

**DNA BARCODING OF DIFFERENT *VENTURIA INAEQUALIS*
STRAINS AND THEIR EFFECT ON PAL BIOCHEMICAL
PATHWAY OF *MALUS DOMESTICA* BORKH**

By

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Thesis submitted to Faculty of Basic Sciences
in partial fulfillment of the requirements
for the degree of

**DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY**

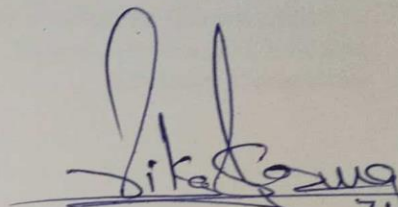


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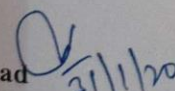
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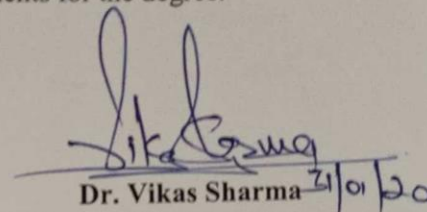
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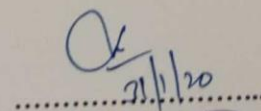
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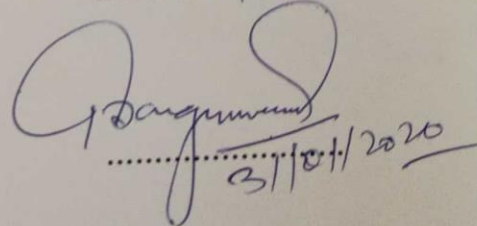
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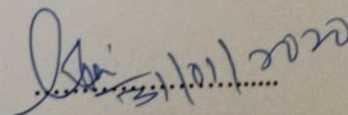
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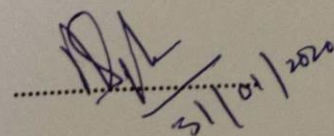
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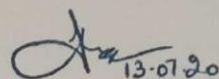


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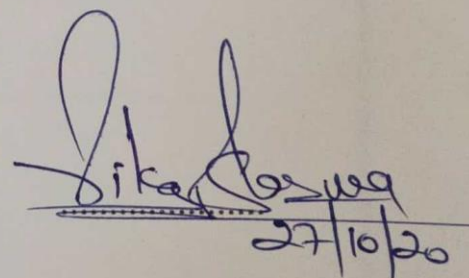
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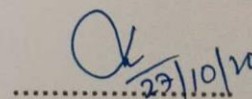


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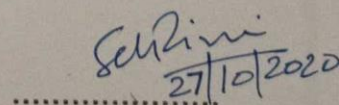
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“This thesis is dedicated to my Parents”

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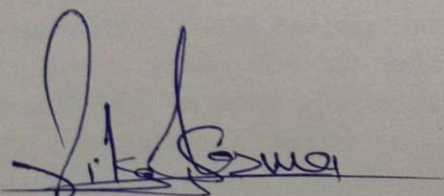
ABSTRACT

Title of the thesis	: DNA barcoding of different <i>Venturia inaequalis</i> strains and their effect on PAL biochemical pathway of <i>Malus domestica</i> Borkh.
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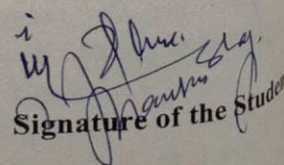
Malus domestica Borkh. (apple) is grown widely in temperate regions of the world for its nutritional and economic importance. Apple is attacked by many diseases, but the common bottleneck for its production is scab disease caused by *Venturia inaequalis*. The present investigation exploits barcoding tool for elucidating the molecular variability and SSR for genetic diversity of the pathogen. The role of different phenolics in response to the *Venturia inaequalis* is studied by expression analysis of different *PAL* genes and production of metabolites by high performance liquid chromatography. Thirty (30) isolates were first validated as *Venturia inaequalis* by simple morphological characterization. Barcoding of *Venturia inaequalis* isolates from twelve (12) districts of two (2) geographical distinct regions of Jammu and Kashmir was carried out using internal transcribed spacer (ITS) region of the ribosomal DNA. ITS based ribotyping of the thirty isolates showed sequence homology with strains/isolates reported from different regions of the world, where as intra sequence comparison has demonstrated high variability among the isolates collected from Bhaderwah (Doda) and Padder (Kishtwar) regions. The DNA barcode sequences were submitted to NCBI GenBank and accession numbers are published. DNA Barcoding based dendrogram revealed that all the accessions formed two main clusters with various degree of sub clustering within the clusters. Analysis based on SSR study reveals that the heterozygosity ranged from 0.0 to 0.5, with an average value of 0.39. The expected heterozygosity or gene diversity ranged from 0.0 to 0.50 with an average of 0.40. The F_{st} value ranges from 0 to 0.6 with an average of 0.194. Diversity within each population (HS) value ranging from 0.26 to 0.33. Average differentiation among populations (GST) was 0.11 and populations were isolated by significant distance ($r^2 = 0.50$, $P < 0.01$). From the analysis of molecular variance (AMOVA), 25 % of variation was observed among population, 9% among individuals and 66% within individuals observed in the population. Structure analysis grouped isolates into two populations. Principle coordinate analysis explained variation of 36.6% in population 1, 14.30% in population

2 and 13.10% in population 3 (admixture) with 64.07% as overall cumulative percentage of variation. Significant levels of genetic diversity were observed based on SSR markers, reflecting existence of polymorphism among selected isolates of *Venturia inaequalis* based on mean genetic diversity indices like Nei and PIC (0.36 and 0.11 respectively). This indicates that extensive short-distance gene flow occurs in Kashmir region, that dispersal over longer distances also appears to occur frequently enough to prevent differentiation due to genetic drift. It is evident that Jammu and Kashmir most likely have *Venturia inaequalis* sub populations linked to diverse climatic conditions of the Jammu region compared to the mountainous inland of Kashmir region. Further HPLC and spectrophotometry-based analysis of total phenols, flavonoids and major marker polyphenolic and flavonoid compounds (ferulic acid, coumaric acid, catechin, sinapic acid, catechin, rutin, phloridzin) were carried out. Pre inoculation to post 1 day (24 hours) in *Venturia inaequalis* treated cultivar showed 16-fold increase in ferulic acid level. Coumaric acid content was found to be 1.15 µg/g (fw) and 7.67 µg/g (fw) before and after 1 day (24 hours) of *Venturia inaequalis* inoculation. Flavanol catechin raised by 16-fold, apigenin 2.4-fold and rutin 2.7-fold after 1 day (24 hours) of inoculation. This was further validated by the expression analysis of polyphenolic genes like PAL, CHS, C3H, F3H. Overall, among all the four genes tested, the highest gene expression level of PAL gene was observed in *Venturia inaequalis* treated cultivar at different stages of inoculation, especially after 1 day and 24 days whereas others showed small differential levels of expression. Relative gene expression/fold change of PAL pathway genes was found in the order as PAL > CHS > C3H > F3H using qRT-PCR. Conclusively, genetic diversity results of present investigation, could help in constructive management of scab. There is a need to increase use of polygenic cultivars so that the fungus has smaller chances of adaptation. Resistance breeding of new varieties that combine resistance genes from different sources is desirable. Modern breeding programmes could be used as a future strategy to introduce the transcription factors for up-regulation of PAL pathway genes, as an adjunct approach to reduce scab apart from disease resistant genes in the development of new apple cultivars.

Key words: *Venturia inaequalis*, apple scab, barcoding, PAL, gene expression



Signature of the Major Advisor



Signature of the Student

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
BI	Before inoculation
bp	Base pair
CE	Catechin equivalent
CHI	Chalcone isomerase
CHS	Chalcone synthase
COX1	Cytochrome c oxidase 1
CTAB	Cetyl tri methyl ammonium bromide
df	Degree of freedom
DNTPs	Deoxynucleotides
DPPH	2-2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic acid
Est.	Var. estimated variance
FC	Folin – Ciocalteu's
Fw	Fresh weight
GAE	Gallic acid equivalent
HCA	Hydroxycinnamic acid
He	Expected heterozygosity
IGS	Intergenic spacer
ISSR	Inter simple sequence repeat
ITS	Internal transcribed spacer
LSU	Largest subunit
M	Molar
MEGA7	Molecular evolutionary genomics analysis
mM	Millimolar
MS	Mean squared deviation
Na	Number of different alleles
NaOH	Sodium hydroxide
Ne	Number of effective alleles
ng	Nanogram
Nm	Nanometer

PAL	Phenylalanine ammonia lyase
PCA	Principal component analysis
PDA	Potato dextrose agar
PI	Post inoculation
PRRs	Pattern recognition receptors
QE	Quercetin equivalent
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
SSR	Simple sequence repeat
SSU	Small subunit
TAE	Tannic acid equivalent
TAE	Tris-acetate-EDTA
TBE	Tris-borate- EDTA
TE	Tris- EDTA
Total var. (%)	Total variance percentage
UV-VIS	Ultraviolet-visible
v/v	Volume by volume
w/v	Weight by volume
μl	Microlitre
μM	Micromole



Introduction

INTRODUCTION

1.1 Apple

Apple (*Malus × domestica* Borkh.) is the most significant fruit grown in the temperate region of the world due to its health / nutritional benefits. In India, it is the fifth most important fruit crop. The Indian apple is largely grown on the North Western Himalayas in the altitude ranges between of 1200-3500 (Sharma, 2010). The commercial cultivation of apple in India is mostly restricted to the Union Territory of Jammu and Kashmir, State of Himachal Pradesh, Uttarakhand and in some parts of Arunachal Pradesh, Sikkim, Nagaland, Meghalaya (Indian Horticulture Database, 2014). Total area under apple production in J&K is 1,62,971 hectares and total annual production of apples in J&K is 17,26,834 metric tons (approx.) in which Kashmir division produces 16,88,413 metric tons and Jammu division produces 38,421 metric tons. Though apple is attacked by many diseases, but scab remains the common bottleneck for its production. Out of all the deciduous fruits, apple is the most important in terms of production and J&K accounts for 70.39% of the total production. In J&K, apple history goes back to as early as 2000 BC.

1.2 Apple scab

Malus × domestica Borkh belonging to family Rosaceae, is one of the most favored main fruit tree crops growing in all the temperate and subtropical countries (Chadha and Awasthi, 2005). Apple scab caused by a dreadful fungus (*Venturia inaequalis*) is a major global bottleneck for horticultural profitability of any apple producing region. *Venturia inaequalis*, a fungal pathogen and a causative agent of apple scab remains the major threat for apple growers / farmers. It is responsible for heavy economic loss to farmers annually. This heterothallic, haploid ascomycete releases ascospore around March in Kashmir and continues for many weeks, later infection is spread via asexual conidiophores (Benaouf and Parisi, 2000). Although, infection may occur on leaves, fruits, stem, green twigs, but the main sites of infection are leaves and fruits. Infection first appears on the underside of the leaf and subsequently spreads to the other parts. Young leaves are more prone to infection than mature / old leaves. The

ascospores constitute the primary source of infection and these become active as soon as spring starts. In Kashmir valley, the release of the ascospores can begin in March and may continue over several weeks (Sharma, 2010). Further, infections occur with asexual conidiospores. Conidia spread from tree to tree in rainsplash and by wind. The pathogen remains active even at very low temperature (Stensvand *et al.*, 1997) 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 (Mills and Laplante, 1951). The disturbing situation is the fast evolution of new race of *V. inaequalis*, which are overcoming scab resistance genes (Patocchiet *et al.*, 2005). Therefore, study of genetic variation in *V. inaequalis* using molecular markers becomes extremely important.

1.3 ITS as fungal DNA barcode

DNA barcoding is a potent complement for specimen recognition that could potentially be useful for identifying and recognizing all fungi and overcoming the diverse sets of conventional criteria used for unfolding diverse groups of fungi. A part of the mitochondrial cytochrome c oxidase 1 (COX1) gene has become the first official animal DNA barcode (Hebert *et al.*, 2003; 2004) and for plants an agreed system is based on the plastid loci *rbcl* and *matK* (Hollingsworth *et al.*, 2009). In 2012, the International Fungal Barcoding Consortium formally recommended that the Internal Transcribed Spacer (ITS) regions of the nuclear ribosomal RNA gene cluster can be used as the primary fungal barcode. In fungi, the complete ITS section is about 600bp long and contains two variable spacers ITS-1 and ITS-2, that are divided by the highly conserved 5.8S rRNA gene (White *et al.*, 1990). The ITS region is flanked by the 18S rRNA gene at the 5'-end of the ITS-1 spacer and by the 28S rRNA gene at the 3'-end of the ITS-2 spacer. The extremely conserved 18S, 5.8S, and 28S rRNA genes allow the design of "universal primers" to amplify the ITS-1, ITS-2 or the entire ITS region in the vast majority of fungi. Another main benefit of using ITS as a barcode is that each haploid genome usually contains multiple tandemly repetitive copies of the ribosomal rRNA gene cluster (including ITS) making it potential to amplify this gene from little amounts of genetic materials. The speedy increase in fungal barcoding studies has been drastically benefited from the availability of reasonably priced DNA sequencing technologies in many parts of the world. These studies have exposed remarkable

undescribed fungal diversity across a broad-spectrum of biological niches (Tedersoo *et al.*, 2014; Grantham *et al.*, 2015).

DNA barcoding in the strict sense is defined as the standardized analysis of an easily amplifiable PCR fragment for sequence-based identification of species. Identifications must be accurate, rapid, cost-effective, culture-independent, universally accessible and usable by nonexperts (Figure # 1). By DNA barcoding, organisms can be identified in life cycle stages not suited for morphological identification (Gilmore *et al.*, 2009). A standardized DNA-based species identification system for fungi would be extremely useful (O'Brien *et al.*, 2005). Identification of many of these, particularly from their vegetative state, will only be possible by molecular methods. Primers have long been available for the nuclear ITS rDNA region (White *et al.*, 1990) which are now commonly used for fungal (Summerbell *et al.*, 2007). Identification by molecular characters has some tradition, particularly for organisms that are very small or where species otherwise are difficult to tell apart. For example, it is now commonly accepted that a new bacterial species has to be published together with a sequence of the nuclear small subunit (SSU/16S) rDNA that distinguishes it from other known species (Scorzettiet *et al.*, 2002).

In a similar approach, the use of a universally recognized gene / a DNA barcode for biological identification was proposed 16 years ago (Hebert *et al.*, 2003). The nuclear ribosomal RNA genes have been constantly discussed as potential candidates for fungal barcoding, because they are most widely used genes for phylogenetic studies for almost two decades. While SSU rDNA sequences are generally used for identification of glomeromycetes (Beck *et al.*, 2007), the ITS region is the most commonly sequenced genetic marker of fungi and it is regularly used to address research questions connecting to systematics, phylogeny, identification of strains and specimens at / below the species level (Smith *et al.*, 2007). The ITS rDNA region is proposed to be the consortium for the barcode of life (CBOL, <http://www.barcoding.si.edu>) as a fungal barcode (Seifert, 2007). The tandem repeat includes two spacer regions, the intergenetic spacer (IGS) and the internal transcribed spacer (ITS). The whole region spanning ITS1, 5.8S and ITS2 can easily be amplified by universal primers in the conserved flanking regions of small subunit and large subunit and is proposed as a DNA barcode for fungi (Figure # 2).

Identification of fungi to species level is a fundamental component of many research efforts in life sciences. This is true in applied as well as in basic research fields. For example, correct species identification of fungal pathogens is elementary for all aspects relating to fungal or plant diseases, both in natural and agricultural ecosystems and might even result in quarantine measures influencing international trade of plants and plant products (McNeil *et al.*, 2004). Moreover, trustworthy species identification plays a pivotal role in all studies involving conservation biology and ecology, because all biological aspects of any given individual in an ecosystem can only be attributed meaningfully *via* an unambiguous identifier like a species name.

Last, but not the least, the concept of biodiversity is fundamentally based on the species unit (*i.e.*, alpha diversity) from which the higher levels of biodiversity (*i.e.*, beta and gamma diversity) are derived (Whittaker, 1970). ITS, rDNA and transcription elongation factor (TEF-1a) primers have been used for rapid detection of *Venturia inaequalis* present in the infected plants and have applications in screening of infected plants as well as in the disease diagnosis. DNA barcode provides a way to identify the species to which a plant, animal or fungus belongs. It enables to identify unknown organisms based on specific DNA regions. ITS region is widely used as a DNA barcoding marker to characterize the diversity and composition of *Venturia species*. ITS region heavily used in both molecular methods and ecological studies of fungi, because of its high degree of interspecific variability, conserved primer sites and multiple copy nature in the genome. ITS is mainly introduced for barcoding of fungal species identification. Approximately, 1,72,000 full lengths fungal ITS sequences are available in GenBank.

The molecular techniques can be used for the identification of species, sub-division of intra-specific strains and analysis of population structure of the pathogen. Previous work investigated morphological and molecular characterization of *Venturia species*. associated with apple using different markers *viz* ITS, ITS-RFLP, IGS, SSR, ISSR, RAPD, URPs, AFLP, *etc.* Various methods like restriction fragment patterns from PCR amplified ITS sequences and direct sequencing of ITS region are considered the major tools in pathological and taxonomic studies at the species level in fungi and a wide range of fungi have been examined by these methods (Scorzettiet *al.*, 2002). The use of resistant variety cannot only be beneficial in reducing the losses due to disease,

but these sources are also useful to minimize the fungicidal toxicity (Roudonis and Valiuskaite, 2009). Further, a study on population genetics of pathogen from various cultivars and in mixed orchard system using SSR markers needs to be assessed.

1.4 Races of *Venturia inaequalis* and apple scab resistant (R) genes

Many races have evolved during past few decades of *Venturia inaequalis* to breach the scab resistance in apple cultivars. Hyper variability in *V. inaequalis* led to eight different races from different parts of the world (Thakur *et al.*, 2013). However, a number of the isolates of *Venturia* are capable of growing on two totally different differential hosts and thus it's tough to classify them to particular race. The projected new terminology by reconsidering the differential host employed in previous studies. Plants have evolved a number of tactics to efficiently fight attack of pathogens, involving a large number of physiological responses including cell wall modifications, hypersensitive reaction, The production of pathogen related proteins and metabolites (Yang *et al.*, 2018). There are numerous examples that by the timing and intensity of a plant's defense responses, the specific vulnerability or resistance as the result of a host–pathogen interaction may be determined. (Tsao *et al.*, 2003).

Several disease resistance (R) genes have been recognized in apples and one of them has been cloned, namely Rvi6 (Vf), which is being investigated for apples resistant to scab engineering (Malnoy *et al.*, 2007). In past decade, researchers were focusing towards screening of various apple cultivars resistant to scab using recombinant technology-based approach *e.g.*, *Mallus floribunda* based *HcrVf2* (Rvi6) gene, has been introgressed into scab susceptible cultivar like Gala via cisgenic approach. Cisgenic approach has been widely used to incorporate scab resistance in desired apple varieties throughout the world which involves the direct transfer of the Vf gene (R-Gene) to apple cultivars from resistant varieties of apple. Since R genes have not been explored to a greater extent in the apple varieties grown in Kashmir, India. Introgression of new R-genes through cisgenesis will help in incorporating resistance to apple scab and prevent huge losses incurred every year. An example of this selective pressure leading to host plant susceptibility was observed in Golden Delicious apple varieties. There are 11 endogenous scab resistant genes (R-genes) reported in different apple cultivars like Va, Vb, Vbj, Vd, Vf, Vg, Vh2, Vh4, Vh8, Vm, and Vr2 (Parisi, 1993; Belfant *et al.*, 2004; Szankowski, 2009). Identification of different genes and mechanism of fungicide

resistance in *V. inaequalis*, the causative agent of apple scab, is essential in order to develop new and highly effective defenses against the spread of disease.

Since commercially important apple scab susceptible cultivars are gaining momentum due to more demand, this has remained a major reason that least commercially important cultivars although possessing endogenous scab resistant potential are not explored to a greater extent. Some traditional apple varieties are available with farmer orchards which show moderate to excellent resistance to apple scab. Most of the disease resistant apple cultivars in Indian belt of Himalaya like *White Dotted Red* are being used as pollinator in apple orchards. Identification of such potential scab resistant R gene sources could act as known repositories for future amelioration of apple cultivars against scab. Gene pyramiding of one or more R-genes through cisgenesis /transgenesis will help in imparting resistance into scab susceptible commercially valuable cultivars (Galliet *et al.*, 2010).

1.5 Role of polyphenols in disease resistance

Phenolic compounds have been shown to be involved in plant defenses against biotic and abiotic stresses (Geibelet *et al.*, 1994). A large number of different phenolic compounds have been reported to occur in various apple tissues (Bazziet *et al.*, 2003). Phenolic compounds in apple, particularly flavonoids, have been claimed to play a role in scab resistance (Treutter, 2005). Furthermore, they possess anti-microbial properties and their concentrations may increase after infection (Michalek *et al.*, 1999). As a large group of bioactive chemicals, phenolics have diverse biological functions and act as phytoalexins (Popa *et al.*, 2008).

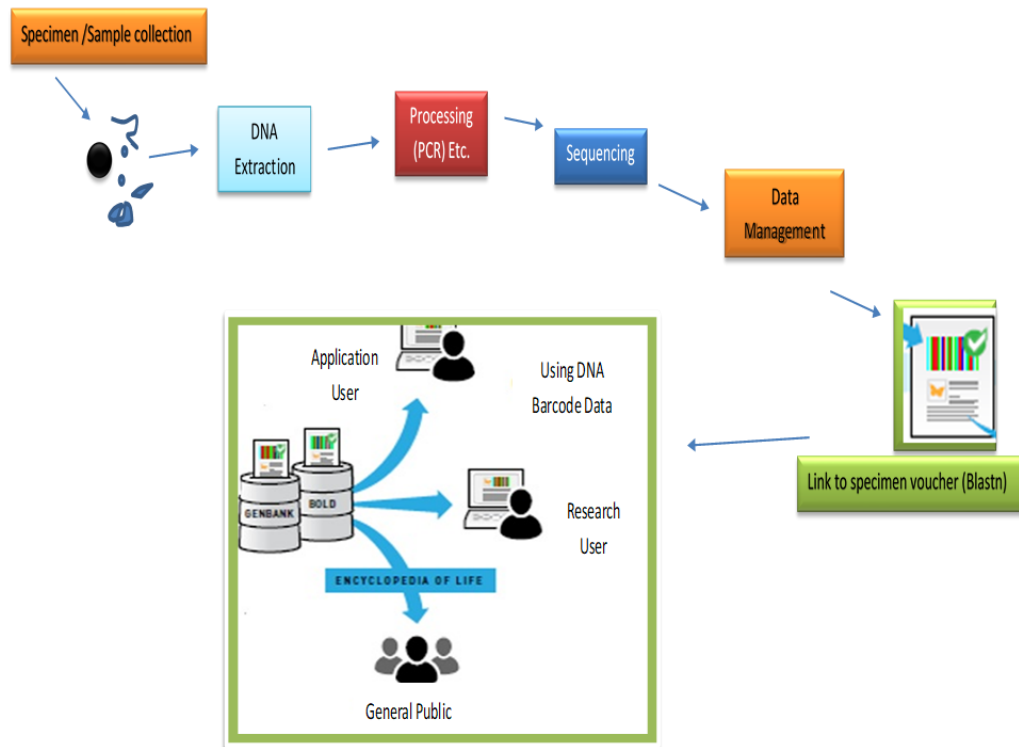
In apple fruit (*Malus × domestica* Borkh.), the flavonoids biosynthetic pathway is most active in the skin with the trans- and cis-flavan-3-ols being catechin and epicatechin respectively, acting as the initiating units for the synthesis of PA polymers. PAL provides the link between primary plant metabolism and the phenyl propanoid pathway. PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid, which is a precursor of many different phenolic compounds. The key enzyme in the flavonoid pathway is chalcone synthase (*CHS*; EC3.2.1.74). *CHS* and chalcone isomerase (*CHI*; EC3.2.1.14) together lead to the formation of flavanones (Mayr and Michalek, 1997). The flavan-3-ols/procyanidins are the most important contributors to the *in vitro*

antioxidant activity of apple and procyanidin B₂ and epicatechin are the most important individual antioxidants in apple. However, hydroxycinnamic acids showed a significant role in the flesh (Tsao *et al.*, 2005). According to the gene expression results, which suggest that lignification plays a role in the defense reaction of the scab resistant cultivar Rewena against *V. inaequalis*. The formerly observed loss of resistance in another scab resistant cultivar (Mayr and Michalek, 1997) by a general blockage of the phenylpropanoid biosynthesis through inhibition of PAL may now be explained as an inhibition of lignification (Picinelli *et al.*, 1995). The strengthened cell wall and cuticular membrane along with sub-cuticular pH of such leaves are speculated to play a role in governing such resistance. A breakdown of ontogenic resistance discovered by restored growth of the infectious agent is determined within the previous senescing leaves of apple.

Therefore, apple scab is the most dreadful disease of apple cultivars, if left uncontrolled, leads to huge economic loss of 30-40% per year globally. When designing management strategies to fight the disease, it is essential to consider the variation among the pathogen population. Detailed research concerning pathogen variation and population genetic structure is needed to understand the biological process, potential and the origin of the pathogen. In a wide range of organisms, ribosomal gene sequencing and internal transcribed spacer (ITS) regions are used to study phylogenetic relationships. As some ribosomal RNA diverges more slowly, studying the ITS regions can reveal more information about the organism. Identifying polymorphisms in nuclear ribosomal DNA is also excellent at assessing the extent of genetic variability in a population. Further, emerging needs for sustainability of food lug us to discover and characterize the resistance mechanism of the plant. Different management capsules however exist, but identifying genes seems indispensable to understand the defense mechanism of host. Detailed studies are required to elucidate the practicality of such resistance and perceive its breakdown mechanism.

1.6 Objectives of the present research work

- Isolation of different *Venturia inaequalis* strains and ITS1 and ITS2 based sequence identification
- Barcoding of DNA sequences
- *Venturia inaequalis* effect on PAL pathway by estimation of phenolics, flavonoids and tannin content and expression analysis of PAL



Figure# 1. Pipeline for DNA barcoding



Figure # 2. Schematic representation of the nuclear ribosomal DNA (barcode) region proposed for fungi. RNA [small subunit (SSU), 5.8S, large subunit (LSU), the intergenetic spacer (IGS) and the internal transcribed spacer (ITS)]

*Review of
Literature*

REVIEW OF LITERATURE

A comprehensive review was conducted to understand the mechanism of disease infection caused by *Venturia inaequalis* and resistance offered by the apple cultivar against *V. inaequalis* in the form of polyphenolic compounds and genes. Further, the study of effect of diversity and variability of *V. inaequalis* was carried out from literature.

2.1 *Malus × domestica* Borkh.

The apple (*Malus × domestica* Borkh.) is a woody perennial angiosperm greatly studied genomically due to its chief economic importance ($2n = 34$), it belongs to family *Rosaceae*, the sub-family *Maloideae*. There are about 25 to 30 species in the genus *Malus* and a number of subspecies of crabapples. The fruits are characterized by two to five carpels situated in a fleshy cover. The main ancestor of the cultivated apple is probably *Malus sieversii* Lodeb. *Malus. roemer*, a species with a high degree of diversity found on the boundary between Western China and the former Soviet Union. The center of origin for this species is Kazakhstan in Central Asia and collection of seeds was recently performed (Forsline *et al.*, 2003; Geibelet *et al.*, 1999). Other species thought to have contributed genes to domestic apples are *M. orientalis* Uglitzk. ex Juz., the European crab (*M. Sylvestris* Miller) and Asian species such as the Siberian crab (*M. baccata* L. Borkh.) (Janick, 2005). Apple species are distributed throughout regions of the world including West Asia, Himalayan Region, Central Asia, India, Western provinces of China, Europe and some parts of America and Africa.

