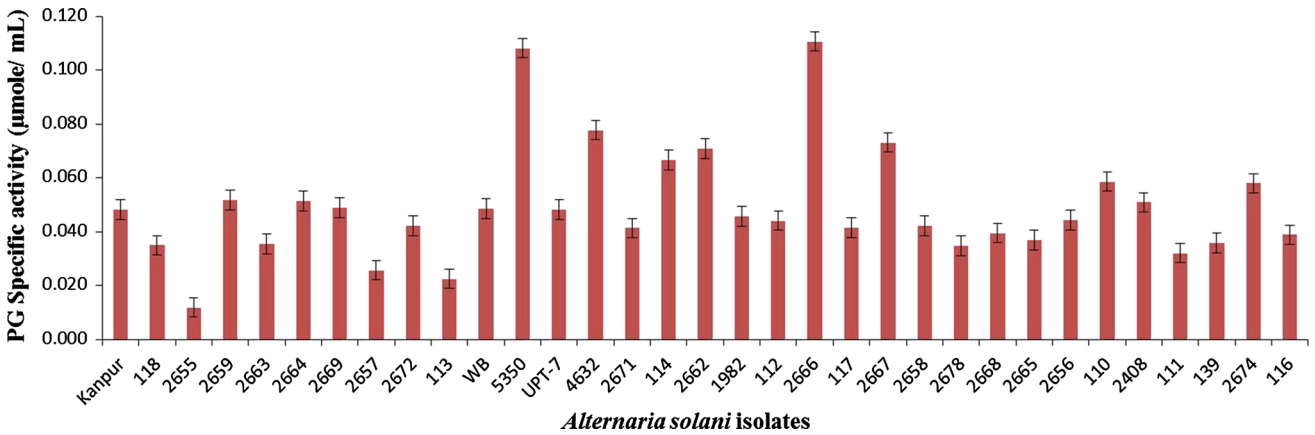

Fig. 3 Specific activity of polygalacturonase (PG) enzyme in thirty-three isolates of *A. solani*

one from New Delhi, one from Chhattisgarh and one from Haryana. The variance in the pathogenicity levels was observed in the dendrogram that consisted of six isolates with lesser virulence and four isolates with more virulence. Three isolates in this group did not produce any symptoms during the detached leaf study. Both the analysis (dendrogram and PCO) revealed that the different isolates possessed diversity among themselves. The grouping among these isolates shows that ISSR primers can reveal diversity in same geographical regions too.

Discussion

Morphological, biochemical and genetic diversity existing in the pathogen population facilitates our understanding for potential appearance of new variants or pathotypes. The study has shown that there is substantial genetic diversity among the isolates. *Alternaria solani*, an asexually propagating fungus, is highly variable at a genetic level [26]. Cultural practices, environmental conditions prevailing in a particular area and plant materials also have contributed to the genetic diversity observed within geographic regions

Table 4 Specific enzyme activities of pectin degrading enzymes of thirty-three *A. solani* Isolates

S. No.	Isolates	Specific PG activity (μmole/ml)	Specific PME activity (Units/ml)
1	Kanpur	0.05	1.71
2	0118	0.03	1.92
3	2655	0.01	2.40
4	2659	0.05	0.84
5	2663	0.04	1.04
6	2664	0.05	1.02
7	2669	0.05	0.71
8	2657	0.03	1.62
9	2672	0.04	0.81
10	0113	0.02	2.02
11	WB	0.05	0.80
12	5350	0.11	1.65
13	UPT-7	0.05	1.15
14	4632	0.08	1.23
15	2671	0.04	1.40
16	0114	0.07	0.78
17	2662	0.07	1.17
18	1982	0.05	1.38
19	0112	0.04	3.42
20	2666	0.11	1.21
21	0117	0.04	1.13
22	2667	0.07	1.02
23	2658	0.04	1.43
24	2678	0.03	1.46
25	2668	0.04	1.22
26	2665	0.04	0.63
27	2656	0.04	0.84
28	0110	0.06	1.88
29	2408	0.05	3.13
30	0111	0.03	1.56
31	0139	0.04	3.54
32	2674	0.06	1.18
33	0116	0.04	2.19

[27]. Several studies have been performed for evaluation of variability in *Alternaria* spp. and its correlation with disease incidence in the laboratory and field condition.

