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Abstract

Apple scab is causing major losses to apple growers throughout the world. The disease is caused by fungal pathogen (Cke.) Wint., which is a heterothallic, haploid ascomycete, reproducing sexually and asexually. Understanding the genetic variability of the pathogen is very important for devising the strategies to control it. DNA based molecular markers have been successfully used for studying the genetic variation in different fungal pathogens. For the present investigation, samples were collected from the different areas of Kashmir valley, during 2004 to 2008. Two molecular markers, namely RAPD and ISSR were used for characterization of isolates, obtained from different cultivars growing in various orchards spread across the valley. The gene diversity revealed by RAPD markers ranged between 0.08 and 0.64, whereas for ISSR, it ranged between 0.1 and 0.8. We conclude that there is high level of genetic diversity within populations of . The isolates were observed WREHLQWHUPL[HGDQGQRWDUHDVSHFLÀFKRZHYHULQVRPHFDVHVFXOWLYDUVSHFLÀFLVRODWHVZHUHLGHQWLÀHG

Ke d : Apple scab; Venturia inaequalis; RAPD; ISSR; Gene diversity

I d c

Apple scab is one of the most serious diseases of apple and is caused by the fungus Venturia inaequalis [1]. e disease causes great economic losses to the growers in particular and apple industry, in general [2]. Although, infection may occur on leaves, fruits, stem or green twigs, the main sites of infection are leaves and fruits. Infection rst appears on the underside of the leaf and subsequently, spreads to the other parts. Young leaves are more prone to infection than mature and old leaves [3]. First visible symptoms are pin head sized water soaked spots. Later, these enlarge and assume dark and smoky appearance. When the disease advances, skin ruptures and the exposed tissue gives brown or black velvet like appearance. Finally, they attain a brownish black colour. If fruits get infected before harvest, the symptoms are not visible until storage [4]. During storage, rough, black and circular lesions may develop. e lesions are formed due to erect, brown conidiophores and numerous conidia produced by the fungus. e ascospores constitute the primary source of infection. ese become active as soon as spring starts [5]. e pathogen increases its biomass considerably within 7 to 10 days, and further infection takes place by asexual spores called conidia.

In Kashmir valley, the release of the ascospores can begin as early as late March, and may continue over several weeks [6]. Further infections occur with asexual conidiospores. Conidia spread from tree to tree in rain–splash and by wind. If the weather conditions are suitable, then the fungus spreads very rapidly and there can be a dramatic increase in the incidence of scab. e pathogen remains active even at very low temperatures [7], and can infect at as low as 1 C within 40 hours. As temperature and wetness are directly related to the incidence of infection, these parameters can be used for prediction of incidence of scab [8]. A modi cation to Mills table has been proposed, which is relevant to Kashmir valley [9]. Even, several models for estimates of ascospore maturity have been developed for controlling the disease [10].

Recently established orchards have been found to be more vulnerable to scab, than those established several decades ago. is can be attributed to commercialization, whereby only one or few types of cultivars are grown in orchards. e situation can become out of control, in case of an epidemic. erefore, it is necessary to understand the genetic diversity of the fungus so that strategies for controlling it can be devised well in time.

In order to control the fungus, various fungicides are being used. is remains the only viable strategy to control the disease, a er the infection has occurred. However, indiscriminate use of fungicides is causing serious environmental and health concerns [5,11]. Even fungicide resistance has been reported in di erent apple growing areas [12]. e long lasting strategy is the cultivation of scab resistant apple cultivars. Major scab resistance genes like Vf, Vbj, Vr, Vm etc., have been identi ed as potential sources of scab resistance in apple [12]. Among these, Vf represents the most extensively studied gene and has been introgressed in many commercial cultivars. However, the disturbing situation is the fast evolution of new races of V. inaequalis, which are overcoming scab resistance genes [13]. erefore, study of genetic variation in V. inaequalis, using molecular markers becomes extremely important.