Apple species have been evaluated by morphological /molecular DNA analysis and have confirmed that *M. sieversii* is the species from which apple domestication started (Forte *et al.*, 2002). These species may have hybridized with *M. prunifolia*, *M. baccata* and *M. sieboldii* to the East and with *M. turkmenorum* and *M. Sylvestris* to the West. Subsequently, well-established apple cultivars were selected and introduced into Europe and especially the Mediterranean regions by the Romans (Harris *et al.*, 2002). Other *Malus* species are occasionally used for introgression of several useful

traits into modern cultivars, but that usually requires several generations of back-crossing to reach acceptable fruit size and quality. The opening of trade routes from China to the Middle East and Europe via Central Asia – “*The Old Silk Road*” played a key role in the evolution and in spreading of cultivated apple (Boudichevskaja *et al.*, 2009). In ancient Greece and Persia, certain techniques (pruning, storage, grafting, precise rootstock usage, selection of distinctive apple clones) of apple cultivation were already used and twenty varieties of cultivated apples in several regions were described (Boudichevskaja *et al.*, 2009). Persia was patently the source of apple and other numerous fruits originated from Central Asia (Janick, 2005). Wild apple fruits were cultivated and frequently spread throughout the Europe and Mideast, which led to the cultivation of thousands of unique and new varieties. Apples have been widely planted all over Europe and consumed as a raw fruit, fermented juice or cider, vinegaras well as cooked and mixed with sugar and sweets. In the 18th and 19th centuries, more than one hundred apple cultivars were described and categorized based on their final application and availability (Harris *et al.*, 2002). Until the introduction of *M. domestica* in the late 19th century, the Chinese pear leaf crab apple *Malus asiatica* Nakai was the major cultivated species in southern and eastern Asia. After *M. domestica* was introduced, breeding started in many parts of the world and the commercial apple production existing today, began.

2.2 Diseases of *Malus × domestica* Borkh.

The most important fungal disease is apple scab, which is described in detail further ahead in the text. Other diseases are powdery mildew (*Podosphaera leucotricha*), monilia (*Monilia laxa*) and apple stem canker (*Nectria galligena*). Storage diseases are also a problem and apart from apple scab, *Gloeosporium* and *Monilia fructigena* cause damages. Other common fungi like grey mould (*Botrytis cinerea*) and green mould (*Penicillium species*) may occur during storage of the fruit. Union territory of J&K is the largest producer of apple in India. In Kashmir valley 45.34% land (1,61,364 hectares) is occupied by apple out of total area of 3,55,921 hectares under fruit. In 2015-16 Kashmir produced 13.54 lakh metric tons of apple, which accounted for 70.39% of the total apple production of India (Table # 1). India accounts for 2.05% of the total world apple production. 1.6% of the apple produced in India gets exported. J&K, despite being a horticulture UT, where the

majority of state economy depends on apple fruit, no ideally effective system to address apple scab has been successful so far.

2.3 Life cycle of *Venturia inaequalis*

Apple scab caused by *Venturia inaequalis*, a filamentous fungus, is one of the most detrimental fungal pathogens that remain a major threat to apple production worldwide. It is among the most noteworthy threats to apple growing areas (orchards) located in cool and precipitated regions of the world (MacHardy, 1996). Causal agent of apple scab *Venturia inaequalis* (Cke) belongs to subdivision Ascomycota, class Loculoascomycetes, order Pleosporales and family Venturiaceae and genus *Venturia*. Saprophytic (sexual state *Venturia inaequalis* (Cke) and parasitic (asexual state *Spilocaea pomi* Fr) are the two states of this fungus (Boone, 1971). Apple scab is the most important disease found in apple (*Malus domestica*) cultivation. Epidemics begin in spring when ascospores are released and infect young emerging apple leaves. Conidia are then produced from these infections and continue the epidemic by infecting leaves and fruits. Although, ascospores and conidia are primarily spread by rain splashing on infected leaves, wind distribution of infected leaves and airborne ascospores can add to the migration of the fungus (Gessler *et al.*, 2006). There are two stages of this disease cycle: sexual cycle and asexual cycle.

2.3.1 Sexual cycle: For sexual reproduction, the presence of two mating types is essential. In the beginning of spring, mating proceeds in infected leaves from the prior season. During winter and early spring, fungus forms fruiting bodies called pseudothecium, which develops in the fallen leaves after the fertilization. The pseudothecium is a cavity located within a dense mat of fungal mycelium called a stroma. Inside this cavity the asci are formed, which are bitunicate, cylindrical shaped, double walled. Each ascus contains eight ascospores which are 11-15 µm long and 4-8 µm wide, brown, two-celled and have a characteristic "footprint" shape (MacHardy, 1996). The development of ascospores continues during the spring and some spores mature and start to increase quickly while the bud breaks and blossom period occur when there are favorable conditions. The ascospores are passively emitted into the air, when the rain wets the fallen leaves (Raudonis and Valiuskaite, 2009). For optimal ascospore release, the presence of light, high humidity and leaf wetness are required.

Developed pseudothecia distend and stick out from the surface of the leaf. Unrestricted ascospores are transferred by rain and wind into the air and when landed on young blossoms, fruitlets, leaves, then ascospores germinate and start to infect the apple tree. Due to temperature, time for the infection period varies and the first symptom may appear approximately after two weeks. The symptoms of primary infection are velvety or olive-brown spots and dark conidia referred to as summer spots occur like bear masses. One cycle of the infection occurs in one season (Jha *et al.*, 2010; Vaillancourt and Hartman, 2000).

2.3.2 Asexual cycle: Once primary infection occurs, secondary spores (conidia) are formed, allowing a tree to continually reinfect itself or neighboring trees as long as environmental conditions are favorable. The disease progresses rapidly and causes severe infections, if plants remain continuously wet for at least a six-hour period with temperatures around 65 to 75 degrees F. The warmer the temperature, the less time the leaf must remain wet continuously for infection to occur. *Venturia inaequalis* overwinters mostly on dead leaves, in which small microscopic flask-formed black fruiting bodies, called pseudothecia, are developed. Ascospores start maturing in early spring and when temperature and moisture are suitable these spores are released forcibly in air. In the spring during the rainy season, fungal spores are released from the infected leaves that remain from the previous year and are carried by the wind to newly emerging leaves and fruits of healthy trees (Gessler *et al.*, 2006). Once primary infection occurs, secondary spores (conidia) are formed, allowing a tree to continually reinfect it or neighboring trees as long as environmental conditions are favorable. Many cycles of conidial production and secondary infection take place in a particular growing period under the suitable weather conditions and late infection in autumn may not be detected. A detailed life cycle of apple scab fungus is shown in Figure # 3. The disease progresses rapidly and causes severe infections if plants remain continuously wet for at least a six-hour period with temperatures around 20 - 25°C. Damage caused by this pathogen are developed in between the cuticle and the cell wall of epidermal layers (MacHardy *et al.*, 2001) that results in the superficial damage which deepens with the passage of time. Moreover, deformation in shape and size of the affected fruits and black velvety lesions make it unsuitable for marketing (Figure # 4).

This disease can have a significant economic impact as diseased fruit are not marketable. The disease negatively affects fruit size and quality (due to blemishes and poor ripening). Over time, repeated defoliation from the disease reduces tree vigor, growth and yield. This outrageous disease leads to premature leaf/fruit fall that results in 70% yield loss and even complete loss is also possible, if steps are not taken in the orchard to reduce infection. This outrageous disease leads to premature leaf/fruit fall that results in 70% yield loss (Thakur *et al.*, 2013). In order to tackle the disease, apple growers spray a number of fungicides throughout the growing season to prevent the disease. The fungicides have better potential to manage the disease however it poses greater threat to environment, humans, aquatic life, even plants itself (Baumgartner *et al.*, 2015). Some fungicide groups for instance trizoles have adverse effect on soil and persist for longer period of time (Shahinasiet *al.*, 2017). Besides environmental threat, the pathogens have ability to develop resistance against fungicides. Thus, renders fungicides ineffective and we have to encounter more virulent strains of the pathogen. Despite the many years of work on scab resistant of apple cultivars, they have yet to gain widespread popularity. Cultivars resistant to scab are reputed to have low fruit quality, poor storability, low yield and lack of market acceptance (MacHardy, 1996).

2.4 Apple scab races

The interest in different apple scab races started early, even though the first authors were more concerned about morphological differences and infection patterns. Three physiological races of *V. inaequalis* were identified (Williams and Shay, 1957). Race 1 was defined as the race commonly found in the USA and other countries; race 2 infected three cultivars: Dolgo, some segregants of the Russian variety R12740-7A and Geneva; and race 3 infected Geneva. It was also discussed whether the origin of race 2 was through import of plant material from Russia. Race 4 was detected in scab resistant seedlings (*M. pumila* R12740- 7A) in Purdue, USA (Williams and Kuc, 1969). Evidence of a new physiological race (race 5) was presented and it was characterized by the capacity to infect plants, giving the pit type of reaction in *M. micromalus* and *M. atrosanguinea*. Supposedly, scab resistant selections from the USA were sent to England, but some selections of *M. micromalus* developed sporulating lesions. Race 6 was first described (Parisiet *al.*, 1993). Scab symptoms on

Prima (Vf) had been noted in Ahrensburg, Germany since 1984. The isolates from Germany proved to infect other Vf-selections, but the progenitor *M. floribunda* 821 remained resistant. The pathogenicity of race 6 was further investigated (Parisiet *al.*, 1993) on apple clones. Race 6 gave symptoms on nearly all of the types of resistance (Vbj, Vr and Va) were not infected. It was found that much of the genetic background for resistance to *V. inaequalis* in wild species had been lost in the breeding process.

The importance of finding polygenic sources of resistance was stressed. The last race to be discovered was the English race or race 7 (Roberts andCrute, 1994). An isolate from a naturally infected *M. floribunda* tree gave lesions on *M. floribunda* 821 and some Vf cultivars, while some other Vf cultivars were not infected. Later, a second dominant gene was discovered in *M. floribunda* 821 (Vfh) and the host-pathogen relationship was discussed. It was confirmed that 'Golden Delicious' (Vg) was resistant to race 7 (Table # 2) (BenaoufandParisi, 2000).

2.5 Disease management of apple scab

There are both indirect and direct control methods for scab. Since apple scab is dependent on humid and cool climate to develop, some locations are better than others for establishing an orchard. The choice of cultivar is also very important. A mixture of cultivars, avoiding the most susceptible ones, can reduce the infection pressure of the fungus. Pruning and fertilizing are two important factors for disease development. If the trees are well pruned, the leaves dry up more quickly and the fungus has less chance of infecting the tree. The level of nitrogen fertilization during the growth period is also of importance. Apple trees do not need high amounts of nitrogen and excess may result in loose growth of the shoots, which are then more susceptible to the disease. Fungicides are important tools for control and managing diseases in: fruits, vegetables, legumes, grasses and other crops. Unlike insecticides and some herbicides which kill established insects or weeds, fungicides are most commonly applied to protect healthy plants from infection by fungal plant pathogens. Among many classes of fungicides, azole or conazole fungicides represent a large group of substances which are widely used in agriculture for protection of crop plants and in pharmaceutical industry in the treatment of various fungal diseases. They are synthetic compounds that can be classified into imidazole or triazole, depending on the number of nitrogen atoms in the five-membered ring (Faro, 2010). To be effective,

fungicides must be applied before infections become established and in a sufficient spray volume to achieve thorough coverage of the plant or treated area.

Management of apple scab on susceptible trees is focused on the prevention of primary infection by ascospores in the spring. Early infection of trees may result in poor fruit set and will result in more secondary inoculum being produced throughout the season. The initial fungicide sprays are therefore timed to coincide with the spring release of primary inoculum. Later, sprays are often targeted at other fungal diseases, in addition to scab, but also are effective against apple scab secondary inoculum. In commercial orchards, airblast sprayers are typically used to apply fungicides. Chemical fungicides for apple scab control are generally of two types, preventive (protectant) and curative. Most growers use a combination of the two for maximum effectiveness. Preventive fungicides are applied to the leaves and fruit before infection. When ascospores or conidia are present on susceptible plant surfaces and leaf moisture / temperature are suitable, the fungicide prevents the fungal spores from germinating or penetrating the host tissue. Good spray coverage and uniform deposition are essential. To ensure coverage of newly emerging tissues and to replace the chemical as it is lost to weathering, fungicides must be re-applied on a regular schedule. Usually, this consists of spraying every 5 to 7 days in the early part of the season and every 7-10 days later in the season(Faro, 2010; Fishel, 2014; Koller *et al.*, 2004).

Curative fungicides have limited systemic activity and if applied soon after infection, are capable of penetrating the leaf and stopping further development of the fungus, thus preventing symptom development. These fungicides are especially useful when growers use a Mills table-based weather monitoring system that tells them when an infection has occurred. Prompt application of the fungicide will stop further disease development. Growers using these systems spray fungicides only when needed and in a season when few infections occur (in a dry spring), they may apply fewer fungicide treatments than a grower spraying preventively. By the time, apples are harvested in summer and fall, fruit fungicide residues are either absent or present at extremely low safe levels (Fishel, 2014). The scab pathogen can infect both leaves and fruit and can cause severe defoliation of apple trees if poorly managed. The disease negatively affects fruit size and quality, due to blemishes and poor ripening.

Overtime, repeated defoliation caused by the disease reduces tree vigor, growth and yield (Nicholson and Beckerman, 2008).

Like other apple growing regions worldwide, scab too is currently being managed by fungicidal sprays from pink bud to harvest (Table # 3) in J&K until recently, it was perceived as the only economical control measure. This perception is changing as a result of the high costs of new molecules such as the strobilurine-based fungicides, increased fungicide resistance in populations of *V. inaequalis* and increased appreciation of environmental costs and consumers negative perceptions of fungicide use. Fungicides are important tools for control and managing diseases in: fruits, vegetables, legumes, grasses and other crops. Unlike insecticides and some herbicides which kill established insects or weeds, fungicides are most commonly applied to protect healthy plants from infection by fungal plant pathogens (Faro, 2010). To be effective, fungicides must be applied before infections become established and in a sufficient spray volume to achieve thorough coverage of the plant or treated area. Poor disease control with fungicides can result from several causes including insufficient application rate, inherently low effectiveness of the fungicide on the target pathogen, improper timing or application method and excessive rainfall.

Despite the many years of work on scab resistant of apple cultivars, there is a need to gain widespread popularity Cultivars resistant to scab are reported to have low fruit quality, poor storability, low yield and lack of market acceptance (MacHardy, 1996). Protection from fungicides is temporary because they are subject to degradation or mineralization over time. Furthermore, in some cases the plant pathogens can be adapted to fungicides. Thus, they have to be reapplied to protect new growth when new disease threatens. Some fungicides such as penconazole increases yield and chlorophyll content. Many other fungicides that are sterol biosynthesis inhibitors for instance triazoles increases the yield by reducing leaf and fruit scab severity about 88.9% and 100% lower than control respectively (Hailey and Percival, 2014).

Whereas, myclobutanil (0.6mL/L) fungicide lead to reduction in disease severity of scab by 80% and have remarkable anti-sporulation activity (Percival, 2010). Triazole fungicides act by inhibiting fungal cytochrome P-450-(CYP)-enzyme used in ergosterol synthesis. Myclobutanil [α -butyl- α -(4-chlorophenyl)-1H-1,2,4-triazole-1-propanenitrile] specifically acts on the 1Terg111T gene responsible for

encoding sterol C14-demethylase (cytochrome P-450 isozyme CYP51), thus inhibits demethylation step in sterol biosynthesis pathway. These sterols are the key components of cell membrane of fungi, its disruption leads to cell death. Other fungicides such as difenoconazole, hexaconazole, mancozeb, dodine, metiram, pyroclostrobin etc have also remarkably reduced scab disease in apple orchards. In spite of its marvelous role in reducing the disease severity, it still has a large number of hitches (Palani, P.V. and Lalithakumari, D. 1999).

Fungicide resistance is a growing issue that evolves pathogen races and newer fungicides are debuting. Day by day more virulent races are surfacing and we have to encounter them with more lethal applications. Many pathogens have developed resistance against myclobutanil. DMI fungicide myclobutanil is considered one of the best chemicals for controlling apple scab, however, its specific mode of action and repetitive use has led to diminished efficacy and resistance (Marine *et al.*, 2007; Shahinas *et al.*, 2017). Most of the fungicides target cytochrome b, a membrane protein that forms the center of the mitochondrial bc₁ complex in the respiratory chain of eukaryotes. The Quinone inhibitor fungicide blocks the electron transfer by binding to the Q_o site on Cytochrome b, through the respiration pathway, that leads to loss of energy and prevention of ATP formation eventually leads to cell death. In many cases, QoI resistance is conferred by a single amino acid change in the *cyt b* gene. In *V. inaequalis*, resistance is conferred by single amino acid substitution at position 143 from glycine to alanine (G143A) (Luo *et al.*, 2010).

2.6 Harmful effects of fungicides

In addition to the high cost, these fungicides have perilous effects on environment. These fungicides are detrimental to diverse ecological components of environment including soils. The high persistence of these fungicides affects the soil quality and becomes unfit for soil flora and fauna. Difenoconazole exhibits moderate to high persistence in soil by the formation of metabolite 1,2,4-triazole. Besides deterioration of soil, it also has harmful effect on living organism. Many fungicides including triazoles are mainly absorbed by gastrointestinal tract (GIT) and accumulated mainly in liver, kidney, adrenal glands and thyroid. The effects of fungicides are mostly respiratory tract irritation, allergy, vomiting, headache and long-term exposures may lead to cancers. Most triazoles have proven to be nontoxic

as well as nonirritant if absorbed by dermal route. However, myclobutanil causes eye irritation (Rouabhi, 2010; Shahinasiet *al.*, 2017). Many studies have shown that triazoles fungicides are extensively absorbed from the gastro-intestinal tract and widely distributed without bioaccumulation in body tissues. The highest levels of these fungicides are detected in liver, kidney, adrenal glands and thyroid. Most of triazole fungicides aren't harmful *via* dermal and inhalation routes; aren't skin infliction or a skin sensitizer. The major a part of triazoles isn't infliction to the eyes, except the myclobutanil that is classified as “irritating to eyes”. The classical approach to the management of infective fungi includes use of chemical compounds that turn out negative facet effects like environmental pollution and development of antifungal agent resistance. *Venturia inaequalis*, the cause of apple scab, quickly develops antifungal agent resistance (Reddel, 2015).

2.7 Genetic resistance of apples against the scab

Scabresistant apples and crabapples are widely available. Fungicide applications to these trees are not needed for scab management, although they may be necessary to control other fungal diseases. Cultivars such as 'Enterprise', 'Freedom', 'Gold Rush', 'Jonafree', 'Liberty', 'Pristine', and 'Redfree' are gaining acceptance by both farmers and consumers. Varieties susceptible to scab such as 'Delicious', 'Fuji', 'Gala', 'Golden Delicious', 'McIntosh' and 'Winesap' are still widely sold because consumers like them and can be grown easily in drier regions where apple scab is not a problem. There are good reasons to plant scab-resistant flowering crabapples such as 'David', 'Harvest Gold', 'Mary Potter' and 'Prairiefire'. These are beautiful landscape plants and provide greater bloom potential because there is no risk of defoliation by apple scab. Another advantage is that application of fungicides for scab management in the landscape will not be needed if resistant cultivars are grown. Cisgenic approach has been widely used to incorporate scab resistance in desired apple varieties throughout the world which involves the direct transfer of the Vf gene (R-Gene) to apple cultivars from resistant varieties of apple. *Venturia inaequalis* has a high degree of genetic variability due to recombination, thus leading to selective pressures on the fungal pathogen to overcome host resistance (Patocchiet *al.*, 1999). An example of this selective pressure leading to host plant susceptibility was observed in Golden Delicious apple varieties. During the 1900s, Golden Delicious apples were highly

resistant to apple scab, whereas today this variety is considered one of the most susceptible apple varieties (Soriano *et al.*, 2009).

There are 16 host genes (Table # 4) responsible for the resistance observed among apple varieties (Belfantiet *al.*, 2004; Szankowskiet *al.*, 2009; Parisi *et al.*, 1993; Petkovseket *al.*, 2007). Eight of the 11 genes were identified as being primarily responsible for host resistance against apple. A range of sources of apple scab resistance have been found (Williams and Kuc, 1969). Several major scab resistance genes originated from small fruited Asiatic *Malus species*. The genes Vbj from *Malus baccata* Jackii, Vb from *M. baccata*, Vm from *M. ×micromalus* and *M. ×atrosanguinea* 804, Vr from *M. pumila* (Russian seedling) R12740-7A, Vf from *M. ×floribunda* 812 have been introgressed into breeding lines and selections to make them available for breeding purposes. The resistance genes differ in their level of resistance. Durable scab resistant cultivars were created combining major resistance genes and polygenic resistance. It is important to identify genes resistance to scab in apple cultivars and determine its significance for breeding. Cultivars with multiple resistance genes can be easily selected with molecular markers associated with the resistance genes. The molecular markers have been developed for identification of gene resistance to scab in apple genome (Soriano *et al.*, 2009; Gessler *et al.*, 2006). To overcome these impediments, the direct transfer of the Vf gene to apple cultivars, thus, is seen as a potential strategy for disease control. Several apple scab resistance genes such as HcrVf (Homologues of *Cladosporium fulvum* resistance genes of Vf region) (Szankowskiet *al.*, 2009), Vr2 (Verma *et al.*, 2014), Vd3 (Patocchiet *al.*, 2004), Vb (Vanblaereet *al.*, 2011), Vbj (Patocchiet *al.*, 1999) have been identified and were mapped on different linkage groups of the apple genome. However, only the Vf locus has been positionally cloned (Xu and Korban, 2002) and proved to consist of a gene cluster with four paralogs, namely HcrVf1, HcrVf2, HcrVf3 and HcrVf4 (Belfantiet *al.*, 2004; Xu and Korban *et al.*, 2002).

Recently a gene Rvi6 (formerly HcrVf2) was derived from the scab-resistant apple 'Florina' which was originally the wild apple *Malus floribunda* 821. Initially, only the HcrVf2 gene was tested by overexpression using the CaMV35S promoter and nos terminator, in scab susceptible apple plants (Parisi *et al.*, 1999) and the transgenic apple plants overexpressing HcrVf2 proved to be resistant to apple scab.

The complete resistance was shown by inserting the HcrVf2 under the regulation of different lengths of native promoters and the nos terminator into susceptible cultivar. 'Gala' and also a mixture of *V. inaequalis* isolates for scab inoculation was used (Szankowskiet *al.*, 2009). Pathogen recognition in plants occurs by a set of genes called R genes (resistance genes) that defend the plants from the common assault of pathogen. Avr factors are present in pathogen and these factors render the pathogen avirulent (Dangl and Jones, 2001). Similarly, resistant genotypes of apple develop incompatible interaction with *V. inaequalis* known as gene-for gene interaction (Boone, 1971).

Several R gene loci have been determined from apple cultivars and many attempts are made to characterize the Avr genes of *V. inaequalis*. A new nomenclature proposed in which scab resistance genes are named as *Rvik* (*R* stands for resistance gene, *vi* stands for *Venturia inaequalis* and *k* stands for differential host) and the corresponding Avr genes of the pathogen are named as *AvrRvik* (Aldwinckle *et al.*, 2007) (Table # 4). Several resistant cultivars are developed either through transgenic or cisgenic approaches. Many transgenic plants developed exhibits resistance against *V. inaequalis* such as McIntosh, Galaxy, Araine *etc.* Expression of endochitinase and N-acetyl- β -D-glucosaminidase (*ech42* and *nag70* respectively) from *Trichoderma atroviride* (gene source) increases resistance against *V. inaequalis*. However, McIntosh cultivar shows resistance against races 1-5 whereas, Galaxy and Araine cultivars against races 1-6 (Bolare *et al.*, 2000, 2001). Another cysteine rich protein called Puroindoline (PinB) is constitutively expressed in transgenic cultivars of Galaxy and Ariane and imparts significant resistance to Race 6 (Faize *et al.*, 2003). About three orthologs (*MpNPR1*-1, 2 and 3) have been identified in apple transgenic cultivars and over expression of *MpNPR1a* exhibits broad resistance against many fungal pathogens, it may be due to enhanced expression of PR proteins (Malnoy *et al.*, 2007). Many different methods are used to control this disease spread and stop growth of this fungus, but not a good success has been achieved so far other than cisgenic approach which is by far most successful approach to tackle this disease.

Cisgenesis rely on the fact that those plants are transformed whose genetic material is derived from species itself or from closely related ones which are capable of sexual hybridization. Moreover, other foreign sequences such as selection genes

and vector-backbone sequences must not be present. Some of the shortcomings of classical breeding have been overcome by cisgenesis. It can be utilized as a fast and rapid tool for the transfer of genetic material between closely related plants (Joshi *et al.*, 2011). Cisgenesis concept, the origin of the cisgene is extended to the gene pool of sexually compatible species and the cisgene is an identical copy of the endogenous gene including the promoter, introns and the terminator in the normal-sense orientation. When using agrobacterium mediated transformation, T DNA border sequences can be used. It is evident that cisgenesis carry some limitations as compared to transgenesis because only traits in the sexually compatible gene pool can be combined and transferred into the crop. Furthermore, the isolation of genomic clones including the endogenous promoters and terminators and the subsequent development of marker and vector-backbone-free plants requires additional expertise and time. The cisgenic concepts can also overcome limitations of classical breeding when it comes to improving traits with limited natural allelic variation within the sexually compatible gene pool. Higher expression level of a trait can be obtained by re-introducing the gene of the trait with its own promoter and terminator. Recently, apple cultivar ‘Gala’ was genetically modified by inserting the apple scab resistance gene *Rvi6* (formerly *HcrVf2*) from the scab resistant cultivar ‘Florina’ (originally from the wild apple accession *Malus floribunda* 821 (Vanblaere *et al.*, 2014). Overall cisgenic approach reduces fungal growth by 80% compared with the scab susceptible ‘Gala’ (Malnoy *et al.*, 2007; Vanblaere *et al.*, 2014). Two subclasses of PR-10 gene (APa and APb) are induced by acibenzolar-s-methyl, a salicylic acid (SA, a plant defense hormone) analog in apple leaves (Mayr *et al.*, 1997; Malnoy *et al.*, 2007).

2.8 DNA barcoding and its significance

Within insects the choice of an appropriate DNA region seemed straightforward, as the ITS and COI (COX1) gene were already in use for phylogenetic inference of insects and had been shown to give high resolution on various taxonomic levels (Hebert *et al.*, 2003). This gene has additionally proven useful in studies on birds and fish (Hubert *et al.*, 2010) and there were high hopes that it would prove equally useful in other groups of organisms as well. Even though molecular data is now widely used in fungal systematic and phylogeny, the valid description of a species still requires morphological characterization according to the

botanical code of nomenclature (McNeil *et al.*, 2004). The use of DNA sequence variation in fungal evolutionary studies started soon after molecular markers began to be used as tools for assessing relationships between organisms in the early 1970s (Dutta and Acharya, 2019). The first to use 5S rRNA gene sequences to analyze the relationships among diverse groups of fungi. However, it was not until the early 1990s that DNA sequences began to be widely used in fungal evolutionary and population genetic studies. The initial adoption of molecular sequences in fungal studies was driven largely by developments in PCR and DNA sequencing technologies as well as the development of a set of fungal DNA primers (White *et al.*, 1990).

The paper (White *et al.*, 1990) has been cited over 11,000 times and many of the primers, including the almost universal fungal ITS primers ITS1 and ITS4 (Table # 5) are still widely used today. By the mid 1990s, PCR and DNA sequencing was routinely used in biology laboratories including for fungal strain and species identifications. These developments have contributed to the rapid expansion of fungal DNA sequences of several DNA fragments of the ribosomal RNA gene cluster to public databases. The first comprehensive dataset of the variable D1/D2 domain of the nuclear large subunit (28S) ribosomal DNA for about 500 species of ascomycetous yeasts was generated (Scorzettiet *al.*, 2002). Comparison of phylogenetic trees derived from the D1/D2 domain and the nuclear small subunit (18S) ribosomal DNA sequences for selected members of the *Saccharomyces* clade showed that the two trees were highly concordant, with similar statistically well-supported branches. A similar dataset for basidiomycete yeasts was established subsequently (Diaz and Fell, 2000; Diaz *et al.*, 2002) and it was found that most basidiomycete yeast species could also be distinguished using the D1/D2 domain sequences of nLSU rRNA. However, they showed that in general the ITS region was better at distinguishing closely related species than the D1/D2 domain. In addition, they found that the intergenic spacer region of the ribosomal gene cluster could also be used for additional differentiation of species and even among strains within the same species. Together, their sequence datasets of the D1/D2 domain and to a lesser extent the ITS and IGS regions, revolutionized the approach to species identification and new species recognition in both the ascomycetous and basidiomycetous yeasts. For example, before these sequences were available, a

taxonomist would need a week to identify a handful of yeast strains using a plethora of physiological, morphological and mating tests that they are required to perform (Kathuria *et al.*, 2015)

Though ITS sequences have been widely used for fungal species identifications for about two decades, whether ITS was the most appropriate fungal DNA barcode across a broad spectrum of fungi was not critically evaluated until 2012. In this study, six DNA regions were evaluated as potential DNA barcodes for fungi by a multinational, multi-laboratory consortium. The six candidate DNA regions included three subunits from the nuclear ribosomal RNA gene cluster (ITS, nSSU, nLSU) and three protein-coding genes [the largest subunit of RNA polymerase II (*RPB1*), second largest subunit of RNA polymerase II (*RPB2*) and the mini chromosome maintenance protein MCM7]. ITS sequences have been used extensively for fungal diversity studies.