In the present study, simple pigmentation, growth characteristics, detached leaf inoculation with an accordant external condition; especially temperature and moisture, specific activity assay of pectin degrading enzymes along with ISSR DNA markers were used to understand the genetic diversity among the *Alternaria solani* isolates belonging to different states of India. The virulence of isolates was scored on the area covered on the leaf after 72 h of inoculation. The lesions caused by the strains varied in size indicate the level of virulence the selected isolates possessed, suggesting that there was significant differentiation in pathogenicity among the isolates of *A. solani* on the used susceptible cultivar of tomato i.e. CO-3. This provides experimental evidence and useful reference data for the reasonable use of disease-resistant varieties of tomato for disease control. Necrotrophic fungus creates a complex physiological system in the plant for their establishment that includes conidial attachment, germination, host penetration, lesion formation and expansion, and tissue maceration followed by sporulation [28]. The plant cell wall is composed of cellulose and hemicellulose in a strengthening network of cohesive pectin matrix cross-linked to lignin and proteins by ionic and covalent bonds. This is the first line of defense for pathogens, bacteria and insects.

In the present study, the authors also tried to corroborate the virulence of isolates with the production of cell wall degrading enzymes i.e. PGs and PMEs. It was found that these enzymes might have a role in the penetration of the fungal isolates in the leaf but it has been observed that many of the isolates which have high activity of these two have lesser virulence or no virulence as compared to the others. The correlation values were calculated for pathogenicity, PG and PME values, and the calculated

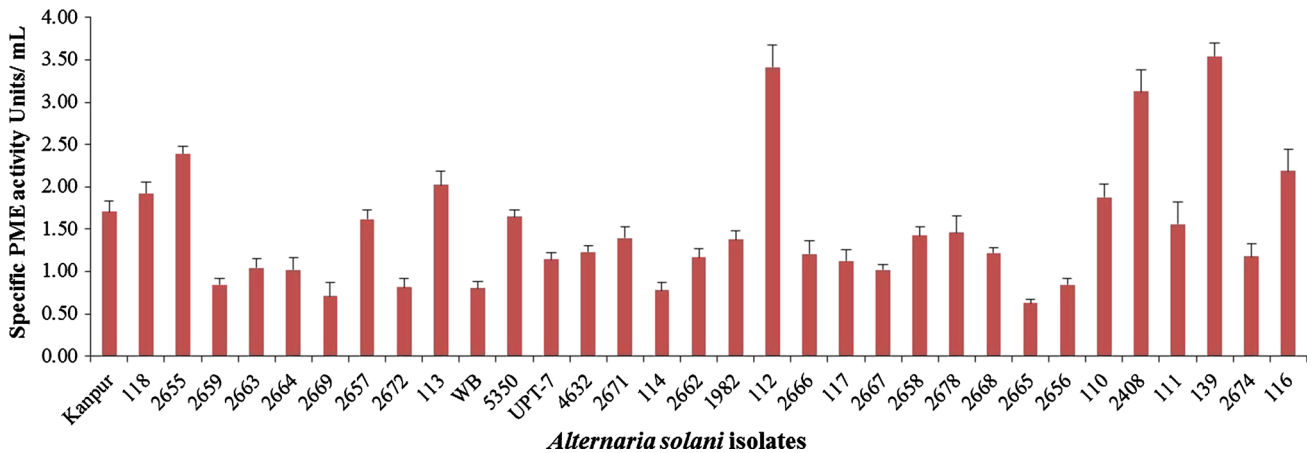

Fig. 4 Specific activity of pectin methyl esterase (PME) enzyme in thirty-three isolates of *A. solani*