DNA based markers have been used to study many pathogenic fungi [14]. RAPD is the simplest method and is generally used for preliminary studies, in unknown populations. RAPD markers have been used for characterization of V. inaequalis [15] in Czech Republic. Characterization of European population has been undertaken to understand the gene diversity, within population [16]. In this study, populations of Venturia inaequalis have been reported to be highly diverse and distributed homogeneously throughout. Kashmir valley represents the main area of cultivation of apple in India; however, no

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information is available on molecular characterization of V. inaequalis. e present investigation aims at lling this lacuna, and seeks to generate information on the genetic diversity among di erent populations of V. inaequalis.

Materials and Methods

Collection of infected leaves

Infected leaf samples were collected from the orchards at di erent time intervals, during 2003 to 2007 (Figure 1a and 1b). A er collection, the leaves were placed between the folds of the newspapers for drying. E orts were made to remove as much moisture as possible, in order to avoid cross contamination by other fungi. e samples were kept in the paper bags and were marked, indicating di erent accession numbers. e bags were wrapped in polythene and kept at 4°C to minimize the degradation, and to prevent the contamination.

Culturing, isolation and preservation of fungus

Potato dextrose agar medium (PDA) is the basic medium for growth of many fungi. Other media tried for the culture of the fungi are apple infusion agar (AIA) and corn meal agar (CMA). e PDA medium worked very well for V. inaequalis. e antibiotic chloramphenicol (50 µg/ ml) was added to the medium, to avoid bacterial contamination.

Leaves were treated with disinfectants to eliminate the bacteria and other non– xed saprophytes from the surface of the leaf. Leaves were rst washed with autoclaved distilled water, dipped in 70% ethanol for 10 seconds, and again washed using autoclaved distilled water. Small pieces from the infected area of the leaf were cut and washed in sterile distilled water. e leaf bits were inoculated on PDA slants and kept at 18°C. e fungal growth was observed a er 10–12 days of inoculation.

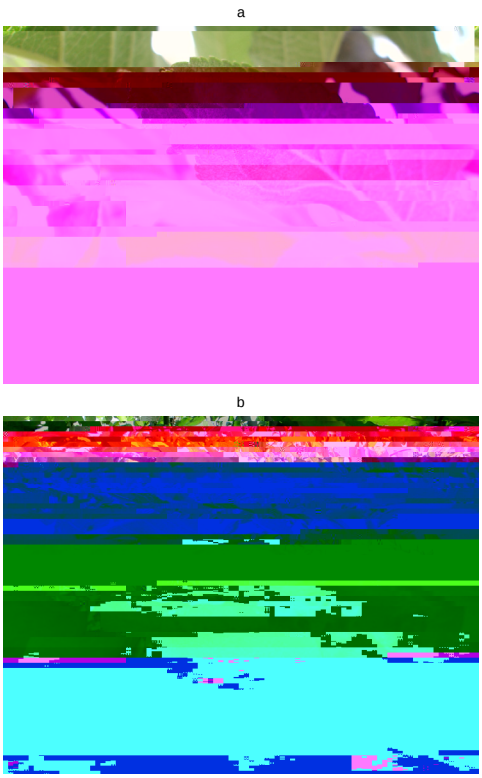

Figure 1 (1a and 1b): Symptoms of apple scab on leaves and fruit. Note the brown or black velvet like appearance of the spots.

e isolates obtained from leaves were again sub-cultured, to obtain pure cultures. Out of several isolates obtained, nally 20 isolates were selected for further studies (Table 1). Single spores were obtained on water agar medium. For long term storage, spores were mixed in 10% glycerol in preservation vials and stored at ultra low temperature (–80°C). ese cultures were stored as stock cultures for future use.