ITS-1 and ITS-2 as independent barcoding loci adding barcode(s) to specific groups of taxa is one way to expand the utility of DNA sequence based fungal identification; however, simplifying ITS to either the ITS-1 spacer or the ITS-2 spacer only has also attracted significant attention for barcoding based research. This is especially true for metabarcoding studies using next-generation sequencing platforms where the read length is typically much shorter than the entire ITS region (~600bp). For example, in metabarcoding studies using next-generation sequencing approaches (Hoggard *et al.*, 2018) used the ITS-1 spacer for studying herbarium fungal samples and human oral mycobiome samples respectively. The ITS-2 spacer for indoor dust and outdoor soil samples was used (Tedersoo *et al.*, 2014). A direct comparison of the utilities of the ITS-1 and ITS-2 spacers as barcodes suggested that these two spacers have comparable discriminating power and barcode gaps and similar to that of the entire ITS region for discriminating many sister species within the UNITE database (Blaalid *et al.*, 2013). Interestingly, the ITS-2 spacer has also been found to be very informative as a secondary barcode for plants and animals. Thus, using ITS-2 for fungal metabarcoding could potentially expand comparative studies among fungi, plants and animals.

2.9 Role of plant secondary metabolites

Phenolic compounds, the secondary plant metabolites, are important determinants in the sensory and nutritional quality of fruits. These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures range from a simple phenolic molecule to a complex high molecular mass polymer. Phenolic compounds have been shown to be involved in plant defence against biotic and abiotic stresses (Geibelet *al.*, 1994). It is well known that phenolic compounds are involved in the natural defensive reactions of apples against various diseases, especially apple scab [*Venturia inaequalis* (Cooke) G. Winter]. The phenolic composition of a plant tissue can determine the level of susceptibility/tolerance to fungal infections and pests. A large number of different phenolic compounds have been reported to occur in various apple tissues (Bazziet *al.*, 2003). To overcome pathogen, plants have developed wide array of defense strategies at structural, biochemical and molecular level. Plant basal immunity in plants is hastened as soon as pathogen is recognized by the host Pattern Recognition Receptors (PRRs) and is effective against large number of pathogens. It includes production of many phenolic compounds, salicylic acid, ethylene, jasmonic acid *etc.* Plant phenolics arising from phenylpropanoid pathway are the secondary metabolites of several classes of structurally diverse products. They play a significant role in pigmentation, growth and especially resistance to pathogens (Lattanzioet *al.*, 2006).

These phenols deposit inside the cell wall and acts as first line of defense thus prevents the fungal penetration and infection (Schwalb and Feucht, 1999). Phenols are having antimicrobial properties against fungi, bacteria and viruses. These polyphenolic compounds for instance chlorogenic acid are well recognized cinnamic acid derivative in fruit. The higher concentration of chlorogenic acid is mostly found in resistant apple cultivars than in susceptible ones (MacHardy, 1996). The content of chlorogenic acid is highest in early season of the crop and declines as the fruit matures from June to September (Ndubizu, 1976; Mayr *et al.*, 1993). Generally diseased leaves accumulate higher concentration of chlorogenic acid (mg/g) to that of healthy apple cultivar at different time period from June to September (Mayr *et al.*, 1995). Flavonoids present in plant tissues play crucial role in plant resistance such as

phloretin found in apple fruits, leaves and confer resistance against *V. inaequalis* (Treutter, 2005).

Large number of fungi including *Venturia inaequalis* hydrolyses phloridzin in to phloretin thus induces defense response. It has been reported that growth of *V. inaequalis* is remarkably reduced by phloroglucinol, phloretic acid and p hydroxybenzoic acid, which are formed by degradation of phloretin. Other compounds such as hydroxycinnamic acids also play a defensive role in apple against *V. inaequalis* among which flavonols (epicatechin, procyanidin B1, catechin) and dihydrochalcones play a key role (Slatnaret *al.*, 2012). However, some hydroxycinnamic acid (HCA) derivatives also exhibit fungitoxicity by inhibiting sporulation of fungi (Sammi and Masud, 2009). Scab infected leaves and fruits accumulate dihydrochalcones in leaves, fruit peel and in the neighboring tissues compared to that of healthy apple peel (Slatnaret *al.*, 2016). In addition, tannins release a large amount of hydrogen ions that is reported to combine with free oxygen radicals (Gu *et al.*, 2008). The high affinity of tannin polymers to proteins is reported to cause the astringency of ciders. The greater the degree of polymerization of tannins, the higher the number of phenolic hydroxyl groups and the stronger the inhibition of free radicals. Moreover, the browning of cut fruits is also reported due to enzymatic oxidation of chlorogenic acid and procyanidins by polyphenoloxidases (PPO) enzymes (Xu *et al.*, 2009) whereas hydroxycinnamic acid pool is linked with the cell wall stiffening of apple (Symoneaux *et al.*, 2014).

Overall phenol biosynthesis plays a pivotal role in the plant basal immunity against apple scab disease (Michaleket *al.*, 1999). Defense role of various phenols, flavonoids and isoflavonoids compounds in plant ranges from preformed or inducible physical and chemical barriers against infection. The higher concentration of chlorogenic acid is mostly found in resistant apple cultivars than in susceptible ones. Phloretin found in apple fruits / leaves and confer resistance against *V. inaequalis*. Other phenolicssuch as phenolic acids, also contribute to healing through increased lignification of damaged areas. Furthermore, they possess anti-microbial properties and their concentration may increase after infection (Michaleket *al.*, 1999). PAL provides the link between primary plant metabolism and the phenyl propanoid pathway (Figure # 5). PALcatalyzes the conversion of phenylalanine to *trans*-

cinnamic acid, which is a precursor of many different phenolic compounds. The key enzyme in the flavanoid pathway is chalcone synthase CHS and chalcone isomerase together lead to the formation of flavanones (Mayr *et al.*, 1997; Fishel, 2014). The flavan-3-ols/procyanidins are the most important contributors to the *in vitro* antioxidant activity of apple and procyanidin B2 and epicatechin are the most important individual antioxidants in apple. However, hydroxycinnamic acids showed a significant role in the flesh. Chlorogenic acid is also reported higher in the flesh than in the peel. Chlorogenic acid is the main phenolic acid present in the apple and has a great ability to scavenge the free radicals found that total flavanol content in the skin of scab resistant varieties was 3-fold higher than in susceptible varieties (Treutter and Feucht, 1990).

Diverse range of virulence factors can potentially target host resistance and it becomes obligatory to understand pathogen virulence strategy. We need to investigate the pathogen virulence factors and its role in disarming the plant defense. Besides nutritional requirements the main fate of pathogen is to suppress defense response of the host. The strengthened cell wall and cuticular membrane along with sub-cuticular pH of such leaves are speculated to play a role in governing such resistance. A breakdown of ontogenic resistance discovered by restored growth of the infectious agent is determined within the previous senescing leaves of apple. Besides race specific and ontogenic resistance, apple employs diverse defense responses which provide resistance, albeit variable and suppress growth and spread of the pathogen. Ultrastructural studies have discovered that though conidia might germinate on apple leaves undergoing defense responses, formation of primary hyphae is delayed and growth of subcuticular stroma is suppressed resulting in reduced conidiation (Picinelli *et al.*, 1995). Phloridzin (a dihydrochalcone glycoside) created by apple in response to *Venturia*, accumulated around the sites of infection, is postulated to get degraded into phloretin by pathogen and hinder its growth (Mayr *et al.*, 1997). Phenolics produced in response to *V. inaequalis* infections in apple are recognized to inhibit infectious agent growth and are attributed to be related to defense mechanisms of scab resistant cultivars (Picinelli and Dapena, 1995). Detailed studies are required to elucidate the mechanism of resistance offered by polyphenols.

Table # 1. District wise production of apple and area in hectares

District	Apple producing area in hectares	Apple production in metric tonnes 2018-19
Srinagar	1674	19231
Ganderbal	7111	91955
Budgam	13777	148276
Baramulla	25231	404089
Bandipora	4969	60358
Kupwara	19281	289215
Anantnag	18336	210619
Kulgam	18106	213653
Shopian	21669	272620
Pulwama	15574	138467
Jammu	00	00
Samba	00	00
Kathua	813	1523
Udhampur	1249	2442
Reasi	1108	1736
Doda	5954	8393
Kishtwar	2961	3891
Ramban	2870	7400
Rajouri	1281	3203
Poonch	2179	2008

Table # 2. Physiological races and their pathological characteristics

Race	Pathological characteristics on apple cultivars
Race 1	Non sporulating lesion on Dolgo, R 12740-7A (a Russian cultivar) and Geneva
Race 2	Sporulating lesions on Dolgo, Geneva and some progenies of R 12740-7A
Race 3	Sporulating lesions on Geneva and non-sporulating lesion on Dolgo, R 12740-7A
Race 4	Non sporulating lesion on Dolgo, Geneva and sporulating lesion on those progenies of R12740-7A on which race 2 isolates cannot sporulate
Race 5	Sporulating lesions on <i>Vm</i> <i>R</i> gene containing cultivars
Race 6	Sporulating lesions on <i>Vf</i> hybrids, but cannot infect <i>Malus floribunda</i> 821 containing <i>Vfh</i> <i>R</i> gene
Race 7	Can infect cultivars having <i>Vf</i> and <i>Vfh</i> <i>R</i> gene, but cannot infect Golden Delicious which contains <i>Vg</i> gene
Race 8	Can infect Golden Delicious, Royal gala and cultivars containing <i>Vh8</i> <i>R</i> gene

Table # 3. Application of fungicidal sprays from green tip stage to harvest stage

S.No.	Tree stage	Name of chemical	Quantity of chemical for 200 litres of
1.	Green tip	Captan	600 g
		Dodine	200 g
		Ziram	600 ml
2.	Pink bud	Mancozeb	600 g
		Propineb	600 g
		Difenoconazole	30 ml
3.	Petal Fall/ Pea Stage	Carbendazim	100 g
		Thiophanate-Methyl	100 g
		Hexaconazole Tebuconazole 50%+Trifloxystrobin 25%WG	100 ml 80 g
4.	Fruit development (walnut size)	Myclobutanil	80 g
		Mancozeb	600 g
		Propineb	600 g
		Dodine	150 g
		Metiram 55%+ Pyraclostrobin 5%WG	300 g
5.	Fruit development (20 days after 4 th spray)	Tebuconazole 50%+Trifloxystrobin 25% WG	80 g
		Propineb	600g
		Zineb	600g
6.	Pre-Harvest (20-25 days before harvest)	Captan	600g
		Ziram	600ml
		Metiram 55%+ Pyraclostrobin 5%WG	300 g
7.	After Post harvest	Copper Oxychloride	600 gm

Table # 4. New and the old names of apple resistance genes along with their differential host

S. No	R-Gene		Source / host (a)	Linkage group	Molecular marker (distance from the gene in cM)
	New Name	Old Name			
1	Rvi1	Vg	Golden Delicious Differential host: h1	LG-12 (Distal end)	MC105 (3.0) CH01D03 (0.5)
2	Rvi2	Vh2	<i>Malus pumila</i> R12740-7A (TSR34T15) Differential host: h2	LG-2 (Distal end)	OPL 19 433(1) Ch02b10 (8)
3	Rvi3	Vh3.1	Differential host: h3	-----	-----
4	Rvi4	Vh4/Vr1	<i>Malus pumila</i> R12740-7A (TSR33T239) Differential host: 4	LG-2 (Distal end)	S22 (4) CH02c02 (5)
5	Rvi5	Vm	<i>Malus micromalus</i> 245-38, <i>Malus atrosanguinea</i> 840 Differential host: h5	LG-17 (Distal end)	OPB12 (6)
6	Rvi6	Vf	“Priscilla” Differential host: h6	LG-1 (Distal end)	M18 (0.2) CH-Vf1 (0.0) AL07 (0.9)
7	Rvi7	Vfh	<i>Malus floribunda</i> 821 Differential host: h7	LG-8	
8	Rvi8	Vh8	<i>Malus sieversii</i> W193B Differential host: 8	LG-2 (Distalend)	OPL19 (1.3)
9	Rvi9	Vdg	J34; Differential host: h9	-----	-----
10	Rvi10	Va	Antonovka Type PI 172623 Differential host: h10	LG-1	B398480 (16)
11	Rvi11	Vbj	<i>Malus baccatajackii</i> Differential host: h11	LG-2 (Distal end)	CH05e03 (0.6) T6 (3.9)
12	Rvi12	Vb	Hansen’s <i>baccata</i> #2 Differential host: h12	LG-12 (Distal end)	Hi02d05 (7.8) Hi07f01(13.7)
13	Rvi13	Vd	Durello di Forli Differential host: h13	LG-10 (Proximal end)	OPAF07-880 (2.0) CH2b07 (9.0)
14	Rvi14	<i>Vdr1</i>	DülmenerRosenapfel Differential host: h14	LG-6 (Proximal end)	HB09TC (5)
15	Rvi15	Vr2	GMAL 2473 Differential host: h15	LG-2 (Proximal end)	CH02c02a (0.0)
16	-----	Vd3	1980-015-025	LG1	CH-Vf1 (1) 67005F17 (7)

a. The apple cultivar from where the gene has been isolated

Table # 5. Selected top-performing loci and primers as primary and secondary fungal DNA barcodes

Locus	Primer name	Oligo nucleotides (5'->3')	Reference
<i>ITS</i>	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	White <i>et al.</i> , 1990
<i>ITS-1</i>	ITS1 ITS2	TCCGTAGGTGAACCTGCGG GCTGCGTTCTTCATCGATGC	White <i>et al.</i> , 1990
<i>ITS-2</i>	ITS3 ITS4	GCATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	White <i>et al.</i> , 1990
<i>TEF1α</i>	EF1-1018F EF1-1620R	GAYTTCATCAAGAACATGAT GACGTTGAADCCRACRTTGTC	Stielow <i>et al.</i> , 2015
<i>60S L10</i>	60S-506F 60S-908R	GHGACAAGCGTTTCTCNGG CTTVAVYTGGAACCTTGATGGT	Stielow <i>et al.</i> , 2015
<i>PGK</i>	PGK-533F PGK-533R	GTYGAYTTCAAYGTYCC ACACCDGGDGGRCCTTCCA	Stielow <i>et al.</i> , 2015
<i>TOP1</i>	TOP1-501F TOP1-501R	ACTGCCAAGGTTTTCCGTACHTACAACGC CCAGTCCTCGTCAACWGACTTRATRGCCCA	Stielow <i>et al.</i> , 2015
<i>LNS2</i>	LNS2-468F LNS2-468R	GGCCATGTGCTGAACATGATCGGHCGWGAYTGGA CGGTTGCCRAAKCCRCATAGAAKGG	Stielow <i>et al.</i> , 2015
<i>TEF3</i>	EF3-3185F EF3-3538R	TCYGGWGGHTGGAAGATGAAG YTTGGTCTTGACACCNTC	Stielow <i>et al.</i> , 2015

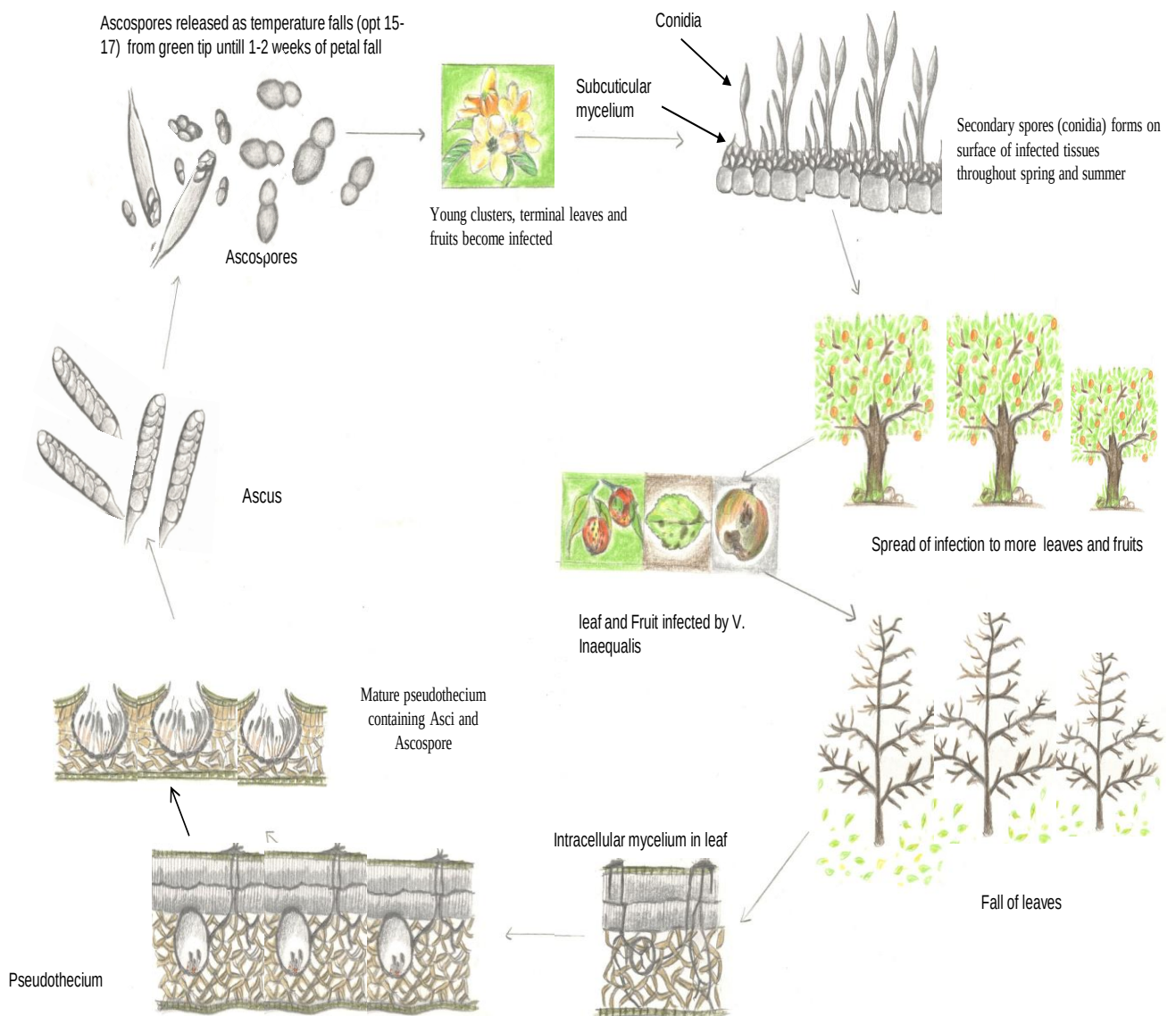


Figure # 3. Life cycle of *Venturia inaequalis* infection



A



B



C

Figure# 4A: At first the infection creates olive green spots with fuzzy margins on both sides of infected leaves

Figure # 4B: The fruit infection is same as it is in leaves. Scars that appear on fruit turn dark brown to black

Figure # 4C: As the fruit development progresses, lesions will have a “scabby” appearance and it will crack as fruit grows larger

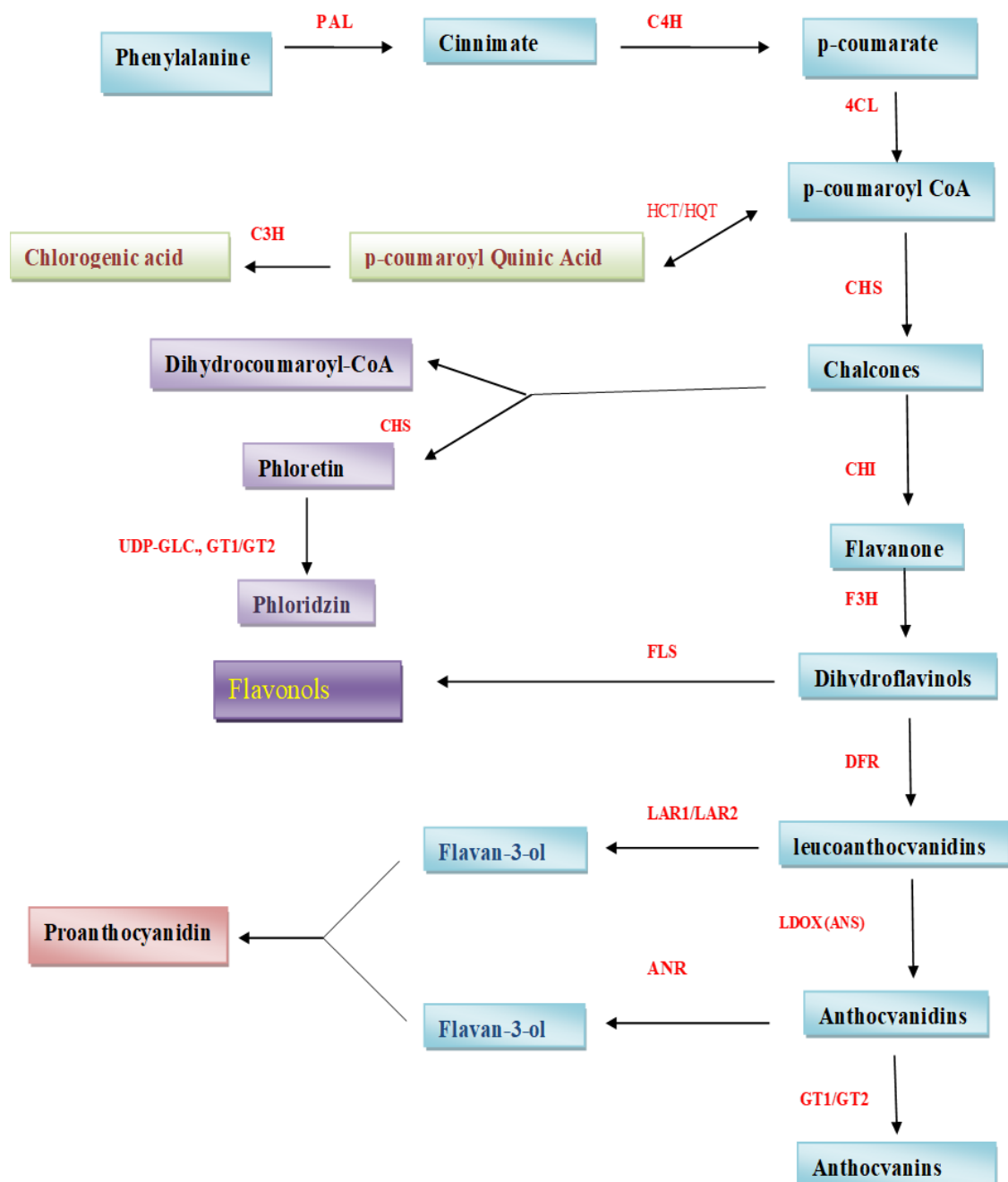


Figure # 5. Scheme of the apple polyphenolic pathway showing structural genes involved in flavonoid biosynthesis in *Malus domestica*. (PAL) Phenylalanine ammonia lyase, (C4H) Cinnamate-4-hydroxymate, (4CL) 4-coumarate:coenzyme A ligase, (CHS) Chalcone synthase, (CHI) Chalcone isomerase, (F3H) Flavanone-3-hydroxylase, (DFR) Dihydroflavonol 4-reductase, (ANS) Anthocyanidin synthase, (UFGT) UDP-glucose flavonoids-3-O-glucosyltransferase, (FLS) Flavonol synthase, (LAR1/2) Leucoanthocyanidin reductase, (ANR) Anthocyanidin reductase, (HQT/HCT) Quinatehydroxycinnamoyl/hydroxycinnamoyl CoA shikimate, (C3H) p-coumarate 3-hydroxylase (GT1/2) Glycosyltransferases



Material & Methods

MATERIAL AND METHODS

The present investigation entitled “DNA barcoding of different *Venturia inaequalis* strains and their effects on *PAL* biochemical pathway of *Malus domestica* Borkh.” was carried out at Division of Biochemistry, Faculty of Basic Sciences, SKUAST-Jammu, Transcriptomics Laboratory, Division of Plant Biotechnology, SKUAST- Kashmir and ICAR-Central Institute of Temperate Horticulture, Rangreth, Srinagar.

3.1 Glassware and equipments

All the glassware and equipments which were used during the present investigation are presented in Table # 6. Glassware was cleaned thoroughly with liquid detergent solution, washed under running tap water, rinsed with distilled water and dried in hot air oven.

3.2 Collection of isolates

Infected samples were wrapped in blotting paper, polybags and kept on ice packs. Samples were collected from 12 applegrowing districts within the two distinct regions of Jammu and Kashmir from May to September 2018 as shown in Table # 7. Disease samples included only apple leaves with scab symptoms mostly from different apple cultivars grown in J&K. Sampling was carried out from trees having at least two to three scab lesions per leaf (Figure # 6). In order to elucidate the molecular and phylogenetic diversity, collection was carried out from two regions by dividing the land into different climatic and topological zones (as J&K is separated by mountain ranges) to determine variability and strain specificity from the two regions (Figure # 7).

3.3 Isolation of fungus

The pathogens were identified under microscope and further were isolated from the infected sample and purified using the monoconidial method on 2% water agar plates (Figure # 8). Single spore isolates were obtained from the fresh lesions of leaves using 2% water agar amended with chloramphenicol(50µg/mL). Conidiospores

from infected leaf samples were dispensed in autoclaved water and diluted by 1/10, 1/100 and 1/1000 before they were poured onto 2% water agar plates and left to dry in a laminar flow cabinet. The plates were incubated at 18°C in a growth chamber for 24 hours with 12-hour day / night light period. After incubation, individual germinated spores were marked (spotted) under microscope were excised with a scalpel blade in a laminar flow cabinet and transferred to potato dextrose agar containing chloramphenicol (50 µg/mL) + PDA). These dishes were incubated at 18°C for more than a month, when the colony was large enough, samples were transferred to fresh PDA medium and PDA slants (Figure # 9).

3.4 Identification, purification and pure culture maintenance of fungal cultures

Venturia inaequalis is slow growing fungus, hence showed growth after 18-20 days, therefore, in case of any contamination it was either removed or was sub-cultured again to maintain pure culture. The cultures were identified by comparing with available literature and maintained for further studies. The spores were also verified using a compound microscope (Olympus) at multiple resolutions (4x to 40x).

3.5 DNA extraction

Cultured fungal isolates were incubated in potato dextrose broth on an incubatorshaker at 19°C for about 20-30 days in continuous dark (Figure # 10). Mycelia was harvested, blot dried, between the paper layers and immediately frozen in liquid nitrogen. After freeze-drying, DNA extraction was carried out using CTAB method (Cenis, 1992). The CTAB buffer contains 200mM Tris HCL pH 8.5, 250 mM NaCl, 25 mM EDTA and 2% CTAB. The mycelium was crushed in extraction buffer using pestle-mortar to make a slurry. The mixture was transferred to a sterilized 2ml microfuge tube and incubated at 65°C in a water bath for 1 hour and was vortexed after every 10 min interval.

After incubation, the lysate was centrifuged at 14000 rpm for 10 mins. Supernatant was transferred into 1.5 ml microfuge tube. After that, 30 µl of 3M sodium acetate, pH 5.2 was added and the tube was incubated at -20°C for 10 mins. The lysate was then centrifuged at 4,000 rpm at 4°C for 15 min and supernatant was transferred to a clean microfuge tube. 4µl of RNase (20mg/ml) was added to cell suspension and again incubated for 10 min at 65°C with intermittent mixing. DNA

was precipitated by the addition of 1 volume of isopropanol, incubated for 15mins, pelleted at 14000 rpm for 10mins and washed with ice cold 70% ethanol to remove salt contaminants, air dried for 30 mins and resuspended in 60 µl of Tris-EDTA buffer, pH 8.0. DNA was quantitatively and qualitatively checked using a nanodrop spectrophotometer (Thermo Scientific) and was further diluted to a working concentration of 30ng/µL and stored at -20°C for further use.

3.6 Primer Design

Primers were designed manually using Oligocalc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>), ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), online software tools, are shown in detail in Table # 8

3.7 PCR amplification

3.7.1 ITS ribosomal DNA amplification

All 30 isolates were amplified using polymerase chain reaction (PCR) in a thermal cycler (Takara, Japan) using 30ng of genomic DNA in a final volume of 25 µL per reaction. The primers used for amplification were ITS1F2 Primer (5'CCTGCGGAGGATCATTA 3'), ITS4R2 Primer (5'TCCTCCGCTTATTGAT3'). The reaction mixture contents are given in Table # 9.