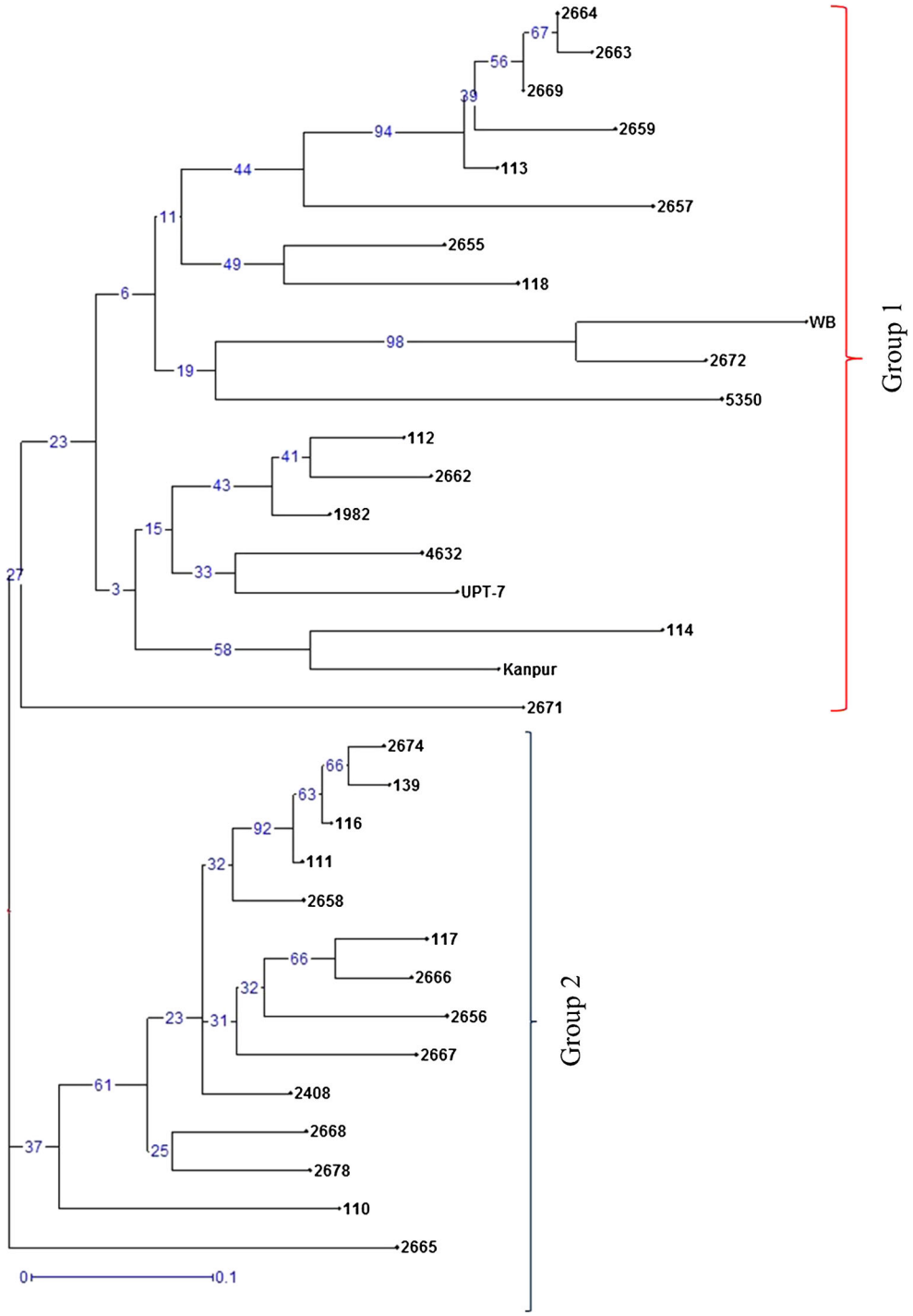

Fig. 5 Neigbhour-joining (NJ) tree illustrating the genetic diversity among 33 isolates of *A. solani,* based on ISSR markers

results have shown a positive correlation between enzyme activity and pathogenicity. However, it may be conferred from the present study that not much correlation was found between the ability to produce the enzymes, in vitro, and pathogenicity. The absence/lesser ability to cause a lesion on the tomato leaf suggests that these enzymes are not concerned with the production of moderate disease symptoms in the tomato plant. The variation in pathogenic potential of sampled isolates ranged from no virulence (0) to moderately virulent (2), suggesting the existence of mild pathogenic variability and also that these have no relation with either geography or their in vitro production of polygalacturonase or pectin methyl esterase activity. The isolates might have expressed pathogenicity and symptoms in field conditions, but this study was performed under in vitro conditions to observe their virulence on the

detached leaf which is quite different from its natural state. In this study, it has been observed that the isolates with high activity of pectin degrading enzymes i.e. PG and PME did not produce disease/symptoms during detached leaf assay e.g. 0139, 2408 have highest PME activity (3.54 and 3.13 Unit/ml) with 0.4 and 0.5 µmol/ml specific activity of PG, respectively. Also, the isolates that showed more virulence did not possess high activity of these two enzymes. This could be understood by a recent study performed in alfalfa [29] that showed the association of disease resistance in the plant with the expression of polygalacturonase-inhibiting proteins. A similar observation can be made in this study, where the isolates showed higher pectin degrading enzyme activity but did not produce any disease symptoms during detached leaf assay. An et al. [30] reported that an inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance. A possible reason for this could be that the machinery for producing disease in live and natural conditions is accompanied with associated biochemical and physiological changes inside the plant. Dendrogram constructed on the basis of ISSR markers did not show grouping of selected isolates. Grouping of the isolates are not in geographical congruency. The isolates that grouped together differed in their morphology, pathogenicity and at biochemical levels. However, subgroup 3 of group III has members with pathogenicity 1. Since *A. solani* is an asexually propagated fungus, genetic mechanisms that could explain such diversity mainly include simple mutations due to external effects. The significant amount of diversity among Indian isolates of *A. solani* can be explained mainly by evolution; resulting from natural and stress induced transposition. These genetic diversity studies can be useful for plant breeding programs for disease resistance as the genetic structure of pathogens reflects the history and the evolutionary potential of a pathogen.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest in this publication.

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