Fungal DNA isolation

Genomic DNA was extracted using the CTAB method with minor modi cations [17]. A er 12–15 days of inoculation, hyphal mass was obtained from asks by ltering the broth through sterile muslin cloth. Fungal mass was then dried between the folds of sterile lter paper. It was powdered in a pre–cooled pestle and mortar, using liquid nitrogen. About 1gm of fungal powder was added to preheated 10 ml CTAB bu er in centrifuge cups, and incubated for 30 minutes at 65°C in a water bath. Samples were mixed every 10 min by inverting centrifuge cups 5–6 times, to make sure that the tissue mixed well with the bu er. 10 ml chloroform: isoamyl alcohol (25:1) was added to each tube. A er proper mixing, the samples were centrifuged to sediment the cell debris and the upper aqueous phase were then transferred to another tube. Later, equal volume of chilled DNA grade ethanol was added, to precipitate DNA. Samples were mixed gently and centrifuged at 8000 rpm for 5 minutes. Supernatant was discarded and pellets were washed 3 times with 70% ethanol, air dried and dissolved in 100 µl TE. e DNA samples were treated with 1µl RNase (10 mg/ml) for three hours at 37°C, to remove RNA.

e RNA free DNA was further puri ed by extracting with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1), followed by centrifugation at 5000g for 10 min. e aqueous phase was collected and mixed with equal volume of chloroform, followed by centrifugation at 5000 g for 10 min. e DNA was precipitated from the aqueous phase, using chilled ethanol. e DNA was obtained as pellet a er centrifugation, which was dissolved in TE and stored at –20°C.

Quanti cation of DNA was done both spectrophotometrically, as well as by agarose gel electrophoresis, using lambda DNA as standard. In the latter case, band intensity of sample DNA was compared with

the band intensity of lambda DNA of various concentrations, and the concentration of sample DNA was thus determined.

RAPD (Ra d A ed P c DNA) a a

PCR was performed in 25 µl volume containing 3 mM $MgCl_2$ × Taq polymerase bu er, 200 µM of dNTPs, 0.8 µM primer, 0.4 units of Taq polymerase and 50 ng of DNA. e thermal cycling conditions consisted of an initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 36°C and extension for 2 min at 72°C,aWabx!£‡8 $¤& Bb)pBx£,— ˜t OÇ—98 ¡t %abx!£‡8 $w“` y6Y b•…‘Py‡@]<ys“•@,At %abx!£‡8 $F)X F

Re a d D c

Culturing of V. inaequalis was a di cult step because of cross contamination of other fungi.Venturia is very slow growing fungus, and takes 10–15 days for growth. Other fungi likeAlternaria are very fast growing and spread on the slant within 3-4 days. We conducted detailed investigations on the morphological features of the fungus. e spores were of characteristic ame shape, in all the isolates. However, the spore size was variable; the size ranged from 13–36 x 6–12 µM (Figure 2).

As pointed out earlier, breeding apple cultivars with durable resistance to V. inaequalis s the need of the hour. However, to achieve this it is extremely important to understand the genetic structure of this pathogen, including its virulence elements and the extent to which evolutionary forces may in uence such elements. We used two molecular markers to undertake detailed investigations on the genetic structure of Venturia. e details are as follows:

RAPD (Ra d A ed P c DNA) a a

e total numbers of fragments ampli ed using 8 RAPD primers

were 84 (Figure 3). e primers generated 84% to 100% polymorphism. e gene diversity ranged from 0.08 to 0.64. Resolving power of primers ranged between 0.59 and 10.6.

ISSR (I e S . . e Se e ce Re ea) a a . . .

8 primers produced a total of 57 scorable markers, among 20 isolates. e size of ampli ed products ranged from 200 bp to 3400 bp. e number of scorable markers produced per primer ranged from 6 to 9 (Figure 4). All primers showed 100% polymorphism, except primer UBC 808 which showed 60% polymorphism. Gene diversity and resolving power ranged from 0.1 to 0.8 and 3.9 to 8.1, respectively.

Cluster analysis was performed by combining the data generated through RAPD and ISSR analyses (Figure 5). Two major clusters were obtained; cluster A and cluster B. Cluster A contains 4 isolates and cluster B contains 16 isolates. Cluster B was further divided into two sub–clusters C and D. Sub–cluster C contains maximum number of isolates, i.e. 13 and sub–cluster D contains three 3 isolates. Sub–cluster C could be further divided into two groups E and F. Group E contains 6 isolates and F contains 7 isolates.

Using microsatellite markers on isolates of *Venturia inaequalis* from ve continents, it has been postulated that the fungus may have originated in Central Asia, from where it has spread to other parts of the world [18]. ese studies have however, revealed high within