3.7.2 PCR cyclic conditions

The amplification reaction was carried out in thermocycler (*Applied Biosystems*). The cycling conditions are shown in Table # 10

3.7.3 Gel electrophoresis

The PCR products were electrophoresed on a 1% agarose gel in 0.5X Tris-Borate-EDTA buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 110V. For detecting amplicon size, a 100 bp DNA molecular ladder was utilized (ABgene, UK) and electrophoresis complete after 1 hour. The fragments were observed under a Gel Doc XR system 170-8170 (Bio-Rad.).

3.8 SSR Amplification

For diversity and structure analysis of selected fungal samples, 28 published SSR primer pairs (Tenzeret *et al.*, 1999; Guerin *et al.*, 2004) were used (Table # 11)

3.8.1 DNA amplification constituents for SSR diversity analysis

The reaction mixture was performed in a 10 µL final volume contents for SSR analysis, as given in Table # 12

3.8.2 PCR cyclic conditions for SSR analysis

The amplification reaction was carried out in thermocycler (*Applied Biosystems*). The cycling conditions are shown in Table # 13

3.8.3 Gel Electrophoresis

The PCR products were electrophoresed on a 2.5% agarose gel in 0.5X Tris-Borate-EDTA buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 110V for 2.3 hours. For detecting amplicon size, a 100 bp DNA molecular ladder was utilized (ABgene, UK). The fragments were observed under a Gel Doc XR system 170-8170 (Bio-Rad.).

3.9 Sequencing, nucleotide alignment and phylogenetic analysis

Amplified PCR products were sequenced at Agri Genome Labs (Infopark Road, Kakkanad, Kerala, India). The same primers utilized for the PCR amplification were used for sequencing. PCR sequences were assembled using the DNA Baser V.4 program to produce complete contigs. These were further aligned using the CLUSTAL W method (Bio-Edit). A search of homologous sequences was performed by BLAST analysis at NCBI (<http://ncbi.nlm.nih.gov/BLAST>). The MEGA7 (Molecular Evolutionary Genomics Analysis Version 7) (Kumar *et al.*, 2016) was used for constructing dendrograms of the 30 isolates from the current study and reference sequences of strains from GenBank, using a bootstrap value of 1000 replications. For validation of results, a non-fungal pathogen *Pseudomonas syringa* was used.

3.10 Plant material and treatment

The apple cultivars chosen for the experiments were scab susceptible Golden Delicious. Therefore, for the artificial *V. inaequalis* inoculation, 2 year old plants grafted on rootstock M9 were grown in a greenhouse. The 2 year old plants grafted on rootstock M9 were shifted in growth chamber where temperature and humidity was maintained (temp 18°C and humidity 78-80%) (Figure # 11)

3.10.1 Preparation of inoculation load

Conidia of *V. inaequalis* pure culture was used for inoculation in a concentration of 5×10^5 conidia/ml measured with the help of Neuberg hemocytometer.

3.10.2 Inoculation conditions and sample collection

The inoculation was performed by spraying the whole plant with the spore solution. In order to provide continuous leaf wetness during the inoculation period of 3 days, the plants were irrigated. In addition, non-inoculated control plants were cultured, mock-sprayed with water under the same conditions. Pooled samples consisting of six to eight leaves from three to four plants were taken pre-inoculation and 1 day, 7 days and 24 days post inoculation, frozen in liquid nitrogen and stored at -80°C for gene expression analyses. Golden Delicious leaves from 25 dpi were additionally sorted according to their macroscopically visible infected leaf area strong infection. Unless otherwise noted, the leaves with highest percentage of infected leaf area were used for expression studies.

3.11 Biochemical analysis

3.11.1 Sample preparation

The apple leaves were taken at different stages *e.g.*, pre-inoculation and 1 day, 7 days and 24 days post-inoculation in a mortar and pestle and were immediately frozen in liquid nitrogen in order to avoid oxidation of the phenolic compounds (Guyot *et al.*, 2002). Homogenization of freeze-dried material without seeds was carried out by crushing in a mortar. An amount of 10 g of the powdered apple was transferred in Oakridge tubes and mixed with 10 mL of methanol or acetone in different concentrations, which was followed by incubation at 10°C for 10 mins. The

mixture was centrifuged ($8000 \times g$, 20 mins at 4°C) (Sigma 3–30 K, Munich, Germany), concentrated by evaporation under vacuum (4°C) in a rotary evaporator (IKA, HB-10, Germany) and freeze dried. The samples were reconstituted with 2 mL of 2.5% acetic acid and methanol (3:1, v/v) and filtered through a 0.22 micron (Nylon, Mumbai, India) syringe filter (Moxcare, Haryana, India) prior to analysis.

3.11.2 Estimation of total phenolic content

Total phenolic content in the methanolic extract was determined by Folin-Ciocalteu's colorimetric method (Singleton and Rossi, 1965), given in Table # 14. Methanolic extract (0.25 ml) diluted with distilled water (2.25 ml) was mixed with Folin-ciocalteu's reagent (0.25 ml) and allowed to stand for reaction (5 mins). This mixture was neutralized by 2.5 ml of 7% sodium carbonate and allowed to stand in the dark ($22 \pm 1^{\circ}\text{C}$, for 90 mins). The absorbance of the resultant blue color was measured at 765 nm using UV–Vis spectrophotometer (U-2001, Hitachi, Japan). Quantification of total phenols was done on the basis of standard curve of gallic acid prepared in 80% (v/v) methanol and results were expressed in mg gallic acid equivalent per gram of fresh weight (mg GAE/g fw).

3.11.3 Estimation of total flavonoids

Flavonoid content in the methanolic extract was determined by aluminum chloride calorimetric method (Chang *et al.*, 2002) as shown in Table # 15. In brief, 0.50 ml of methanolic extract of sample was diluted with 1.50 ml of distilled water and 0.50 ml of 10% (w/v) aluminum chloride added along with 0.10 ml of 1M potassium acetate and 2.80 ml of distilled water. This mixture was incubated at room temperature for 30 mins. The absorbance of resulting reaction mixture was measured at 415 nm UV-VIS spectrophotometer. Quantification of total flavonoids was done on the basis of standard curve of catechin prepared in 80% (v/v) methanol and results were expressed in mg catechin equivalent per gram of fresh weight (mg CE/g fw).

3.11.4 Estimation of total tannin content

Total tannin content was estimated following the method (Nwinuka *et al.*, 2005) with minor modifications as shown in Table # 16. Methanolic extract (5 ml) was added to Folin-Ciocalteu's reagent (0.5 ml) and saturated with 1 ml sodium

carbonate solution. The solution was diluted up to 10 ml with distilled water and allowed to stand ($22\pm 1^{\circ}\text{C}$, for 20 mins). The absorbance of resulting greenish blue color was measured at 700 nm and quantification of tannin was done on the basis of standard curve of tannic acid prepared in 80% (v/v) methanol. Results were expressed in mg tannic acid equivalent per gram fresh weight (mg TAE /g fw).

3.12 HPLC analysis

The HPLC analysis of samples was carried out in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps, a degasser coupled to a photo-diode-array detector and an injection valve with a 20 μL loop. An injection volume of 20 μL and a flow rate of 1.0 mL min^{-1} with 1 hour of run time was used for the separation process. The analysis was carried out in triplicate for each sample. Chromatographic separations were performed on C18 (250 μ - 4.6 mm) with a 5 μm column using a solvent system in gradient mode followed by isocratic run, as represented in Table # 17. The filtration of the mobile phase was done through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and was subjected to 40 mins ultrasonication. Instrument control, data acquisition and data processing were done by using Class WP software (version 6.1) from Shimadzu (Columbia, SC, USA). Quantitative determinations were made by taking into account the respective peak areas of standards at a particular retention time versus the concentration and are expressed in mg/g. as shown in Table # 17, Figure # 12

3.13 Expression analysis

3.13.1 RNA isolation

Prior to RNA isolation, microfuge tubes, tips, tip boxes, mortarpestle, spatula, forceps were DEPC treated (0.1%) overnight and then autoclaved followed by drying in hot air oven. Total RNA was extracted from frozen leaf samples of inoculated apple cultivars before inoculation and 24 hours, 7days and 24 days post inoculation using cetyltrimethylammonium bromide (CTAB) method (Asif *et al.*, 2006). Extraction buffer 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine (free acid) (HRS), 2% β -mercaptoethanol (added just before use) with several modifications to extract apple RNA fast and method is as follows.

- Tissue samples (100mg) were grinded in a pestle-mortar and in liquid nitrogen and transferred to a fresh microfuge tube.
- 1ml of pre warmed at 60 °C CTAB buffer, 200 µl of 2% β-mercaptoethanol was added to the homogenized sample and mixed thoroughly and incubated at 60°C for half an hour and vortexed after every 10mins.
- Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added and immediately vortexed for 2 mins
- Centrifugation of the samples at $12,000 \times g$ for 10 mins at 4° C was done.
- Following centrifugation, the mixture separated into lower red, an interphase and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was carefully transferred into a fresh 1.5ml tube without disturbing the interphase. Re-extraction was carried out with an equal volume of Chloroform: Isoamyl alcohol and centrifuged at $10,000g$ for 10 mins at 4°C
- The upper aqueous phase was carefully transferred into a fresh 1.5ml tube without disturbing the interphase.
- The aqueous phase was processed for RNA precipitation
- 1/3 volume of 8M LiCl was added to the aqueous phase, mixed by inversion and stored at 20°C at least for 2 hours and for better yield overnight.
- Centrifugation was done at $15,000 \times g$ for 20 mins
- The supernatant was removed from the tube leaving only the RNA pellet.
- The pellet was washed with 1 ml of 75% DEPC ethanol.
- The samples were vortexed briefly and then the tubes were centrifuged at $7500 \times g$ for 5 mins at 4° C. The wash was discarded.
- The RNA pellet was air dried for 5-10 mins.

- The RNA pellet was resuspended in RNase free water (DEPC water).

After dissolving, samples were run in 1% agarose gel to check quality of RNA and presence of any DNA contamination, if present was removed by method given below:

3.13.2 DNase treatment

- RNA sample was added with 5 µl of 10x Buffer.
- 1 µl of DNase-1 was added.
- Sample was incubated at 37° C for 30 mins
- Finally, either 1 µl of EDTA (stop solution) was added or mixture was incubated at 65° C for 10 mins

The concentration of RNA and its integrity was verified by an optical density measurement ratio of OD260/OD280 and also by nanodrop.

3.13.3 cDNA synthesis

First strand cDNA was synthesized from isolated RNA using oligo-dT and superscript II (Invitrogen) in a reaction volume of total 20 µl. cDNA synthesis confirmation was determined using GAPDH (Guerriero *et al.*, 2012) and cDNA (50 ng) was further capitalized for PCR amplification. cDNA synthesis was done using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit using oligodTprimers. The first strand cDNA was synthesized by using manufacturer's protocol as follows. The cDNA synthesized was stored at -20°C. The protocol for synthesis of first strand is given below

- The following reagents were added in an RNase free tube on ice as per the indicated order.

Template RNA	1-2 μ l
Oligo(dT) ₁₈ primer	1 μ l
5X Buffer	4 μ l
Nuclease free water	7-8 μ l

- The components were mixed gently and a brief spin was given.
- Incubation at 65°C for 5 mins was given.
- The tubes were chilled on ice and brief spin was given.
- The tubes were again placed on ice and the following components were added as per the indicated order.

RiboLock RNase Inhibitor (20U/ μ l)	1 μ l
10 mM dNTP Mix	2 μ l
RevertAid M-MuLV RT (200U/ μ l)	2 μ l

- The components were mixed gently and a brief spin was given.
- The components were incubated at 42°C for 40 mins.
- The reaction was terminated by heating at 70°C for 5 mins.

3.13.4 Validation of cDNA and primers using PCR:

To validate cDNA, polymerase chain reaction (PCR) was carried out using primer of GAPDH gene.

3.13.5 PCR reaction mixture:

PCR amplification for GAPDH gene was carried out in a reaction volume of 20 μ l in 0.2 ml PCR tubes. The reaction mixture contents are given in Table # 18

3.13.6 PCR cyclic conditions

The amplification reaction was carried out in thermocycler (*Applied Biosystems*). The cycling conditions are shown in Table # 19

3.13.7 Gel electrophoresis

The amplified products of GAPDH genes were electrophoresed on 1% agarose gel and compared with 100 bp DNA ladder respectively.

3.13.8 cDNA Quantification

cDNA quantification was done using Qubit[®] 2.0 Fluorometer (Invitrogen) and nanodrop.

3.13.9 Relative gene expression studies of PAL pathway genes

The relative gene expression was performed according to manufacturer's instructions (BIORAD, Californian, USA) as given in Table # 20. The PCR conditions were same as specified (95°C for 3 mins, 40 cycles each at 95°C for 30 secs and 50-62°C for 30 secs). The reference gene used was actin to calculate relative gene expression of candidate PAL pathway genes. Actin gene is constitutively expressed in most of plant tissues, thus is commonly used in internal real-time PCR endogenous base line control in plants (Suknoet *al.*, 2007). All the reactions were carried out in three technical triplicates and no-template controls (NTC). The quantification of target PAL pathway genes was determined relative to actin using the $\Delta\Delta C_t$ method and expressed as $2^{-\Delta\Delta C_t}$ for graphic representation (Livak and Schmittgen, 2001). Relative gene expressions were determined according to the Livak and Schmittgen, 2001:

Relative Gene Expression = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = [\Delta] C_{t \text{ sample}} - [\Delta] C_{t \text{ reference}}$

[Δ] $C_{t \text{ sample}}$ - C_t value for any sample normalized to the endogenous housekeeping gene
[Δ] $C_{t \text{ reference}}$ - C_t value for the reference sample normalized to the endogenous housekeeping gene

3.14 Statistical Analysis

Phylogenetic analysis, pairwise alignment, multiple sequence alignment analysis was carried out by Mega 7 version 8.0 software. SSR based analysis was carried out using Popgene and Genalex version 6 and scoring was done as base pair scoring and binary scoring in which bands were scored as '1' (for presence) and '0' (absence) (Yeh, 1999). Popgene and Genalex version 6.5 were used for calculating genetic diversity, homozygosity, heterozygosity, Nei's genetic distance and proportion of shared alleles, Gene flow F_{ST} , N_m , AMOVA respectively (Peakall and Smouse, 2006), Index of association r_d statistics was applied to examine associations of alleles among different loci (Brown *et al.*, 1980; Smith *et al.*, 1993), which is a comprehensive measure of multilocus linkage disequilibrium (Brown *et al.*, 1980). DARwin software version 5.0.158 was used in phylogenetic analysis (Perrier and Jacquemoud, 2006). Population descent and individual clustering (K) was done by means of Structure software version 2.3.4 (Pritchard *et al.*, 2000), ΔK method (Evanno *et al.*, 2005) was applied to best estimate K and was computed using Structure Harvester version 0.56.3 (Frantz *et al.*, 2006; Earl, 2012).

Biochemical data and expression data were subjected to analysis of variance, Pearson correlation coefficient and multivariate analysis using Graph pad Prism Version 8.0. 2. The treatment significance of pre and post inoculated leaves was assessed using the RM (ANOVA), Data are presented as mean values $\pm$ standard deviation (SD) of 3 replicates for each analysis and differences among different treatments were analyzed using Tukey's test at 5% level.

Table # 6. Glassware and equipments used during the study

Glassware & Small Equipments	Equipments
Conical flasks	Microscope
Petri plates	Autoclave
Beakers	Hot air oven
Funnels	BOD incubator
Measuring cylinder	Laminar air flow
Test Tubes	Centrifuge
Oakridge tubes	Mortar and pestle
Conical Flasks	Thermocycler
Glass slides	Gel electrophoresis
Glass rods	Gel doc system
Eppendorf tubes	Real Time PCR
Scalpel	PCR
Cork borer	Rotary evaporator
Razor	Incubator shaker
Spatula	Water bath
Filter paper	Haemocytometer
Plastic bag	Spectrophotometer
Muslin cloth	HPLC
Inoculation needle	Refrigerators (-20 & -80)
Inoculation loop	
Syringe	
Filters	

Table # 7. Samples collected from two regions of J&K with geographic location, latitude and longitude from various apple growing areas

S.No	Location	District	Latitude	Longitude	Region
1	Trehgam	Kupwara	34.52°N	74.184°E	Kashmir
2	Bankoot	Bandipora	34.42°N	74.650°E	
3	Charar-i-sharief	Budgam	33.86°N	74.766°E	
4	Syedpora	Shopian	33.72°N	74.83°E	
5	Beerva	Budgam	34.01°N	74.5953°E	
6	Chadoora	Budgam	33.80°N	75.100°E	
7	Batpora	Srinagar	34.93°N	74.464°E	
8	Hajin	Bandipora	34.09°N	74.79°E	
9	Yaripora	Kulgam	33.70 °N	75.0°E	
10	Handwara	Kupwara	34.40°N	74.28°E	
11	Kakapora	Pulwama	33.88°N	74.92°E	
12	Wakura	Ganderbal	34.05°N	74.47°E	
13	Gutlibagh	Ganderbal	34.09°N	74.09°E	
14	Naidkhai	Bandipora	34.09°N	74.79°E	
15	Gantmulla	Baramulla	34.08 °N	74.033°E	
16	Pinjura	Shopian	33.72°N	74.83°E	
17	Pattan	Baramulla	34.85°N	74.37°E	
18	Sogam	Budgam	34.02 °N	74.780°E	
19	Mattan	Anantnag	33.70 °N	75.285°E	
20	Uri	Baramula	34.08 °N	74.05°E	
21	Pranu Baderwah	Doda	32.58°N	75.538°E.	Jammu
22	Nalthi Baderwah	Doda	32.93°N	75.712°E	
23	Doda	Doda	33.13°N	75.57°E	
24	Kishtwar	Kishtwar	33.32°N	75.77°E	
25	Tund	Kishtwar	33.32°N	75.77°E	
26	Faisal Abad	Kishtwar	33.32°N	75.77°E	
27	Padder	Kishtwar	33.13°N	75.09°E	
28	Padder	Kishtwar	33.13°N	75.220°E	
29	Koti Bhaderwah	Doda	33.14 °N	75.547°E	
30	Bhaderwah	Doda	33.13°N	75.57°E	

Table# 8. Primers for amplification of ITS1-5.8S-ITS4 regions

S.NO	Primer name	Primer sequence	Size of amplicon	Tm °C
1	K-Lab- ITS1F2 Primer	5'CCTGCGGAGGATCATTA 3'	520bp	63.7
2	K-Lab-ITS4R2 Primer	5'TCCTCCGCTTATTGAT3'		53.6

Table# 9. PCR reaction mixture for amplification of ITS1- 5.8S-ITS4 region

Constituents	Volume
Autoclaved water	17.8 µl
PCR buffer (1x)	2.5 µl
MgCl ₂ (25 mM)	1µl
dNTPs (25 mM)	0.4 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq Polymerase (5 U/µl)	0.3 µl
DNA sample	1 µl
Total volume	25 µl

Table # 10. Thermal cycling conditions for amplification of ITS1-5.8S-ITS4 region.

Steps	Temperature (°C)	Time	Number of cycles
1. Initial denaturation	94 ⁰ C	5 min.	1
2. Denaturation	94 ⁰ C	30 sec.	35
3. Annealing	53 ⁰ C	45 sec.	
4. Extension	72 ⁰ C	1 min.	
5. Final extension	72 ⁰ C	15 min.	1

Table # 11. List of 28 SSR primers with forward (F) and reverse(R) sequence, allele size and annealing temperature

S. No	Locus	Primer sequence 5' – 3'	Allele size (bp)	Annealing temperature
1	Vitc1/2	F: CTTACCTCTCACTTGCTAAC R: GTTCTGACAAGACTGTGTTG	173-241	58 ⁰ C
2	Vitc1/82	F: ACTGTCTCTAGGCGAAAG R: ACTTGAAGCTCGCTAAG	227-247	58 ⁰ C
3	Vitc1/130	F: GATTGGTGACGCATGTGT R: GCTGGAGATTGCGTAGAC	132-15	58 ⁰ C
4	Vitc2/D	F: GCTCCTTCTGGGTAAGA R: CTCTACATCTCATCCCATC	184-278	58 ⁰ C
5	Viaggt8/1	F: GTGCGGAATATCCAAGTA R: CCAGACTCCTCTACTACAACC	188-196	58 ⁰ C
6	Vica9/152	F: GCACCTGCTCTGTCTATCTC R: AAGGTTCAAGCACTGGAG	167-191	58 ⁰ C
7	Vitca7/P)	F: GAATACTTCCAAGTGCACAG R: GTGACGCGGATATGGTAC	192-224	58 ⁰ C
8	Vitg11/70	F: GAAGAGGTTGGAGTGGTTG R: GAACCGAATCTGTACAGGAC	184-196	60 ⁰ C
9	Vica9/134	F: ATACAGGAGTGAACAGCAGG R: ATACTGCTCACTTGCGTGC	228-236	60 ⁰ C
10	Vigtg10/95	F: AGGTGTTGCTGTCTTGGAG R: CGATAGTGTCATTTCCAATCC	134-169	58 ⁰ C
11	Vicacg8/42	F: TGTCAGCCACGCTAGAAG R: CACCGGACGAATCATGC	196-232	60 ⁰ C
12	Vigt8/146	F: TGGAGAGAAGAGAAGAGTGG R: GGCAACATCCAATAATCG	128–134	60 ⁰ C
13	Vitc2/16	F: ACATTGACGAAGACGAGC	147–165	58 ⁰ C

		R: TACAATTGAGGCGTGTCC		
14	Viga3/Z	F: ACGCCTCCTTACTTCTTG R: CCCTCGTATTACGTTCTC	87–97	58°C
15	Viga7/116	F: GCCTGGTTGTGGATCTGTC R: ATCCTGCTACATCGACCTTC	159–173	60°C
16	Vitg9/99	F: CCGTGTCGAGACCTAATATC R: TGGCTCTTCGTAAGTCCTTC	155–167	58°C
17	Vica9/X	F: TCGCGCATCACTATCTACAC R: AGACAGGAATGTGGTGGAAG	225–239	58°C
18	Vica10/154	F: CCTCCTTCCTATTACTCTCG R: CTGAAGCGAACCTATGTCC	108–172	58°C
19	ViaacS10	F: ATTCCAAGCCTTACAACACCC R: TCCACTTCACCCATCGTTC	180–186	58°C
20	Vigt10/ε	F: GCAGTGCAGGAATAGTAAGG R: GCTGTGATACCAGAGAACGA	171–173	60°C
21	Vitg9/129	F: CTAATTCAACTCGCTGCGTC R: TTTCAGCCAGCTAACCTAGG	267–285	58°C
22	1tc1a	F: TCGAGATCCTCAAACCTTCCTT R: TTTTAACTGTGCGGCCTG	109–187	54°C
23	1tc1b	F: CGATTGGGGATATGAAGACTT R: TTAGTAATCAAATCGCACCCA	149–210	54°C
24	1tc1g	F: TCACTCAACAATACAGTTTCTTAG R: TTTCACGGTAGCGATAGGAG	111–185	57°C
25	1aac3b	F: AGCGCTAGGTCGTGAAATC R: TTTCTGAAGTGTGTGGGACAT	118–174	55°C
26	1aac4b	F: GGTGAGGAGGGAGACGAG R: CATCACGCCCCTATCAAAC	166–177	58°C
27	1aac4f	F: CTTGACAGACCACAGCGAC R: CTGACTGAGAGTGGCATCG	96–116	58°C
28	1aac4h	F: TCGTTCATCGTTCGTTTTTTCG R: AATAGTGCGTACCCATATATCCA	198–201	56°C

Table# 12. PCR reaction mixture for amplification of SSR region

Constituents	Volume
Autoclaved water	3.5 µl
PCR buffer (1x)	2 µl
MgCl ₂ (25 mM)	0.5 µl
dNTPs (25 mM)	0.5 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq Polymerase (5 U/µl)	0.5 µl
DNA sample	1 µl
Total volume	10 µl

Table# 13. PCR reaction cycle conditions for amplification of SSR region

Steps	Temperature (°C)	Time	Number of cycles
1. Initial denaturation	94 ⁰ C	5 min.	1
2. Denaturation	94 ⁰ C	30 sec.	35
3. Annealing	53 ⁰ C	45 sec.	
4. Extension	72 ⁰ C	1 min.	
5. Final extension	72 ⁰ C	15 min.	1

Table# 14. Composition mixture for Folin-ciocalteu's colorimetric method

Constituents	Volume
Extract	0.25 ml
Distilled water	2.5 ml
Folin–Ciocalteu's reagent	0.25 ml

7% sodium carbonate	2.5 ml
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Table# 15. Composition mixture for aluminum chloride colorimetric method

Constituents	Volume
Extract	0.50 ml
Distilled water	4.30 ml
10% (w/v) Aluminium chloride	0.50 ml
1M potassium acetate	0.10 ml

Table# 16. Composition mixture for tannin estimation by Nwinuka *et al.*, method

Constituents	Volume
Extract	5 ml
Folin-ciocalteu's reagent	0.5 ml
Sodium carbonate solution.	1 ml
Distilled water	3.5 ml

Table# 17. Mobile phase used for determining standard peak area at particular retention time for quantitative determination

Compound	Mobile Phase	Gradient	Lambda max
Catechin	Solvent A 2.5% Acetic acid Solvent B Aceto-nitrile	3–9% B (0–5 min.) 16–36.4% B (15–33 min.) 100% B (5 min.)	280 nm
Phloridzin			326 nm
Coumaric acid			254 nm
Apigenin			280 nm
Rutin			254 nm
Ferulic acid			322 nm

Table # 18. PCR reaction mixture for amplification of GAPDH genes

Constituents	Volume
Autoclaved water	13.6 µl
PCR buffer(1x)	2 µl
MgCl ₂ (25 mM)	1.2 µl
DNTPs(25 mM)	0.4 µl
Forward primer (10 µM)	1 µl
Reverse primer(10 µM)	1 µl
Taq Polymerase(5 U/ µl)	0.3 µl
cDNA sample	0.5 µl
Total volume	20 µl

Table # 19. Thermal cycling conditions for amplification of GAPDH genes

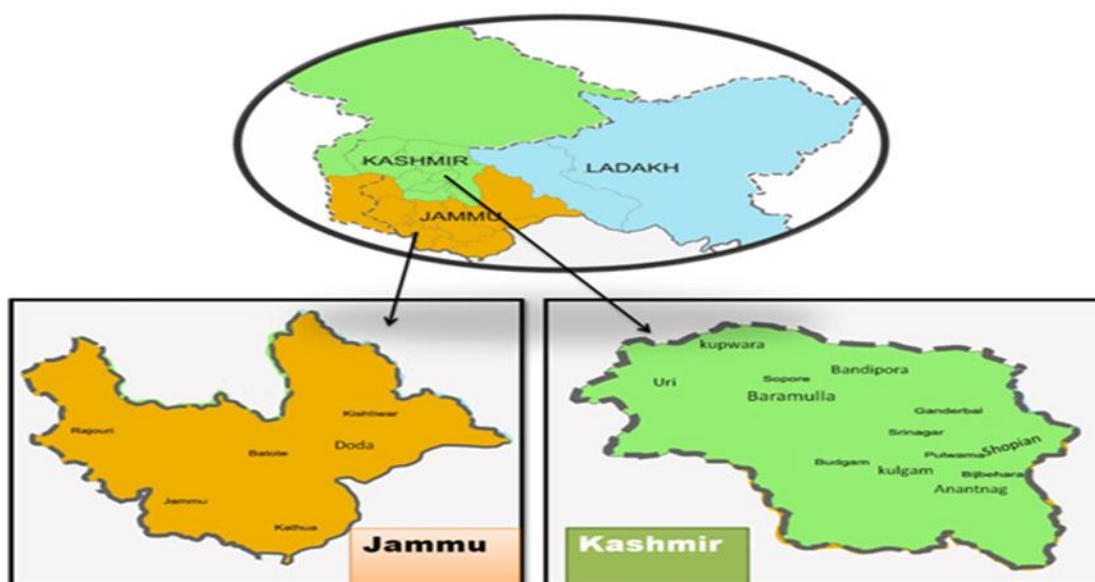
Steps	Temperature °C	Time	Number of cycles
1. Initial denaturation	94 ⁰ C	2 min.	1
2. Denaturation	94 ⁰ C	10 sec.	35
3. Annealing	61 ⁰ C	30 sec.	
4. Extension	72 ⁰ C	2 min.	
5. Final extension	72 ⁰ C	10 min.	1

Table # 20. Real time PCR reaction mixture for relative gene expression study

Constituents	Volume
SYBR green supermix (2x)	5 µl
Primer mix (10µM)	1 µl
cDNA template	1 µl
Nuclease free water	3 µl
Total volume	10 µl



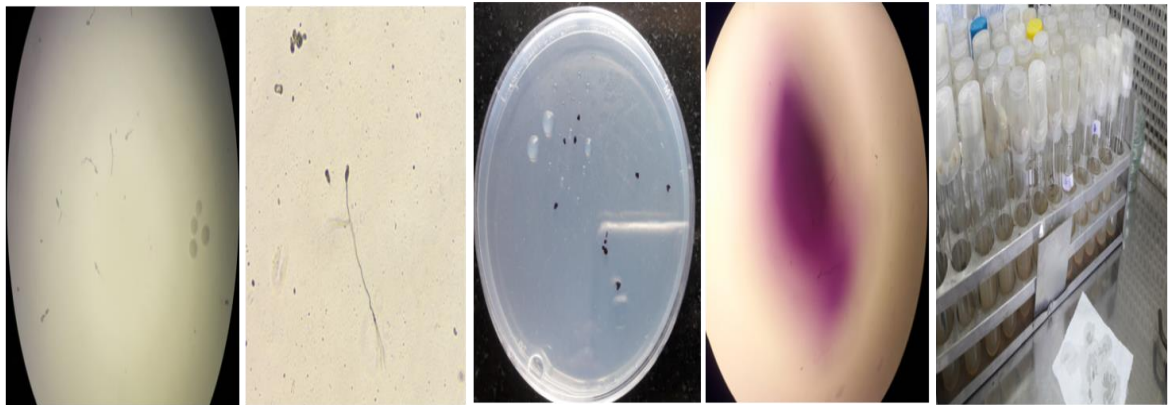
Figure# 6. Scab infected leaf samples having multiple spots of scab lesions



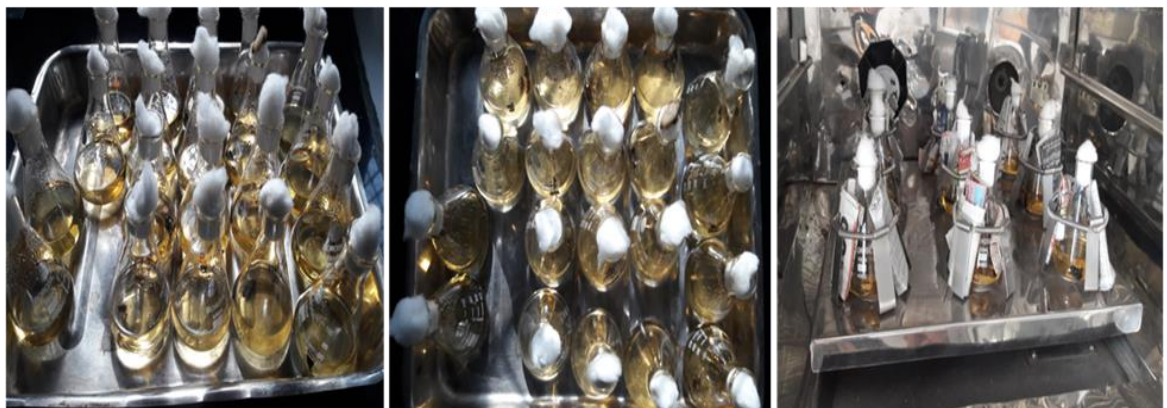
Figure# 7. Maps showing the hotspots of J&K from where sample collection was carried out



Figure #8. The pathogens (spore) identification under microscope



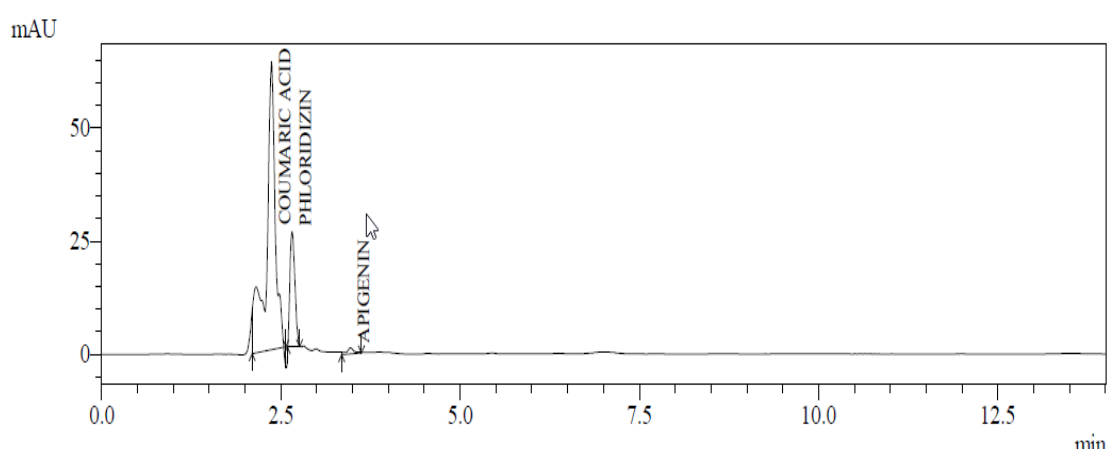
Figure# 9. Marking of individual germinated spores (spotted) under microscope



Figure# 10. Incubation for mycelium harvest in an incubator-shaker at 18-19°C in continuous dark



Figure# 11. Two year old plants grafted on rootstock M9 grown in a greenhouse and shifted to growth chamber where temperature and humidity was maintained



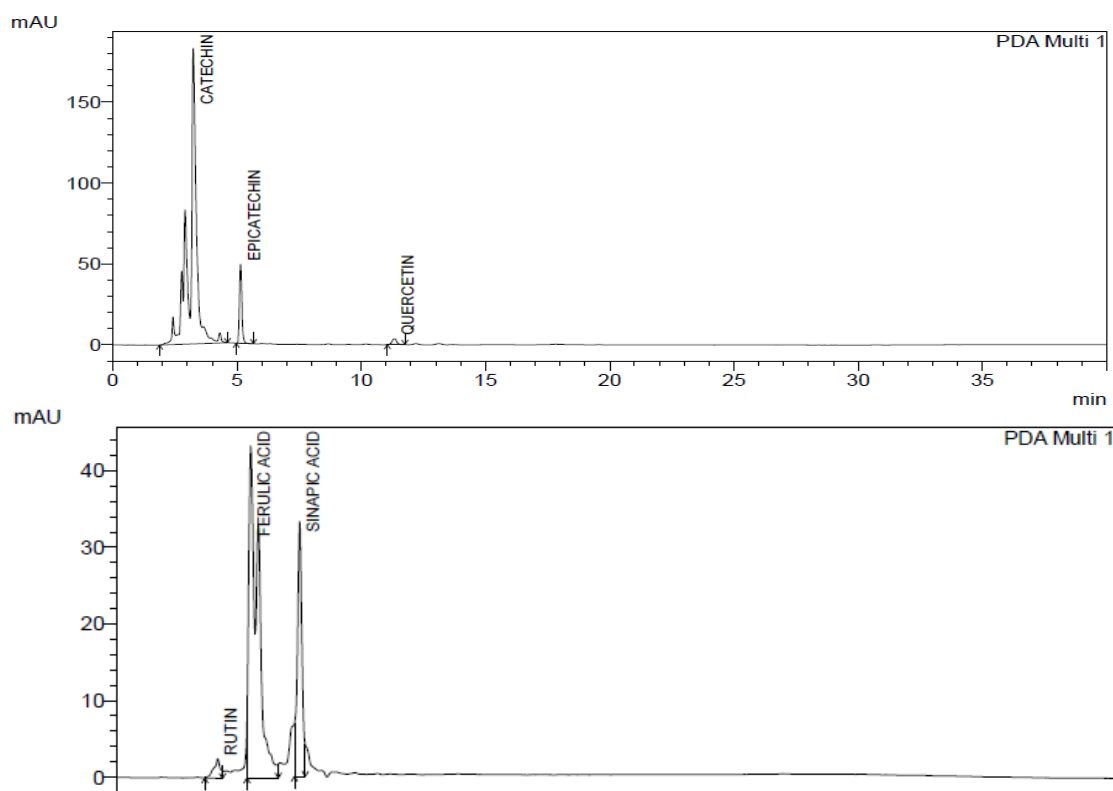


Figure # 12: HPLC chromatograms of standards



Results

RESULTS

For DNA barcoding of different *Venturia inaequalis* strains and determining the effect of *Venturia inaequalis* on PAL biochemical pathway of *Malus domestica* Borkh, 30 isolates were collected from different regions of Jammu and Kashmir. The single spore isolation was carried out, isolates were cultured and sub-cultured for pure culture maintenance and then pure cultures were verified based on morphological characteristics of spores. Later on DNA was extracted from the mycelia and PCR amplification, using internal transcribed spacers (ITS) primers was carried out. The product obtained was sent for sequencing and later sequences were analyzed using NCBI BLAST tool, published in NCBI GenBank. Further, evaluation of genetic diversity and population structure in *Venturia inaequalis*, associated with apple scab disease, was carried out using 28 SSR markers.

To study role of different PAL pathway genes and polyphenolics, the pure cultures were used for inoculation purpose and apple plants (Golden Delicious) were grown in green house, later shifted to growth chamber to maintain proper conditions. Plants were inoculated with *Venturia inaequalis* and others were mock sprayed with water that acted as control. Samples were collected at different time period of inoculation. Further, RNA extraction, cDNA synthesis and real time PCR was carried out. The methanolic extracts were prepared at different inoculation stages for HPLC and total phenolics, flavonoids and tannins were analyzed.

The investigation was carried out at Division of Biochemistry, Faculty of Basic Sciences, SKUAST-Jammu, Transcriptomics Laboratory, Division of Plant Biotechnology, SKUAST-Kashmir and ICAR-Central Institute of Temperate Horticulture, Rangreth, Srinagar. Standard procedures/protocols followed during the course of research work are given in Chapter 3 namely “*Material and Methods*”.

The results thus obtained are given in Tables (**Table#21-33**) along with the important observations summarized in Figures (**Figure#13-44**)

Table # 21. Isolate name with accession numbers provided by NCBI GenBank

<i>Venturia</i> isolate name	Accession number
Trehgam	MK478885.1
Bankoot	MK359025.1
Charar-i-sharief	MK504436.1
Syedpora	MK478887.1
Beerva	MK359026.1
Chadoora	MK504428.1
Batpora	MK504429.1
Hajin	MK359032.1
Yaripora	MK530205.1
Handwara	MK359027.1
Kakapora	MK359028.1
Wakura	MK367580.1
Gutlibagh	MK504434.1
Naidkhai	MK359029.1
Gantmulla	MK504430.1
Pinjura	MK504437.1
Pattan	MK504431.1
Sogam	MK504435.1
Mattan	MK359031.1
PranuBhaderwah	MK359030.1
NalthiBhaderwah	MK583539.1
Doda	MK504432.1
Kishtwar	MK504433.1
Tund	MK532037.1
Faisal Abad	MK532036.1
Uri	MK532035.1
Padder	MK532034.1
Padder	MK532033.1
KotiBhaderwah	MK532032.1

Bhaderwah	MK532031.1
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Table# 22. Account of the genetic investigation of *Venturia inaequalis* isolates with 28 SSR loci

Locus	Ne	I	Ob_He	Exp_Ho	Exp_He	Nei	Obs_Hom	Ave_Het	Pic	Fst
Vitc1/2	1.25	0.36	0.23	0.79	0.20	0.19	0.76	0.20	0.20	0.15
Vitc1/82	1.18	0.28	0.16	0.84	0.15	0.17	0.83	0.15	0.15	0.08
Vitc1/130	1.92	0.67	0.66	0.51	0.48	0.45	0.33	0.48	0.10	0.32
Vitc2/D	1.38	0.45	0.33	0.71	0.28	0.25	0.66	0.27	0.25	0.21
Viaggt8/1	1.99	0.69	0.83	0.49	0.50	0.47	0.16	0.49	0.16	0.64
Vica9/152	1.99	0.69	0.86	0.49	0.50	0.43	0.13	0.49	0.32	0.07
Vitcca7/P	2.00	0.69	1.00	0.49	0.50	0.48	0.00	0.50	0.52	0.00
Vitg11/70	1.86	0.65	0.73	0.52	0.47	0.42	0.26	0.46	0.20	0.12
Vica9/134	1.94	0.67	0.76	0.50	0.49	0.43	0.23	0.48	0.16	0.00
Vigtg10/95	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.07	0.24
Vicacg8/42	1.99	0.69	0.96	0.49	0.50	0.47	0.03	0.49	0.16	0.00
Vigt8/146	1.94	0.67	0.43	0.50	0.49	0.48	0.56	0.48	0.16	0.00
Vitc2/16	1.98	0.68	0.63	0.49	0.50	0.49	0.36	0.49	0.16	0.15
Viga3/Z	1.14	0.24	0.13	0.87	0.12	0.15	0.86	0.12	0.16	0.00
Viga7/116	1.96	0.68	0.86	0.50	0.49	0.49	0.13	0.49	0.16	0.00
Vitg9/99	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.16	0.00
Vica9/X	1.94	0.67	0.83	0.50	0.49	0.48	0.16	0.48	0.16	0.04
Vica10/154	2.00	0.69	1.00	0.49	0.54	0.50	0.00	0.50	0.16	0.00
ViaacS10	1.96	0.68	0.86	0.50	0.48	0.47	0.13	0.49	0.30	0.00
Vigt10/ε	1.99	0.69	0.96	0.49	0.50	0.45	0.03	0.49	0.10	0.00
Vitg9/129	1.64	0.57	0.53	0.60	0.39	0.40	0.46	0.39	0.25	0.00
1tc1a	1.00	0.00	0.00	1.00	0.00	0.36	1.00	0.00	0.15	0.00
1tc1b	2.00	0.69	1.00	0.49	0.50	0.48	0.00	0.50	0.12	0.00
1tc1g	1.30	0.39	0.26	0.76	0.23	0.21	0.73	0.23	0.10	0.00
1aac3b	1.76	0.62	0.63	0.55	0.44	0.41	0.36	0.43	0.13	0.00
1aac4b	1.86	0.65	0.73	0.52	0.47	0.43	0.26	0.46	0.15	0.00
1aac4f	1.06	0.14	0.06	0.93	0.06	0.21	0.93	0.06	0.10	0.18
1aac4h	1.96	0.68	0.73	0.50	0.49	0.49	0.26	0.49	0.15	0.00
Mean	1.74	0.57	0.64	0.59	0.40	0.36	0.35	0.39	0.18	0.19
Standard deviation	0.34	0.19	0.31	0.15	0.15	0.15	0.31	0.15	0.08	0.03

Legend: (Exp_Ho) Expected homozygosity, (Exp_He) heterozygosity, (Nei) Nei's, Na= Observed number of alleles, Ne = Effective number of alleles, I = Shannon's

Information index, (Ob_He) observed heterozygosity, (Ob_Ho) homozygosity, Fst=genetic differentiation

Table # 23. Evanno output table

k	lnpk	stdevlnpk	Ln'k	Delta K
1	0.667	0.960	NA	0.00
2	0.233	1.429	72.833	34.138
3	0.200	15.616	24.033	0.806

Table # 24. Genetic variation among and within populations using AMOVA

Source	df	SS	MS	Est. Var.	%
Among Pops	2	21.720	10.860	0.554	25%
Among Indiv	27	50.980	1.888	0.211	9%
Within Indiv	30	44.000	1.467	1.467	66%
Total	59	116.700		2.231	100%
F-Statistics	Value	P(rand >= data)			
Fst	0.248	0.001			
Fis	0.126	0.040			
Fit	0.343	0.001			

Df: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares, Est.Var: Estimated variance

Table # 25. Variation in total phenolic and flavonoid content in apple cultivar after different time periods of inoculation

Total Phenols	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	0.24±0.03	0.30±0.05	0.88±0.01	1.07±0.07
Inoculated	0.20±0.04	1.23±0.07	1.88±0.07	2.39±0.08
Total Flavonoids				
Non-Inoculated	0.11±0.08	0.17±0.09	0.24±0.08	1.03±0.05
Inoculated	0.15±0.08	1.74±0.06	2.70±0.09	4.89±0.07

Values are mean ± standard deviation; total phenolics are expressed as mg GAE/g fw and total flavonoids are expressed as mg CE/g fw BI (before inoculation), DPI (days post inoculation)

Table # 26. Variation in total tannin content in apple cultivar after different time periods of inoculation

Total Tannins	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	1.01±0.29	1.92±0.06	2.10±0.17	2.63±0.42
Inoculated	1.17±0.14	2.05±0.25	4.83±0.12	5.32±0.32

Values are mean ± standard deviation; total tannins are expressed as mg TAE/g fw, BI (before inoculation), DPI (days post inoculation)

Table # 27. Variation in ferulic acid and coumaric acid content in apple cultivar after different time periods of inoculation

Ferulic acid	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	0.26±0.06	0.31 ±0.08	1.91±0.08	2.60±0.04
Inoculated	0.50±0.08	8.41±0.05	14.55±0.21	23.94±0.06
Coumaric acid				
Non-inoculated	1.02±0.01	1.14±0.54	2.12±0.44	5.22 ±0.21
Inoculated	1.15±0.02	7.67±0.07	14.91±0.42	25.72±0.69

Values are mean ± standard deviation; ferulic acid and coumaric acid are expressed as µg/g (fw), BI (before inoculation), DPI (days post inoculation)

Table# 28.Variation in phloridzin content in apple cultivar, after different time periodsof inoculation

Phloridzin	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	1.83±0.26	2.00±0.53	2.87±0.84	3.18±0.61
Inoculated	2.06±0.07	11.88±0.74	17.44±0.92	39.24±0.49

Values are mean ± standard deviation; phloridzin is expressed as µg/g (fw), BI (before inoculation), DPI (days post inoculation).

Table # 29. Variation in catechin, apigenin and rutin content in apple cultivar, after different time periods of inoculation

Catechin	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean ± SD	Mean ± SD	Mean ± SD
Non-inoculated	0.83±0.09	1.45±0.06	3.35±0.06	6.74±0.81
Inoculated	0.93±0.08	14.96±0.09	17.26±1.06	33.64±0.46
Apigenin				
Non-inoculated	2.07±0.02	2.96±0.21	3.42±0.8	7.22±0.07
Inoculated	2.45±0.07	6.00±0.19	11.87±0.24	18.89±0.07
Rutin				
Non-inoculated	1.20±0.54	2.03±0.21	5.62±0.01	9.05±0.79
Inoculated	2.70±0.07	7.32±0.69	25.10±0.88	31.31±0.72

Values are mean ± standard deviation; catechin, apigenin and rutin are expressed as µg/g (fw), BI (before inoculation), DPI (days post inoculation)

Table# 30. RNA extracted at different stages after inoculation and quality check by spectrophotometer & concentration measured by nanodrop. Finally normalized for cDNA synthesis

Sample	A260/280	Conc.(ng/µL)	Normalization
Non-inoculated BI	2.0	219	100 ng/µL
Inoculated BI	2.0	221	
Non-inoculated 1DPI	2.0	439	
Inoculated 1 DPI	2.4	717	
Non-inoculated7-DPI	2.3	635	
Inoculated7-DPI	2.1	439	
Non-inoculated 24 DPI	2	214.6	
Inoculated 24 DPI	2.1	218	

Table # 31. Relative gene expression level of PAL gene after different stages of inoculation

PAL Expression	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	1.11±0.15	1.85±0.26	2.38±0.84	3.36±0.13
Inoculated	1.83±0.25	14.08±0.39	24.67±0.54	32.51 ±0.83

Values are mean ± standard deviation; whereas, BI (before inoculation), DPI (days post inoculation).

Table# 32. Relative gene expression level of CHS gene after different stages of inoculation

CHS Expression	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	0.12±0.00	0.32±0.02	1.03±0.52	3.42±0.31
Inoculated	0.19±0.00	4.11±0.55	10.1±0.42	16.67±0.82

Values are mean ± standard deviation; whereasBI (before inoculation), DPI (days post inoculation)

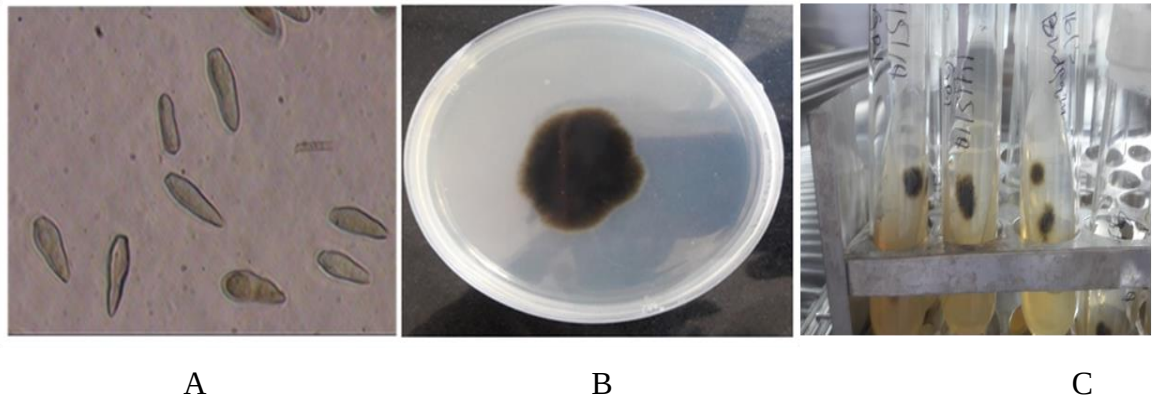
Table # 33. Relative gene expression level of F3H and C3H after different stages of inoculation

F3H Expression	BI	1DPI	7DPI	24DPI
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Non-inoculated	0.00±0.00	0.03±0.02	0.90±0.00	1.48±0.01
Inoculated	0.04±0.00	0.80±0.03	2.18±0.01	6.52±0.21
C3H				
Non-inoculated	0.00±0.00	0.14±0.13	0.73±0.83	1.12±0.27
Inoculated	0.01±0.00	0.32±0.54	2.04±0.42	10.64±0.82

Values are mean ± standard deviation; whereas BI (before inoculation), DPI (days post inoculation).

Table# 34. Fold change of PAL, CHS, C3H and F3H genes at different stages

Fold change	Stage	PAL	CHS	C3H	F3H
	Inoculated-BI	1.82	1.58	1.40	0.60
	Inoculated -1DPI	43.92	12.86	2.28	2.58
	Inoculated -7DPI	94.96	39.29	5.32	8.67
	Inoculated -24DPI	2750.45	292.51	85.12	11.80



Figure# 13. The spores verified under compound microscope (Olympus) at 10x to 40x.

- A. Conidia observed under 40 x resolutions**
- B. Pure culture of *Venturia inaequalis***
- C. Pure culture of *Venturia inaequalis***

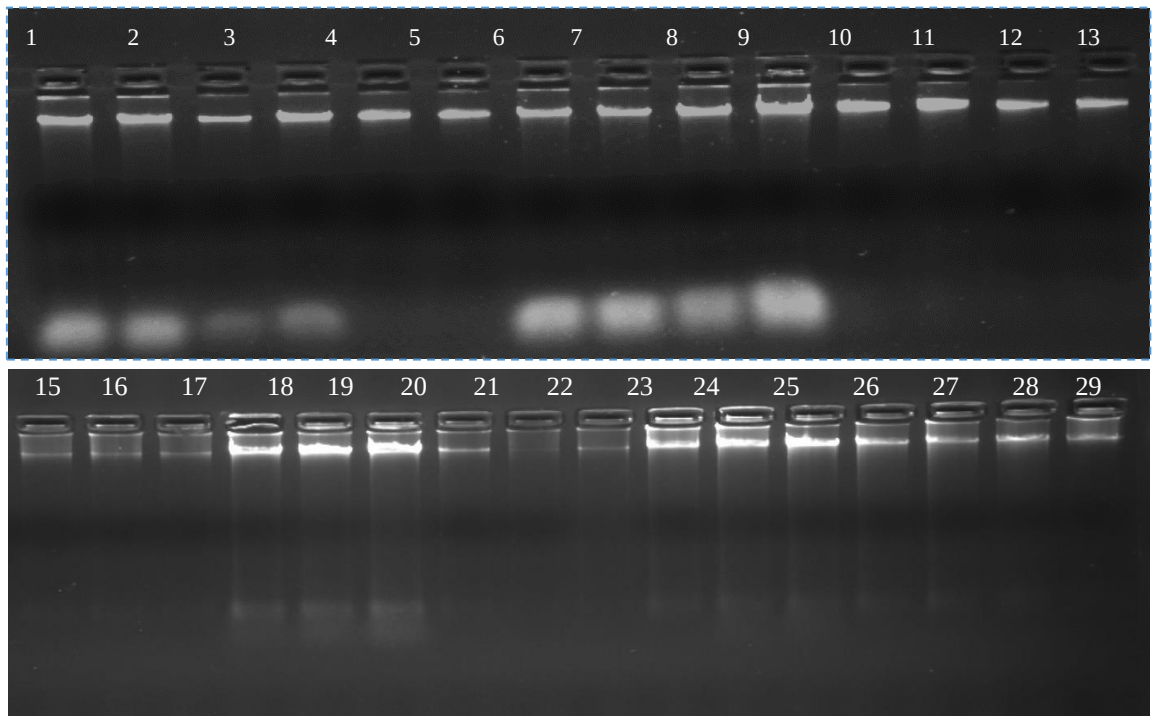
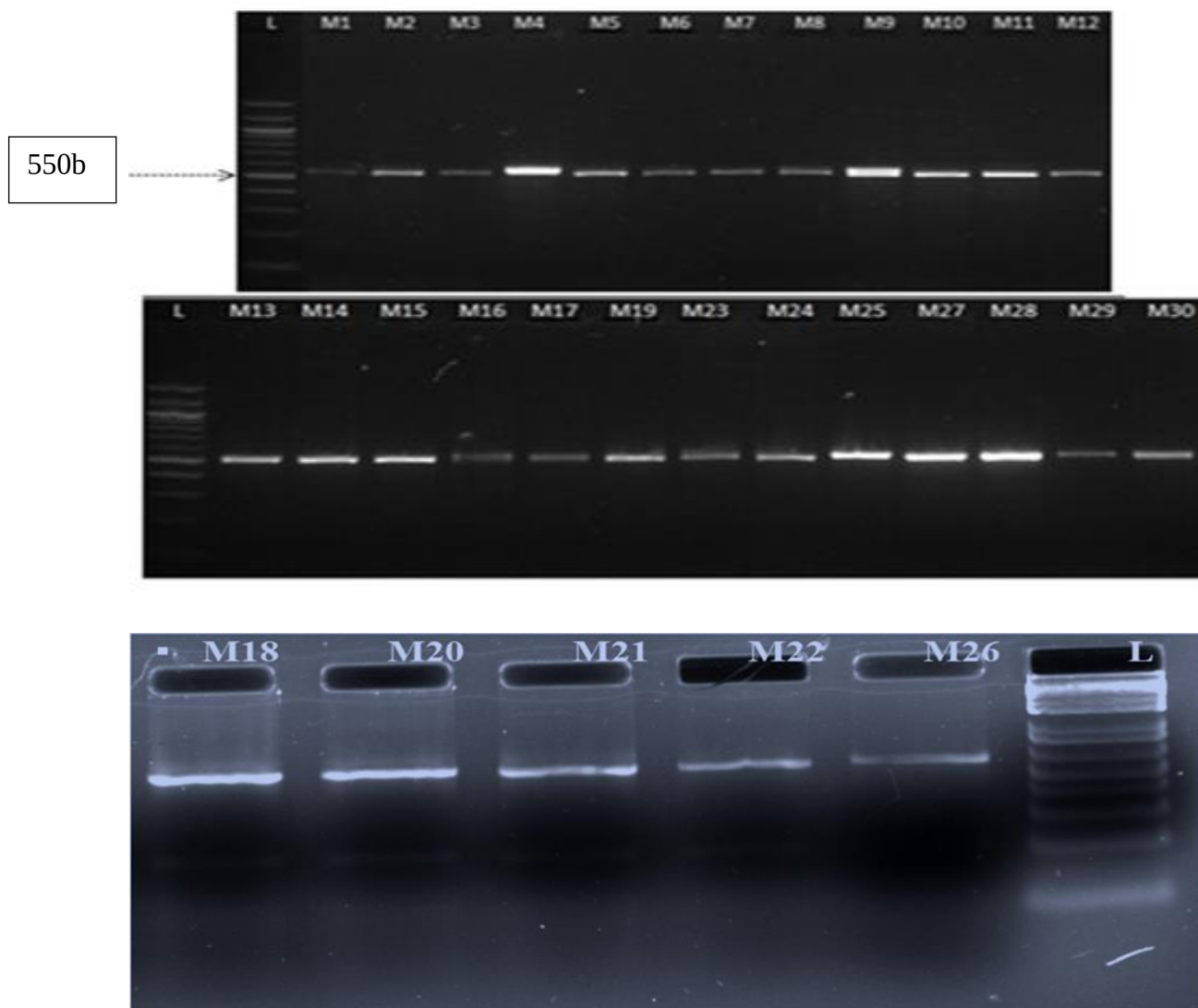


Figure # 14. Prominent, intact and brighter DNA bands were obtained



Figure# 15. PCR product (550bp) amplified from ITS region in *V. inaequalis*. 100bp ladder (L), significant amplification of expected band size at 550bp

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Venturia inaequalis strain CBS 594.70 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	993	993	100%	0.0	100.00%	KF156040.1
Venturia inaequalis strain CBS 309.31 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	993	993	100%	0.0	100.00%	EU035437.1
Venturia inaequalis strain CBS 535.76 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	988	988	100%	0.0	99.82%	EU035460.1

Figure# 16. Analyzing with BLASTn, all sequences showing 99%-100% sequence with GenBank submitted sequences for *Venturia inaequalis*

- ☐ [Venturia inaequalis isolate Tund Kishtwar J&K internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence](#)
483 bp linear DNA
Accession: MK532037.1 GI: 1575750857
[GenBank](#) [FASTA](#) [Graphics](#)
- ☐ [Venturia inaequalis isolate Faisal Abad Kishtwar internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence](#)
482 bp linear DNA
Accession: MK532036.1 GI: 1575750856
[GenBank](#) [FASTA](#) [Graphics](#)
- ☐ [Venturia inaequalis isolate Uri Baramulla internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence](#)
481 bp linear DNA

Venturia inaequalis isolate SMS13 internal transcribed spacer 1, partial sequence 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK367580.1

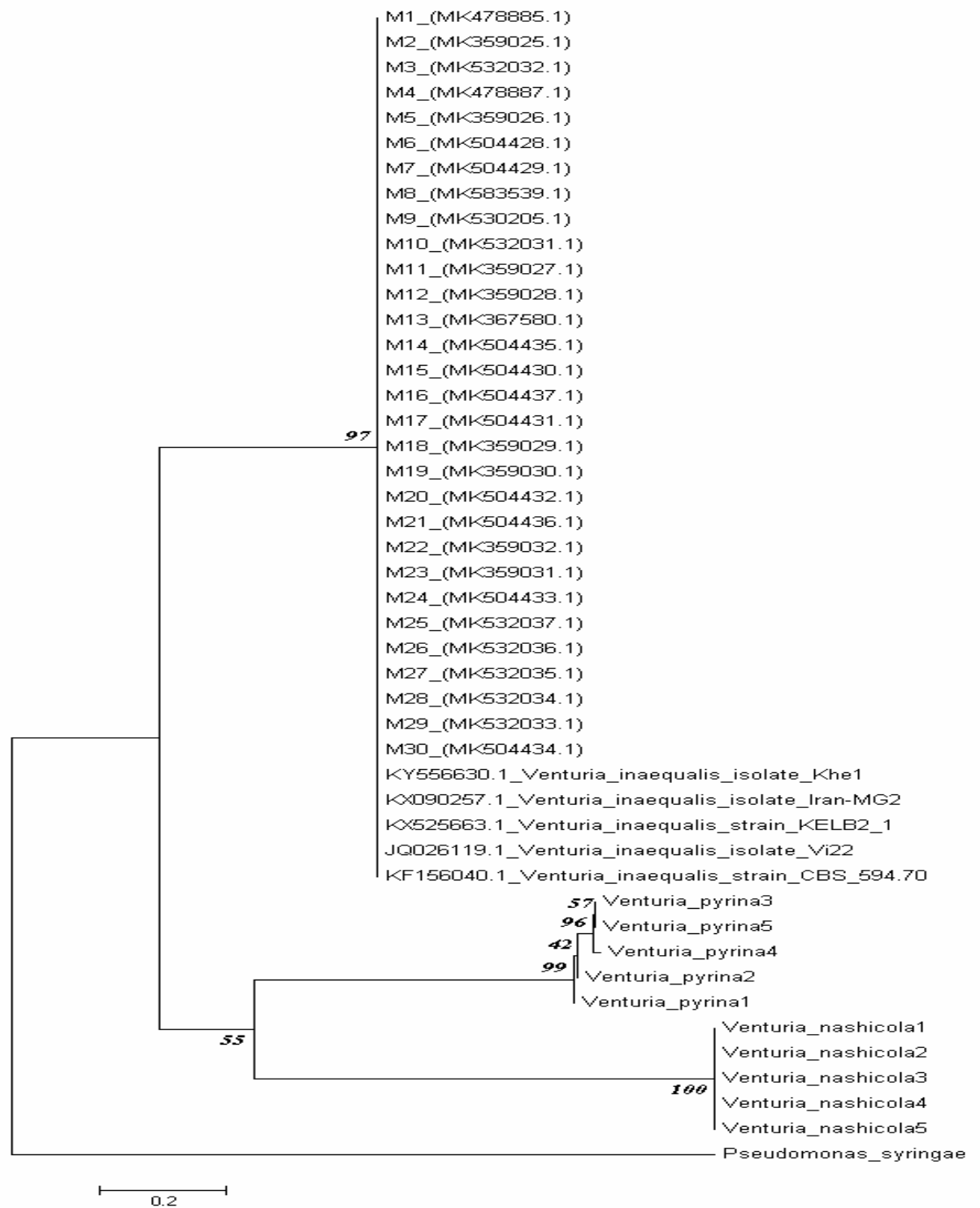
[FASTA](#) [Graphics](#)

Go to: 

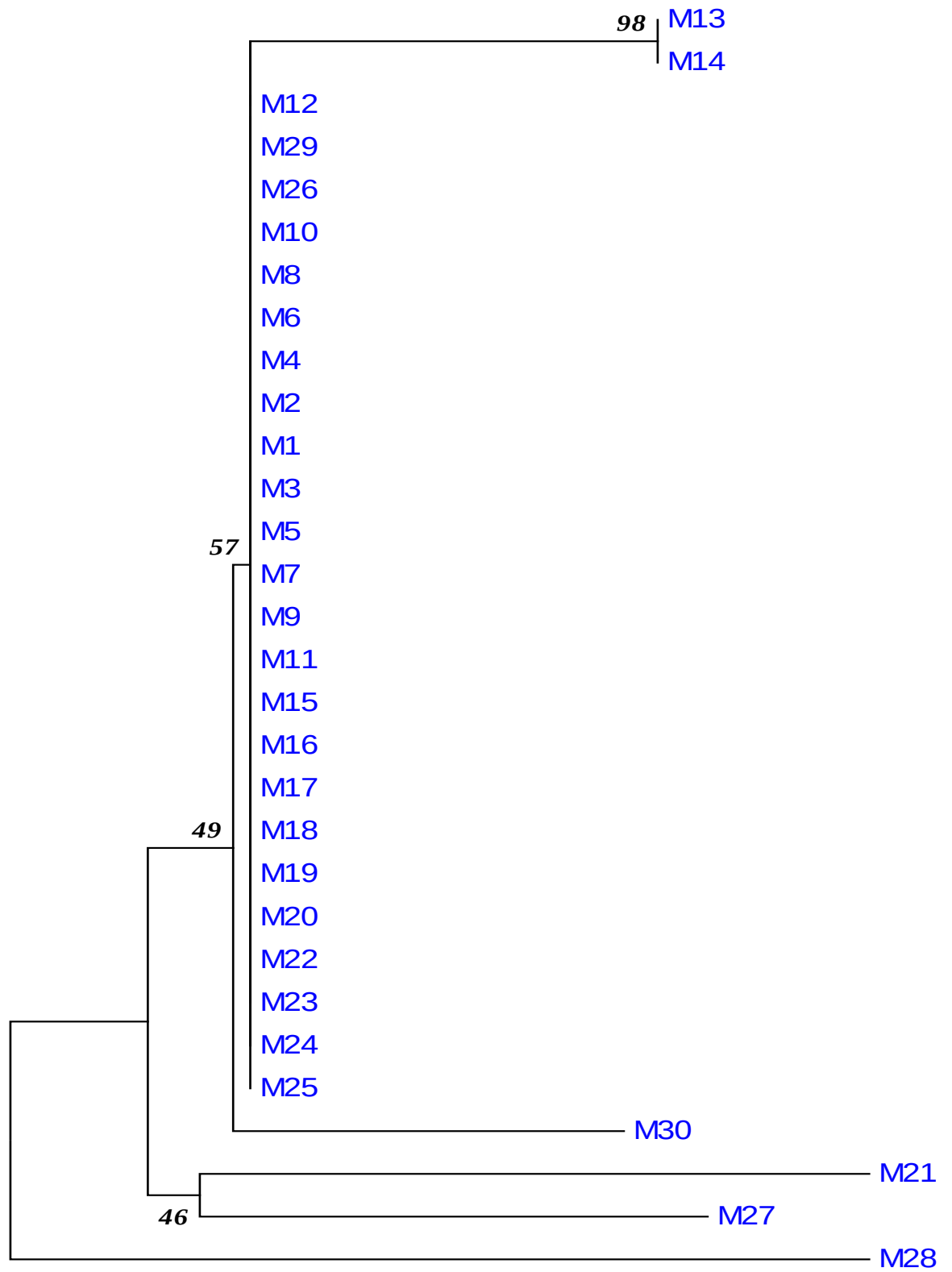
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LOCUS      MK367580          410 bp    DNA     linear   PLN 11-JAN-2019
DEFINITION Venturia inaequalis isolate SMS13 internal transcribed spacer 1,
partial sequence; 5.8S ribosomal RNA gene, complete sequence; and
internal transcribed spacer 2, partial sequence.
ACCESSION  MK367580
VERSION    MK367580.1
KEYWORDS   .
SOURCE     Venturia inaequalis
  ORGANISM Venturia inaequalis
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetes incertae sedis; Venturiales;
            Venturiaceae; Venturia.
REFERENCE  1. (bases 1 to 410)
  AUTHORS  Mansoor,S.S., Sharma,V., Ahmad,N., Moni,G., Guleria,S. and
  Masoodi,K.Z.
  TITLE    Barcode of Venturia inaequalis
  JOURNAL  Unpublished
REFERENCE  2. (bases 1 to 410)
  AUTHORS  Mansoor,S.S., Sharma,V., Ahmad,N., Moni,G., Guleria,S. and
  Masoodi,K.Z.
  TITLE    Direct Submission
  JOURNAL  Submitted (05-JAN-2019) Plant Biotechnology, SKUAST-Kashmir,
  Shalimar, Srinagar, J&K 190025, India
COMMENT    ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES   Location/Qualifiers
    source          1..410
  
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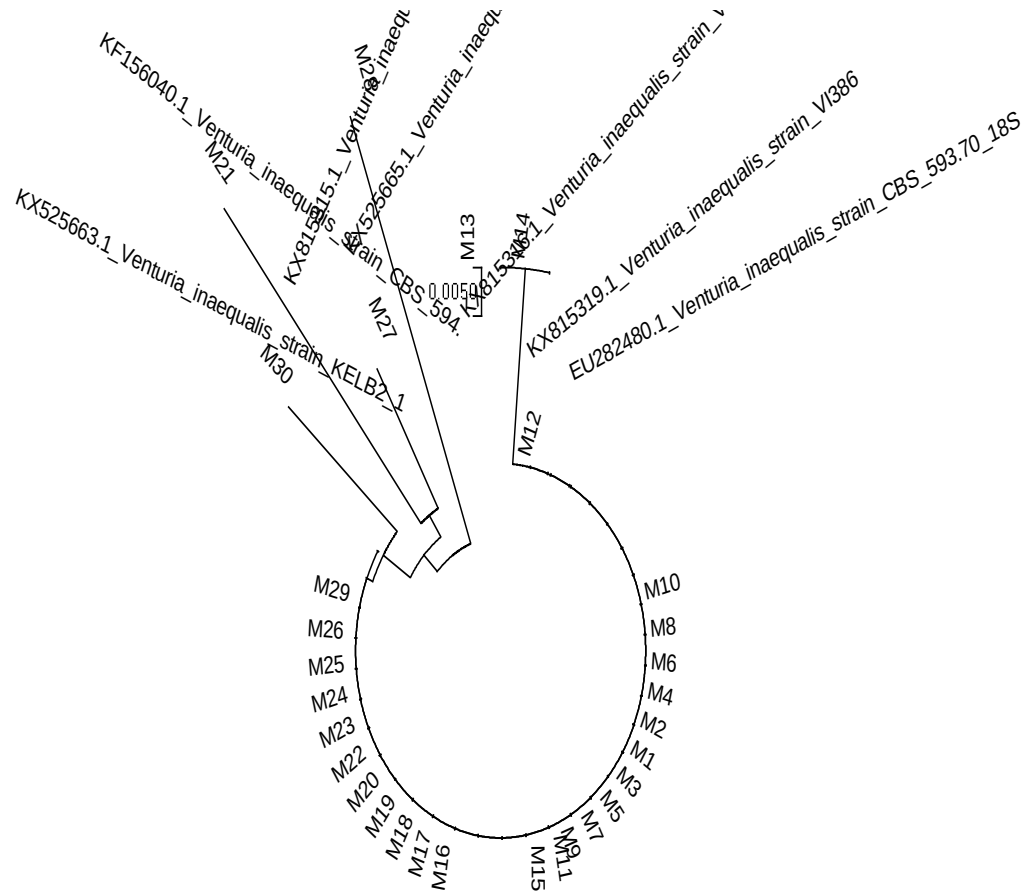
Figure# 17. Representative sequence flat files of the NCBI GenBank published sequences



Figure# 18. Phylogenetic relationship of *Venturia inaequalis* using the internal transcribed spacer (ITS) region gene nucleotide sequence alignment with bootstrap values at nodes



Figure# 19. Phylogenetic analysis (Local clustering)



Figure# 20. Phylogenetic analysis (Local clustering)

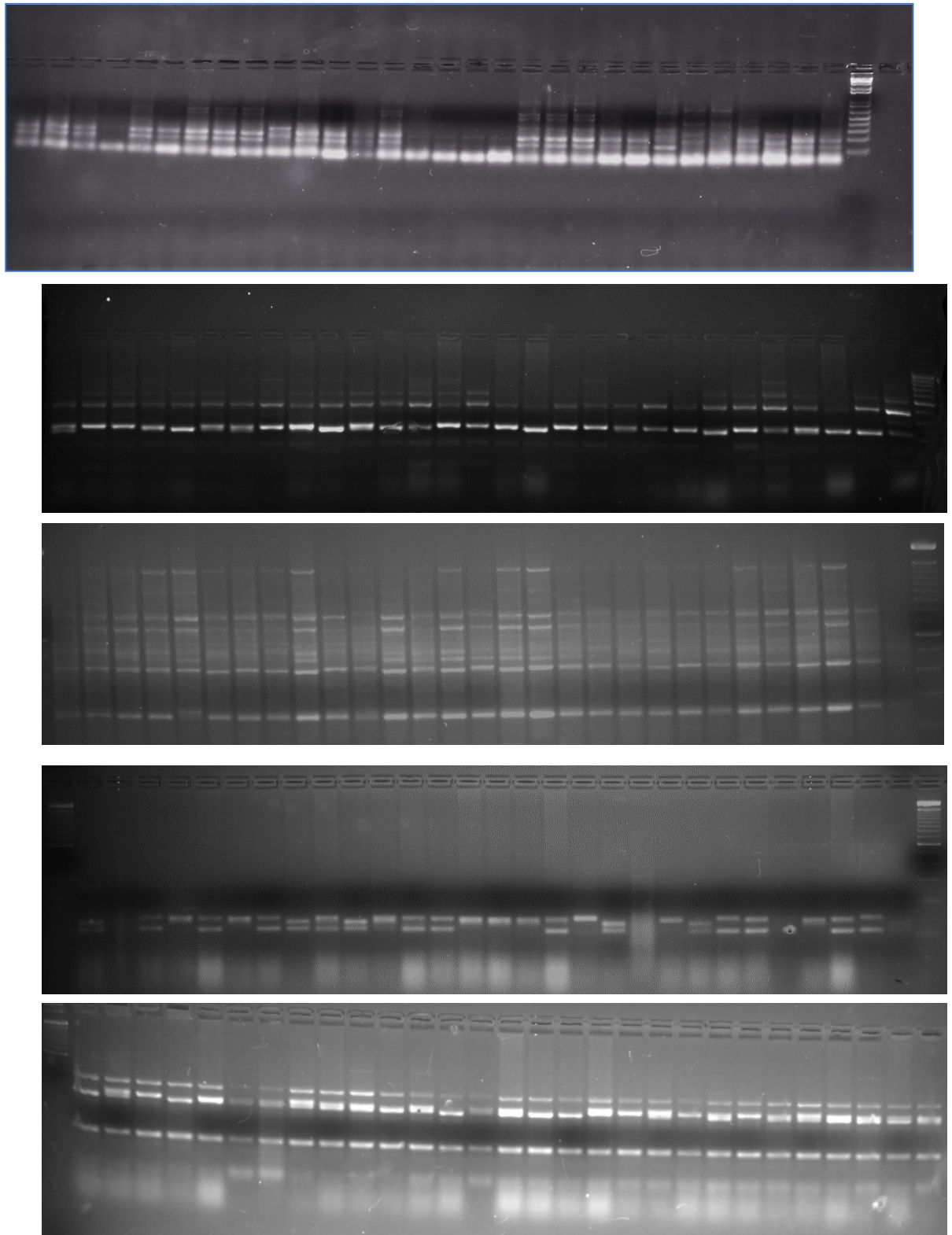
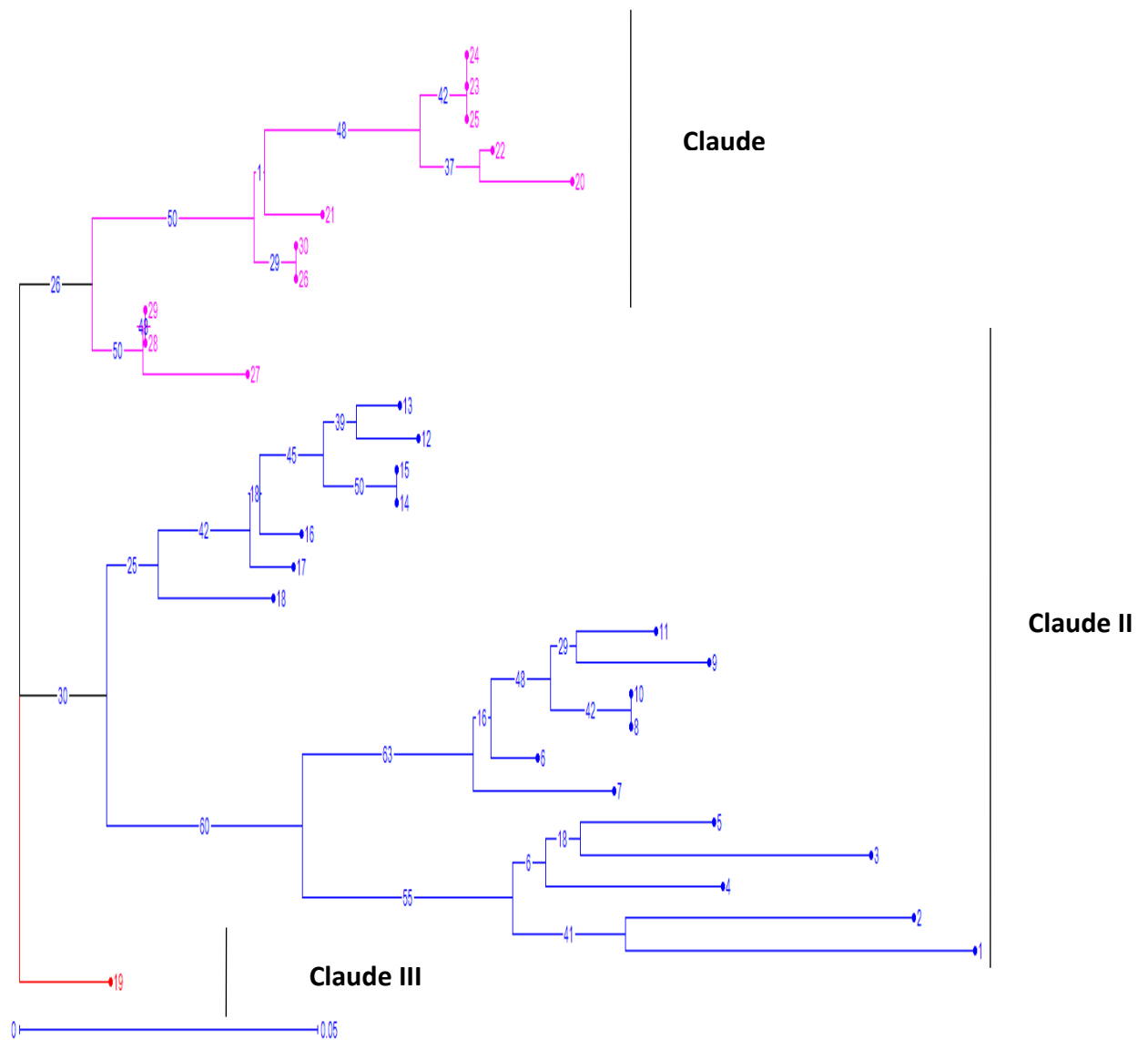
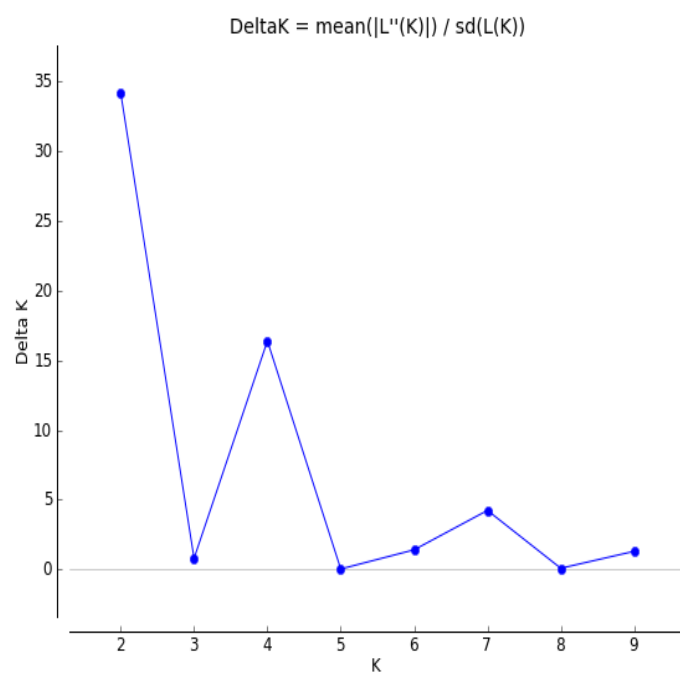


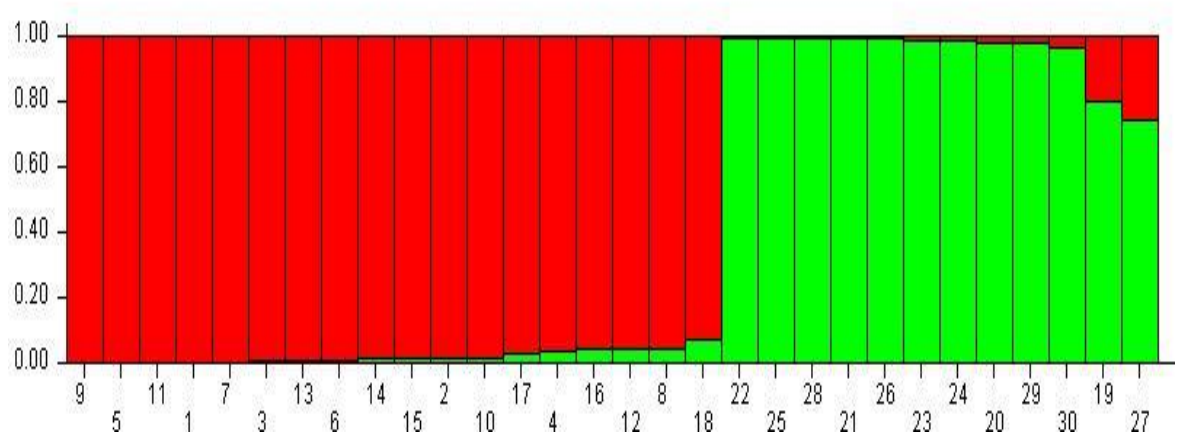
Figure # 21. SSR representative gel pictures



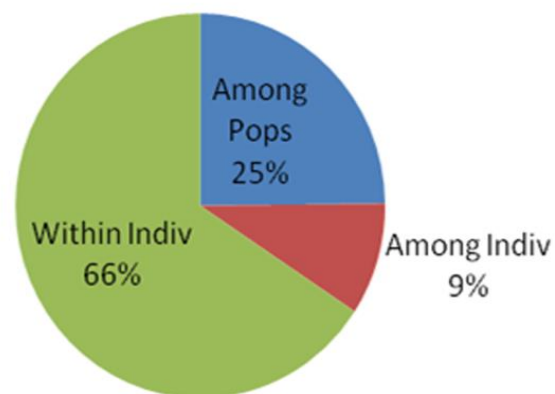
Figure# 22. The cluster analysis (dendrogram) of 30 isolates revealing diversity within *Venturia inaequalis* populations



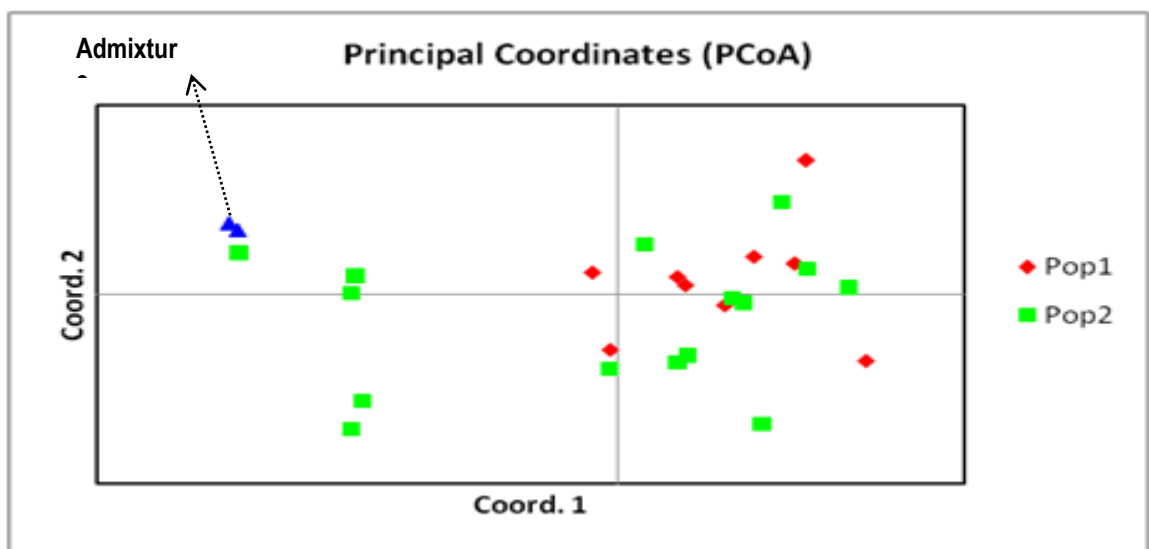
Figure# 23. ΔK peak value of 34.6 among the assumed K



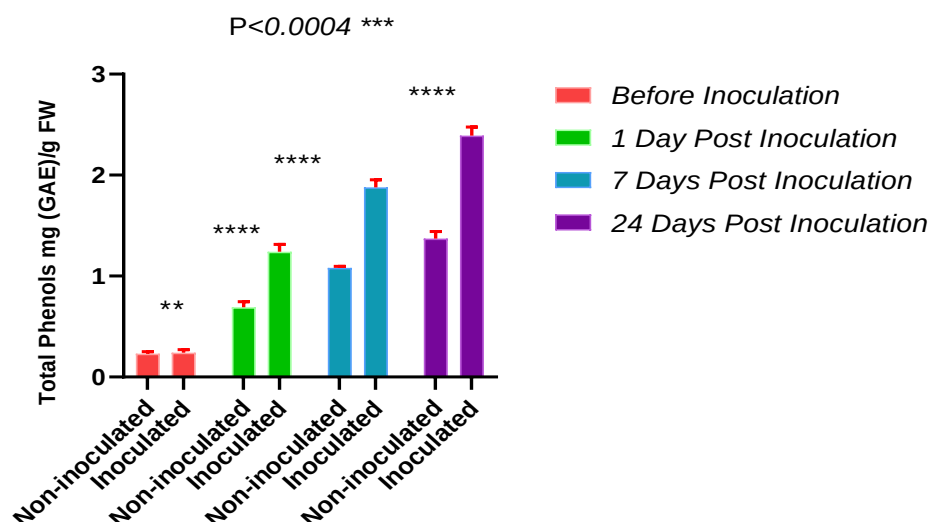
Figure# 24: Population distribution using STRUCTURE analysis software, the isolates were grouped into two major populations with a small admixture (19 and 27only)



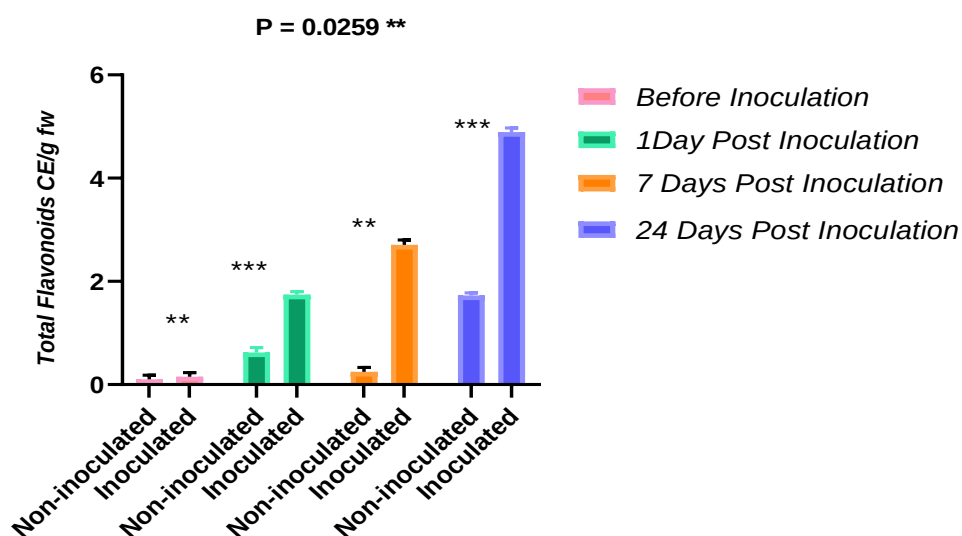
Figure# 25. Percentages of molecular variance



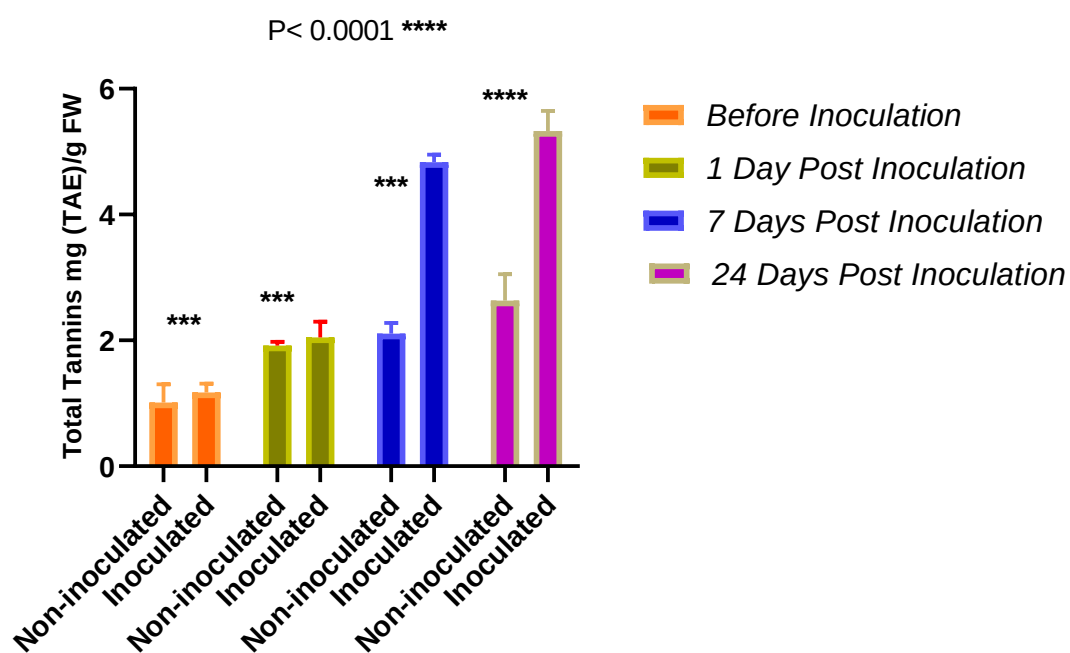
Figure# 26. Principal coordinate study of two *Venturia inaequalis* populations and an admixture population from diverse apple producing regions in the Jammu and Kashmir



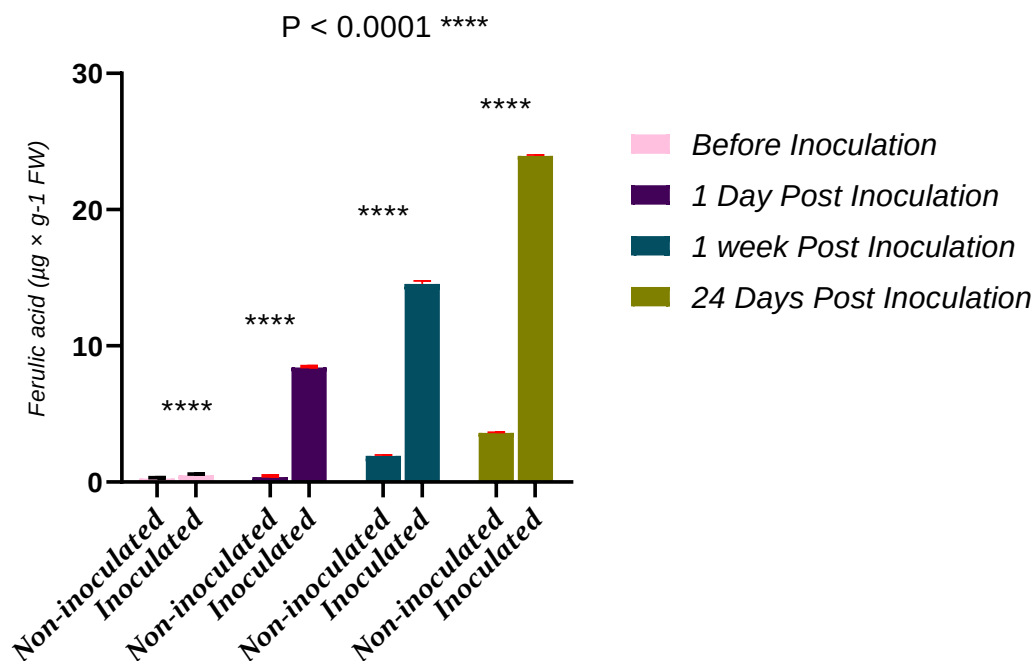
Figure# 27. Total phenolic content in apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; total phenolics are expressed as mg GAE/g fw



Figure# 28. Total flavonoid content in apple cultivars after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; total flavonoid are expressed as mg CE/g fw



Figure# 29. Total tannin content in apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; total tannins are expressed as mg TAE/g fw



Figure# 30. Ferulic acid content apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; ferulic acid is expressed as $\mu\text{g/g}$ (fw)

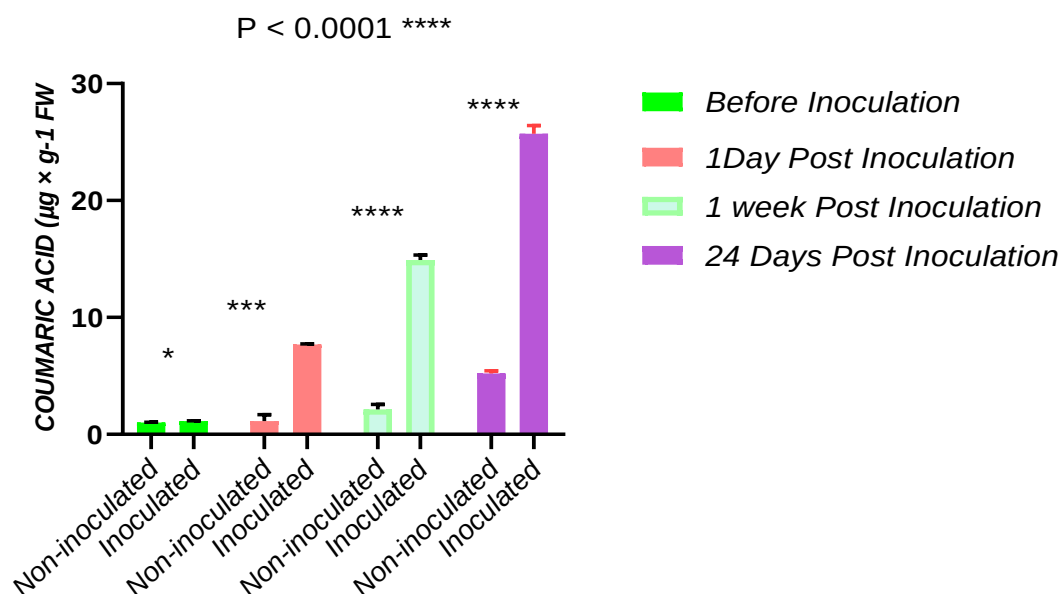
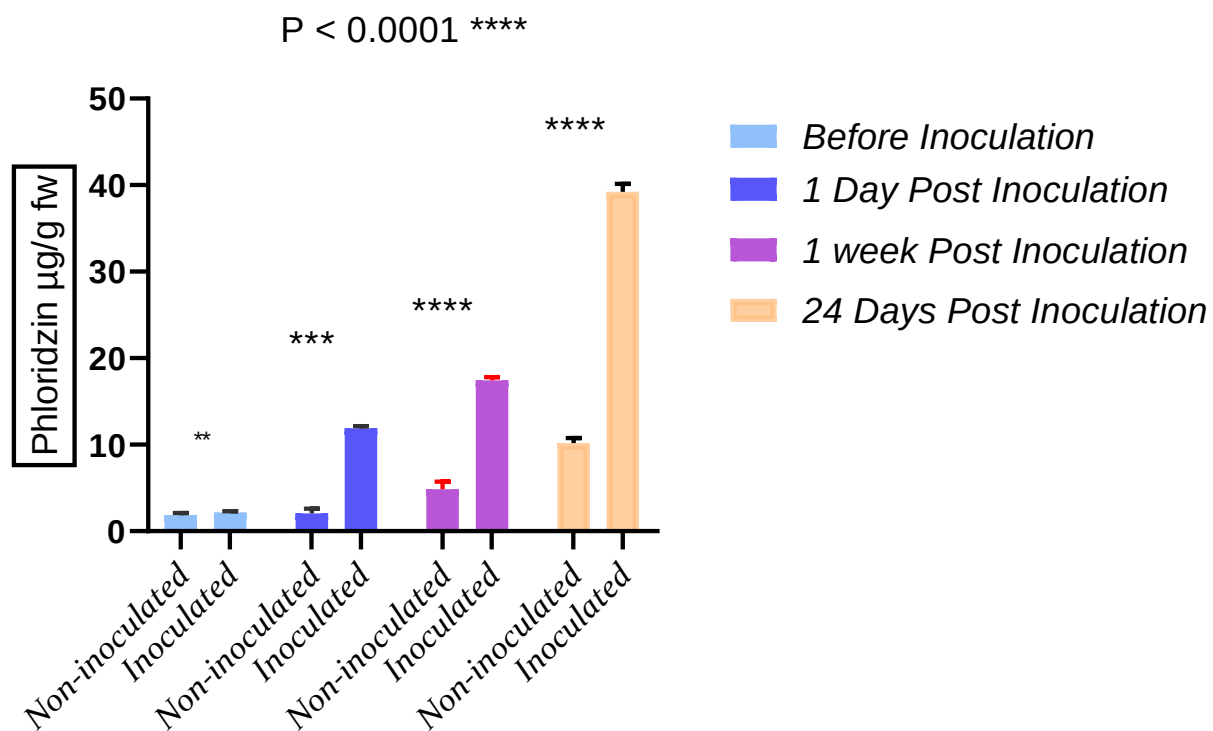
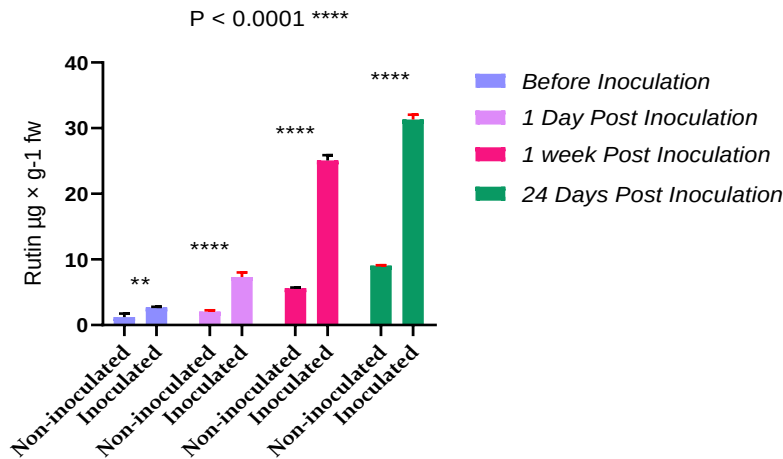
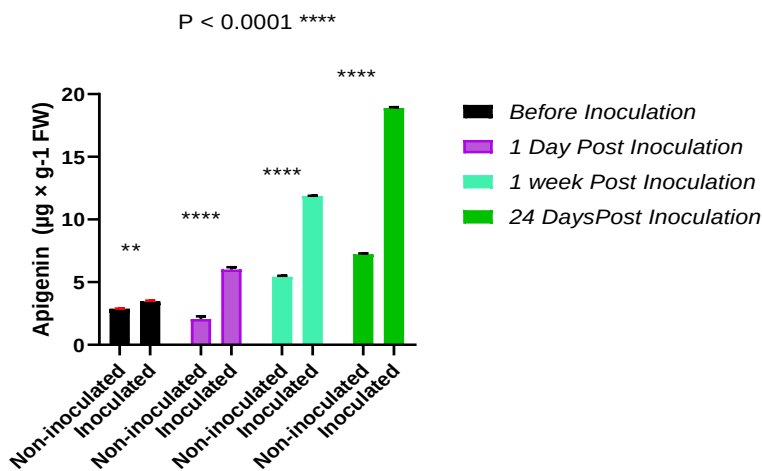
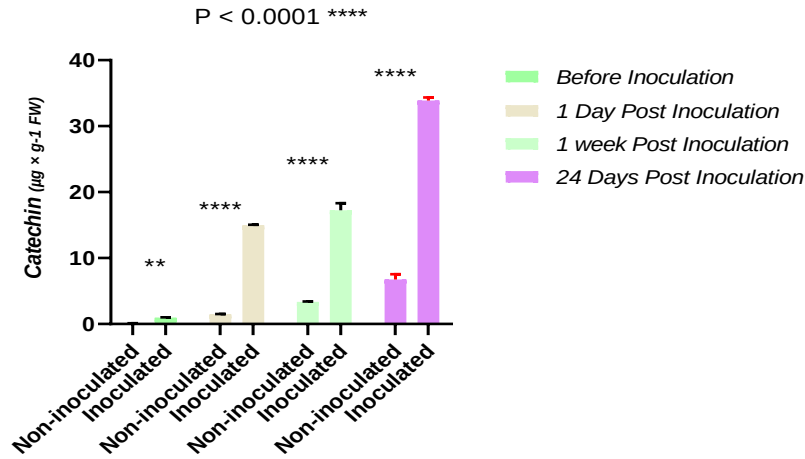


Figure #31. Coumaric acid content in apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; coumaric acid is expressed as $\mu\text{g/g}$ (fw)



Figure# 32. Phloridzin content in apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; Phloridzin is expressed as $\mu\text{g/g}$ (fw)



Figure# 33. Catechin, apigenin & rutin content apple cultivar after different stages of inoculation. Whereas * indicate significant variation ($p < 0.05$). Values are mean $\pm$ standard deviation; rutin is expressed as $\mu\text{g/g fw}$.

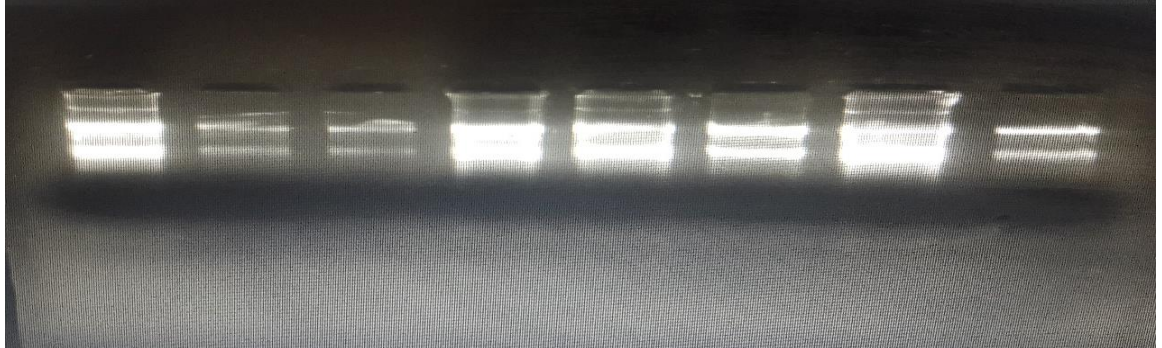
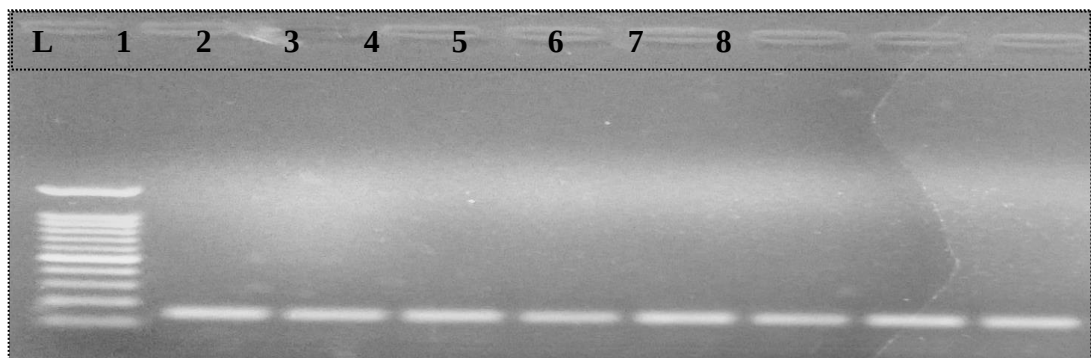


Figure #34. RNA gel picture, extracted at different stages after inoculation



Figure# 35. cDNA confirmation of isolated RNA from leaves of apple cultivars using GAPDH primer (Band at 181 bp). L indicates marker DNA ladder (1Kb).

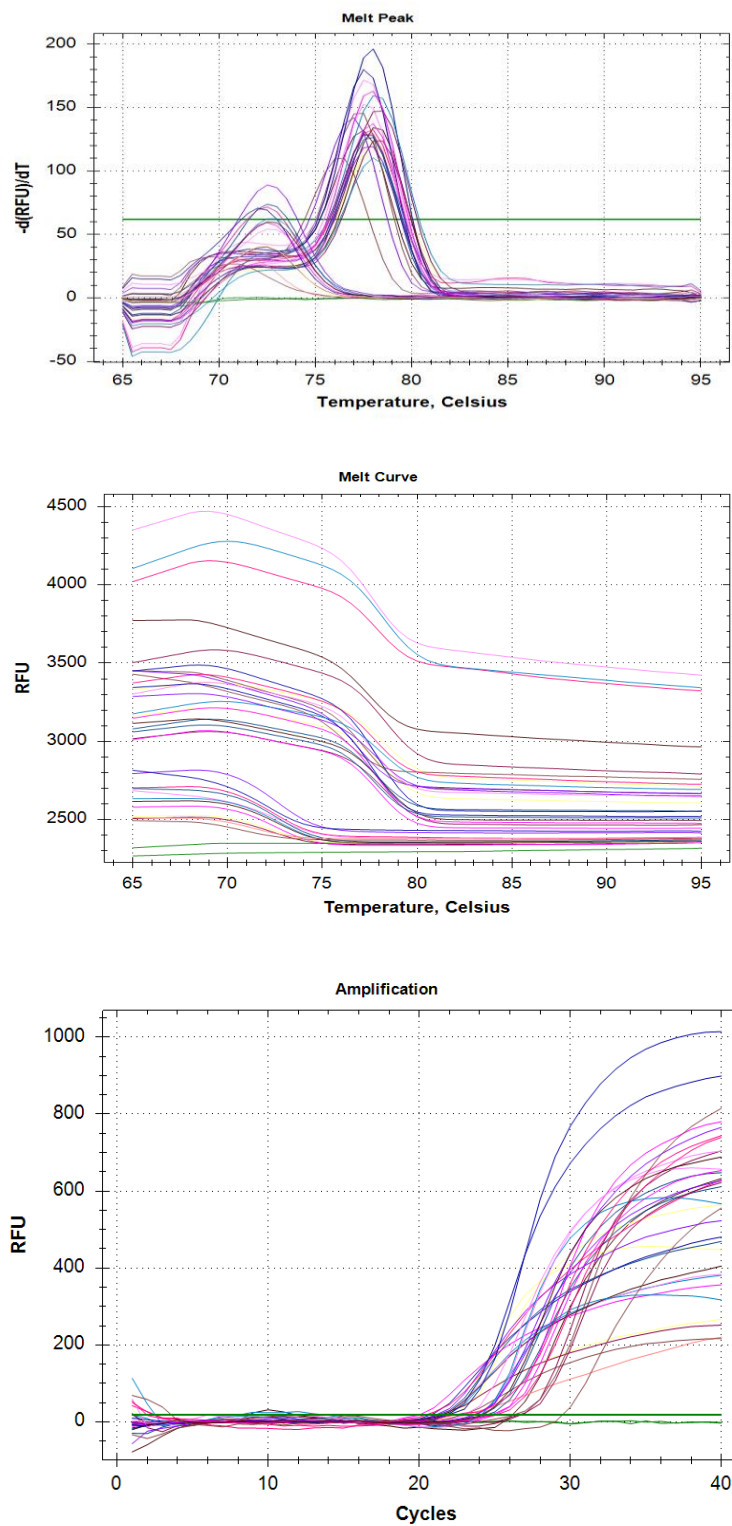
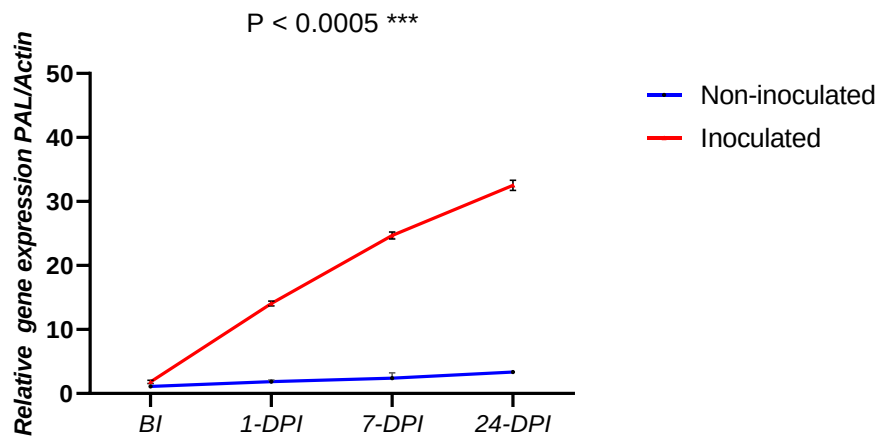
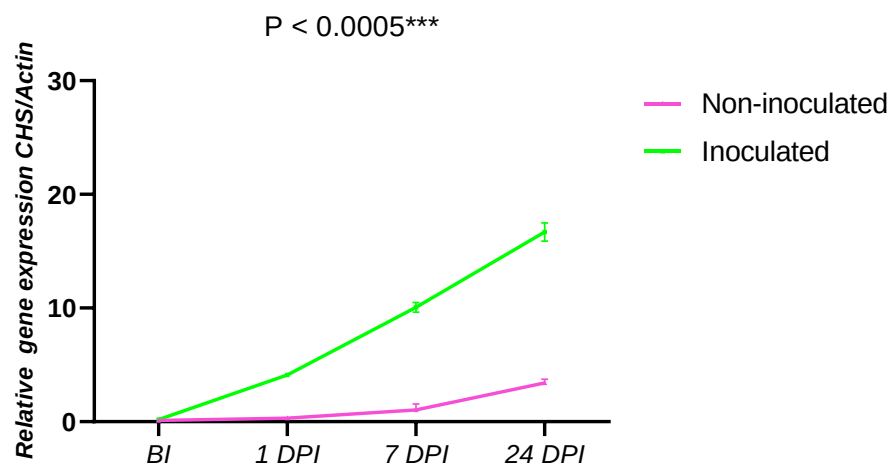


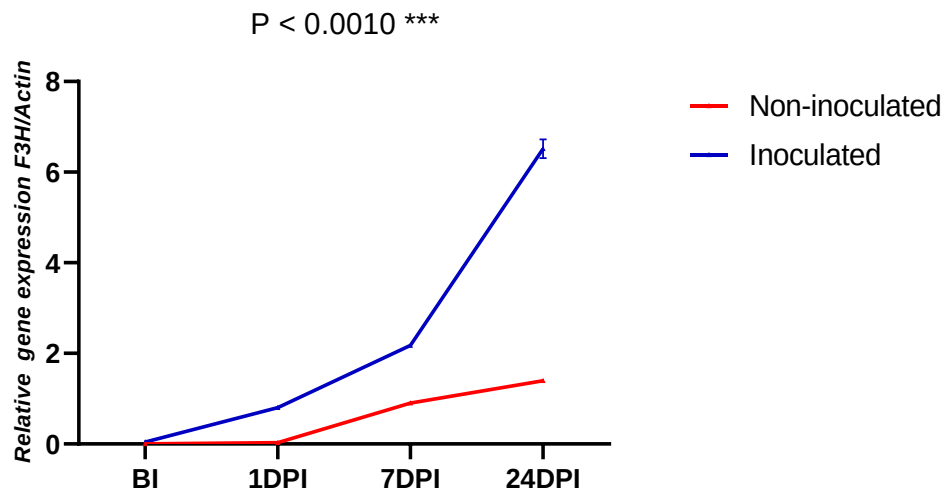
Figure #36. Melt peaks, melting curves and sharp amplification observed post completion of qRT-PCR



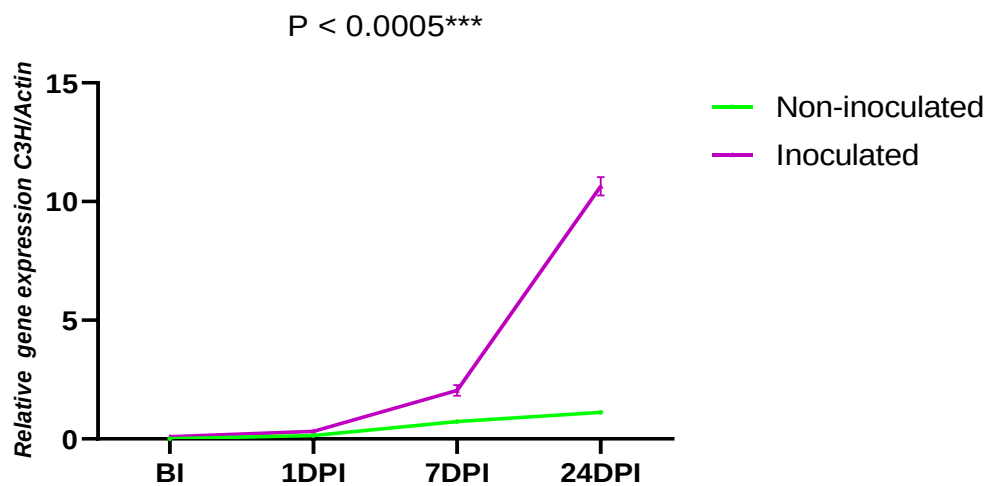
Figure# 37. Relative gene expression of PAL gene in apple cultivar under various post *Venturia* inoculation events by qRT-PCR, where $P < 0.0001$ ** Extremely significant, BI-before inoculation and DPI- days post inoculation.**



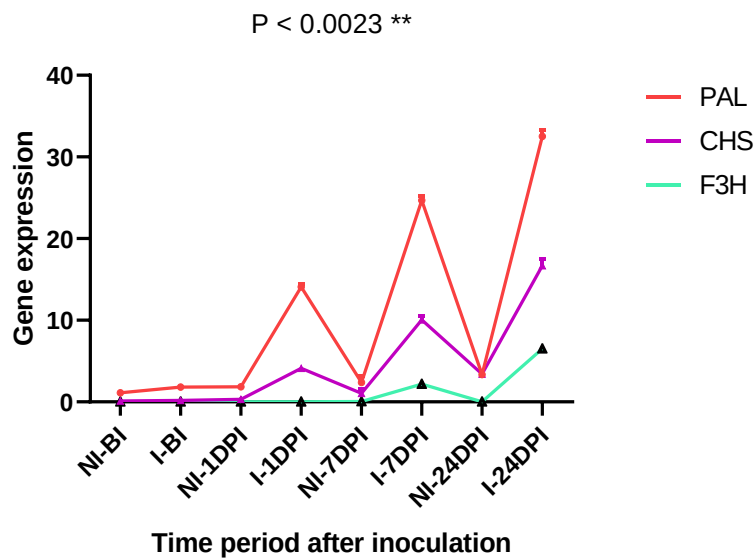
Figure# 38. Relative gene expression of CHS gene in apple cultivar under various post *Venturia* inoculation events by qRT-PCR, where $P < 0.0001$ ** Extremely significant BI-before inoculation and DPI- days post inoculation**



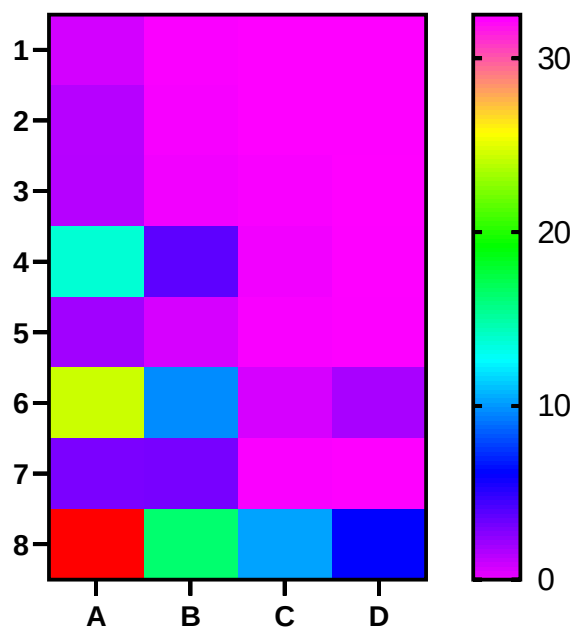
Figure# 39. Relative gene expression of *F3H* in apple cultivar under various post *Venturia* inoculation events by qRT-PCR, where $P < 0.0001$ **** Extremely significant, BI-before inoculation and DPI- days post inoculation



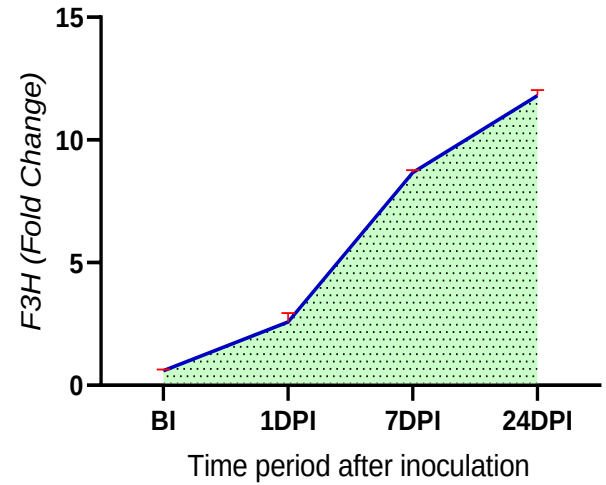
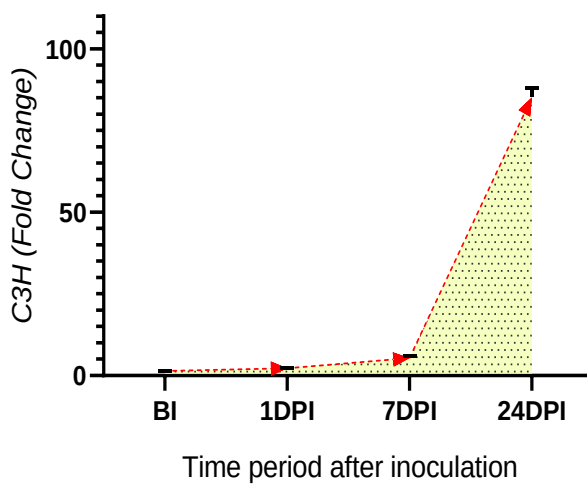
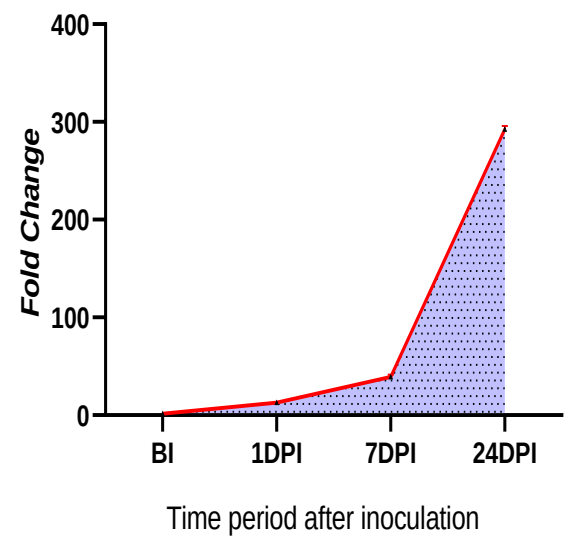
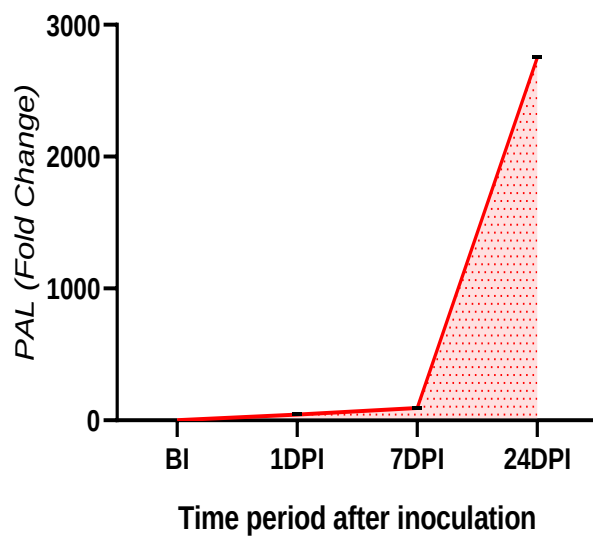
Figure# 40. Relative gene expression of *C3H* gene among three different apple cultivars under various post *Venturia* inoculation events by qRT-PCR, whereas $P < 0.0001$ **** Extremely significant, BI-before inoculation and DPI- days post inoculation



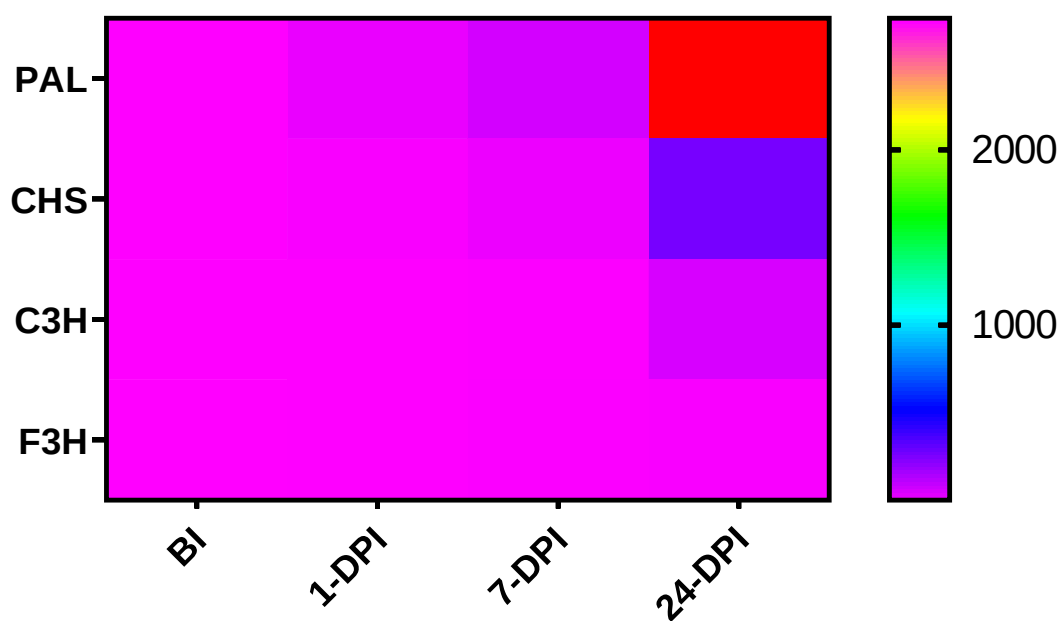
Figure# 41. Differential levels of gene expression of PAL, CHS, C3H and F3H at different stages before and post inoculation events when compared to corresponding house-keeping gene ACTIN



Figure# 42. Heatmap for PAL (A), CHS (B), C3H (C) and F3H (D) genes representing relative levels of gene expression at different stages before and post inoculation events compared to corresponding house-keeping gene ACTIN



Figure# 43. Gene fold change of PAL, CHS, C3H and F3H genes in apple cultivar under various post *Venturia inaequalis* inoculation events by qRT-PCR, where $P < 0.0001$ ** Extremely significant, BI-before inoculation and DPI- days post inoculation**



Figure# 44. Heatmap for PAL, CHS, C3H and F3H gene fold change at different stages before and post inoculation events, where BI-before inoculation and DPI-days post inoculation

4.1 Validation of *Venturia inaequalis* by morphological characterization

Thirty isolates were validated as *Venturia inaequalis* by simple morphological characterization. The conidia from the *V. inaequalis* fungal culture were single cell organisms and narrower at one end than the other (Figure # 13). Collectively, the conidia appeared brown or olive. However, once viewed underneath the microscope in a single cell suspension, they appeared lighter in color. Conidia ranged from 6-12 μm wide and 12-22 μm long and were created by specialized short hyphae known as conidiophore. The characteristics observed were similar to those described by Winka *et al.*, 1998.

4.2 DNA extraction and ITS based molecular characterization

Prominent, intact and brighter DNA bands were observed under UV-transilluminator (Figure # 14). Thirty isolates were amplified with the ITS primers. An approximately 550 bp amplicon was observed from all isolates (Figure # 15). After sequencing the PCR product and analyzing with BLASTn, all sequences showed 96%-98% sequence homology with GenBank submitted sequences for *V. inaequalis* (Figure # 16). Sequences were submitted to NCBI GenBank (Figure # 17) and accession numbers were obtained (Table # 21). *Sequence flat files are presented in appendix 1.*

4.3 Phylogenetic analysis

Phylogenetic analysis revealed that our isolates clustered along with another submitted *V. inaequalis* isolates from GenBank. The sequences of other isolates *V. pirina* and *V. natchicola* formed separate subclusters. *Pseudomonas syringae* formed a different cluster (outgroup) in the dendrogram (Figure # 18). Local clustering of sequences has revealed variability in ITS barcode sequences of *V. inaequalis* collected isolates. More variability was observed among isolates collected from Bhaderwah and Padder (Kishtwar) regions (Figure # 19; Figure # 20). Using ITS ribotyping of the 30 isolates collected from two regions of J&K displayed sequence homology with isolates and strains reported from different regions particularly Iran (Khe1 and MG2), South Africa (Strain_KELB2), India (Vi22) and Netherlands (Strain CBS 594.70).

4.4 SSR genotyping and genetic diversity analysis

The 30 *V. inaequalis* isolates were genotyped using 28 SSR markers (Figure # 21). The results obtained through Popgene software analysis for genetic diversity parameters are presented in Table # 22. The number of alleles per marker varied from one to fifteen with an average of four per locus. The highest number of 15 alleles was from Vitc1/2 and Vitc1/130 in the set of 30 isolates. The mean polymorphism information content value was 0.073 with a minimum value of 0.014 (Vitc1/130) and a maximum of 0.195 (Vitg11/70). The average heterozygosity ranged between 0.00 and 0.50 with an average of 0.39. The expected heterozygosity or gene diversity ranged from 0.00 to 0.50 with an average of 0.40. The effective number of alleles ranged from 1.00 to 2.00 with an average of 1.74. The Shannon index, an estimate of diversity ranged from 0.00 to 0.60 with an average of 0.57. The F_{ST} value ranged from 0.000 to 0.600 with an average of 0.194.

4.5 Cluster analysis

After using the neighbor joining method (DARwin software) cluster analysis, the 30 *V. inaequalis* isolates were categorized into three major clusters (I, II, and III). Eleven isolates (M20 to M30) were in cluster I. Eighteen isolates (M1 to M18) were in cluster II. Only one isolate (M19) was in cluster III. Both cluster I and II were further categorized into two sub clusters (Figure # 22). The isolates were grouped into separate clusters based on geographical distribution.

4.6 Isolates were grouped together into two major populations

The *V. inaequalis* isolates collected from different regions within J&K were grouped into two major populations. The assumed value of probable subpopulations (K) was determined by selecting a higher ΔK value with regards to the amount of clusters inferred by Structure software As per the Evanno table output (Table # 23), $K = 2$ was observed to be the best due to the high ΔK peak value of 34.6 among the probable K value (Figure # 23). Isolates from the same latitude within the Jammu region were grouped as subpopulation 1 (isolates M1-M18). Similarly, isolates from the same latitude within the Kashmir region were grouped as subpopulation 2 (isolates

M20-M26 and M28-M30). There were two isolates (M19 and M27) that were determined to be admixtures and grouped as subpopulation 3 (Figure # 24). Moreover, STRUCTURE based analysis sorted two populations (6.6% of the overall isolates) with a Q admixture proportion to the second cluster with the likelihood of 0.2 and 0.8, suggesting a considerable level of genetic flow between the two clusters.

4.7 Analysis of molecular variance reveals genetic variation

The two subpopulations along with admixture subpopulation generated from the structure analysis were analyzed for genetic variation among and within the subpopulations using the analysis of molecular variance (Table # 25). However, in the admixture subpopulation the sample size was less than 5, which couldn't be considered as a population. From the analysis, 25% of variation was observed among the subpopulations, 9% among individuals and 66% within individuals observed in the same subpopulation. Wright's F statistic was estimated to determine the deviation from Hardy-Weinberg equilibrium in the isolate population. Pair-wise F_{ST} values showed significant differentiation among all the pairs of subpopulations ranging from 0.248 to 0.881. This suggested that the three groups are significantly different from each other. The F_{IS} for all the 28 marker loci was 0.126, while F_{IT} was 0.343 across the clusters. The F_{ST} values and their distribution pattern show clear differentiation of subpopulations from each other (Figure # 25). Principal coordinate analysis also revealed the same, thus validating the results (Figure # 26) where coordinate 1 and 2 explained variation of 36.60% in subpopulation 1, 14.30% in subpopulation 2 and 13.10% in subpopulation 3. The overall cumulative percentage of variation was 64.07%.

4.8 Total phenols, total flavonoids and total tannins estimation using spectrophotometer

In general, there is an increasing trend of total polyphenolics after different stages of inoculation. The *Venturia inaequalis* inoculated cultivar has demonstrated statistically differential contents of total polyphenolic content ranging between 0.205-2.395 mg GAE/g fw. There was a 4-fold increase in total phenolics post 1 day (24-hours) of inoculation and from 1 day to 7 days content increased by 1.3fold while as from pre inoculation to post 24 days of inoculation, phenolic content increased 12

fold. In control, no such increase in the phenolic contents at different stages of post treatment was found. The highest content in control was found 24 days post inoculation as 1.871 mg GAE/g fw (Table # 25; Figure # 27). Total flavonoids content was again highest in inoculated cultivar, where the content reached to 4.89 mg CE/g fw at 24-day post inoculation. Before inoculation phenolic content was 0.15 mg CE/g fw, followed by 1.74, 2.70 & 4.89 mg CE/g fw and highest content reached at 24-DPI when symptoms in susceptible were clear visible. Pre inoculation to post 1 day (24 hours), the flavonoid content increased 11.6 fold and from 24 hours to 7 days, 1.6-fold increase was found whereas from 7 days to 24 days there was 1.8-fold increase in flavonoid content. After 24- days post inoculation, the flavonoid content was 1.03 mg CE/g fw (Table # 25; Figure # 28).

Total tannins also showed same increasing trend as shown by total phenols and flavonoids. The content was found higher from pre inoculation till 24-days after treatment in inoculated cultivar. Initially in control, tannin content was 1.01 mg TAE/g fw compared to susceptible 1.17 mg TAE/g fw. After 1-day (24-hours) post inoculation tannin content remained in average range of 1-2.63 mg TAE/g fw in control, while as in inoculated total tannins increased 2.8 fold from 1 day (24-hours) to 7 days. No significant increase was found from 7 days to 24 days of inoculation. From pre inoculation to post 24-day inoculation, total tannins increased by 2-fold in control and 5 fold in *V. inaequalis* inoculated cultivar (Table # 26; Figure # 29).

4.9 HPLC based quantification of different phenolic compounds

In the present investigation, the following phenolic compounds were identified in the leaves of inoculated apple cultivars: apigenin, p-coumaric acid, (+) - catechin, phloridzin, rutin and ferulic acid. Furthermore, HPLC chromatograms are given in appendix II. The current investigation has revealed that among selected apple cultivar, the highest ferulic acid content was shown by inoculated apple cultivar ranging between 0.50- 23.94 µg/g fw with significant increase at each post treatment stage. Pre inoculation to post 1 day (24 hours) in treated cultivar, there was a 16-fold increase in ferulic acid content whereas in control (water sprayed) 1.3-fold increase was found at first stage post treatment (Figure # 30).

Coumaric acid content was found to be 1.15 $\mu\text{g/g}$ fw before *V. inaequalis* inoculation and after 1 day (24 hours) the content reached to 7.67 $\mu\text{g/g}$ fw which shows a 1.6-fold increase in content whereas in other stages, a 2- fold increase was found. In case of control, the content remained in constant range till 7-days *i.e.*, 1.02 $\mu\text{g/g}$ fw to 1.91 $\mu\text{g/g}$ fw. whereas after 24-days the content reached up to 5.22 $\mu\text{g/g}$ fw. Thus, a 2-fold increase was seen from 7 to 24 days post inoculation in control (Table # 27; Figure # 31). In susceptible *V. inaequalis* inoculated cultivar, both of the hydroxycinnamic acids rapidly increased with time period. Within dihydrochalcones phloridzin content ranged from 2.06 $\mu\text{g/g}$ fw to 39.24 $\mu\text{g/g}$ fw in *V. inaequalis* treated cultivar and in mock /water treated, the content was in range of 1.83 $\mu\text{g/g}$ fw to 3.18 $\mu\text{g/g}$ fw. In case of control phloridzin, initially increased 1.2-fold whereas in *V. inaequalis* treated, the increase was 5.7 fold after 1-day (24 hours) of inoculation. From pre inoculation till 24-days inoculation, total increase in control was 2.2-fold and in *V. inaequalis* treated, there was 19-fold increase (Table # 28; Figure # 32).

Flavanol catechin production showed drastic increases in *V. inaequalis* treated cultivar, the content of catechin raised by 16-fold, apigenin 2.4-fold and rutin 2.7-fold after 1-day (24 hours) of inoculation whereas in control, catechin content increased by 1.7 fold, apigenin 1.4 fold and rutin by 1.7 fold after 1-day (24 hours) of mock/waterinoculation. After 24 days of post inoculation, the content of catechin was found highest in *V. inaequalis* treated cultivar 33.64 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ and at this point of time infection was severe and spots were prominent (Figure # 33). Isoflavonoid apigenin content before inoculation was 2.45 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ which increased after inoculation and had a peak value of 11.87 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ and 18.89 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ at 7-days and 24-days respectively. Rutin content increased from 2.70 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ (BI) to 31.31 $\mu\text{g} \times \text{g}^{-1}\text{fw}$ (24-Days PI) in *Ventura inaequalis* treated cultivar. In control, all the three flavonoids showed a small increase from pre inoculation stage to 24-days post inoculation stage (Table # 29). The phenolic contents overall showed a marked increase in *Ventura inaequalis* treated susceptible cultivar than in control. In control, phenolic content either remained constant or increased only marginally along with time period.

4.10 Expression analysis of PAL, CHS, C3H and F3H genes

4.10.1 RNA isolation and cDNA preparation:

The total RNA was isolated from the mentioned samples (treated and untreated), the intactness, size and quality of RNA extracted was checked on 1.5% agarose gel electrophoresis (Figure # 34). The RNA gel showed distinctly separated sharp ribosomal RNA bands (28S, 18S) with thickness of 28S rRNA twice than that of 18S. The quality RNA index in the form of absorbance at 260/280 was found closer to 2.0, indicating that the quality of isolated RNA was excellent and intact for further gene expression studies (Table # 30). The total RNA isolated was quantified having concentration between 200-717.2 ng/ μ l. (Table # 30). Using housekeeping gene GAPDH, cDNA synthesis confirmation was seen based on GAPDH amplification at 181 bp region (Figure # 35).

4.10.2 Relative gene expression / fold change of PAL, CHS, C3H, F3H genes

Relative levels of gene expression of polyphenolics biosynthetic genes namely PAL, CHS, C3H and F3H among apple cultivars, each in triplicates (n=3) was detected by their mean Ct values. Sharp amplification, melt peaks and melting curves were observed post completion of qRT-PCR (Figure # 36). The sharp amplification peaks rose up once cycle number reached near 20 cycles corresponding to relative fluorescence units (RFU) indicating correctness of PCR conditions. Using Ct values of samples as well as housekeeping gene ACTIN, the relative gene expression levels of each sample was determined through $\Delta\Delta C_T$ approach. The relative gene expression of PAL gene with respect to ACTIN among scab susceptible *V. inaequalis* treated apple cultivars was found statistically significantly higher as compared to control. Among susceptible *Venturia* treated cultivar, there was significant increase in the PAL expression levels *i.e.*, 1.83 ± 0.25 pre inoculation and 14.08 ± 0.39 post 1-day (24 hours) of inoculation, which showed a 7.8-fold increase, whereas in control, no such significant change was found. The level of gene expression in control was in 1.11 ± 0.15 to 3.36 ± 0.13 range which demonstrated lower insignificant expression of PAL gene. Therefore, PAL gene expression levels were found highest in *V. inaequalis* treated after 24 days post inoculation 32.51 ± 0.83 , thus indicating the highest PAL expression levels observed 24 days post inoculation (Table # 31 and Figure # 37).

Another important gene in the form of CHS was found up-regulated in *Venturia inaequalis* treated cultivar at different stages *i.e.*, 4.11 ± 0.55 (1DPI), 10.1 ± 0.42 (7-Days PI) and 16.67 ± 0.82 (24 days PI). From pre inoculation to post 1-day

inoculation in *V. inaequalis* treated cultivar, there was a 23-fold increase in gene expression level whereas from 1-day to 7-days, 2.5-fold and 7-days to 24-days, 1.7-fold increase was observed in CHS expression level and the lowest insignificant relative gene expression was found in control samples (Table # 32 ; Figure # 38). The moderate levels of F3H and C3H gene expression were observed in *V. inaequalis* treated apple cultivar at different stages, which ranged from 0.04 to 6.52 and 0.01 to 10.64 from pre inoculation to post 24-days inoculation in F3H and C3H respectively. Before inoculation to post 1-day F3H was highly upregulated *i.e.*, a 20-fold increase in F3H and 30-fold increase in C3H whereas no such upregulation was observed in control (Figure # 39; Figure # 40; Table # 33). *Further, melt peak, melt curve and amplification curve of PAL, F3H, CHS, C3H is present in appendix-III.*

To conclude, among all the four genes tested, the highest gene expression level of PAL gene was observed in *Ventura inaequalis* treated cultivar at different stages of inoculation, especially after 1-day whereas others showed small differential levels of expression (Figure # 41). The heatmap was constructed indicating differential gene expression levels of 4 different genes namely PAL, F3H, CHS, C3H (Figure # 42). Different color codes of heatmap demonstrated diverse gene expression levels *e.g.*, reddish (highest expression). Fold change of PAL, CHS, C3H and F3H genes are presented in Table # 34 and Figure # 43. The gene fold change of non-treated / control samples was always considered as 1. The results demonstrated highest fold change of PAL gene (2750.45) in *V. inaequalis* treated cultivar at different stages of inoculation. The order of fold change in inoculated cultivar is as $PAL > CHS > C3H > F3H$. The heatmap was constructed indicating fold change levels of 4 different genes - PAL, F3H, CHS, C3H (Figure # 44).



Discussion

DISCUSSION

Apple is grown in high altitude areas of India, particularly J&K. It is the primary cultivated crop in J&K as the climate conditions are distinct (sub temperate to true temperate) and are very suitable for the cultivation of apples (Sheikh *et al.*, 2017). This crop suffers huge losses both quantitatively and qualitatively due to frequent epidemics of scab disease (Ebrahimi *et al.*, 2016) that is caused by *Venturia inaequalis*. Therefore, in the present investigation, the molecular characterization through ITS and diversity analysis through SSR of *Venturia inaequalis* was undertaken in order to provide better management strategies for this disease.

Thirty isolates collected from two distinct regions were used for elucidating genetic molecular variability, diversity and population structure as the noncoding ribosomal DNA ITS sequences don't change more rapidly than the coding sequences and may diverge between species and populations. Thus, analysing ITS regions has become one of the primary methods for identification and characterization of a fungal strain or species and in 2012, the international fungal barcoding consortium formally recommended that the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster be used as the primary fungal barcode (Liyanage *et al.*, 1992; Monjeet *et al.*, 2013; Iwenet *et al.*, 2002). In fungi, the complete ITS section is about 600 bp long and contains two variable spacers ITS-1 and ITS-2 that are divided by the highly conserved 5.8S rRNA gene (Xu, 2016; White *et al.*, 1990) and another main benefit of using ITS as a barcode is that each haploid genome usually contains multiple tandemly repetitive copies of the ribosomal rRNA gene cluster (including ITS), making it potential to amplify this gene from little amounts of genetic material. The speedy increase in fungal barcoding studies has been drastically benefited from the availability of reasonably priced DNA sequencing technologies in many parts of the world. These studies have exposed remarkable undescribed fungal diversity across a broad-spectrum of biological niches (Hajibabaei *et al.*, 2007b; Tedersoo *et al.*, 2014; Grantham *et al.*, 2015).

DNA based approaches to the appraisal of fungi, thus, opens a window to an area of diversity. The most important molecular methods are reasonably objective and

tend to be clear cut to apply when compared to morphological, anatomical, ultrastructural or chemical procedures (Brasier, 1996; Crous *et al.*, 2007). Sequence homology was observed with isolates reported from different regions of the world particularly Iran, South Africa, Netherlands and Canada. As expected, after phylogenetic analysis of 45 *Venturia* sequences (35 *Venturia inaequalis*, 5 *Venturia pirina* and 5 *Venturia nashicola*), two clades emerged, one with all the *Venturia inaequalis* sequences and another with the *Venturia pirina* and *Venturia nashicola* sequences that further separated into two subclades. The similar results were produced by Padder and coworkers in the year 2011. The current study identified taxonomic relationships or differences between thirty *Venturia inaequalis* isolates, which can help to identify characteristics such as resistance or susceptibility towards a particular anti-fungal agent.

Microsatellites or SSRs are very useful markers for population genetics analysis because of their high specificity, polymorphism and reproducibility. These are major advantages of using SSR markers over random amplification of polymorphic DNA. The SSR markers used in this study were highly variable and results generally corresponded with previous population genetics studies conducted in Europe. However, in this study the markers 1tc1a, 1tc1b, 1tc1g, 1aac3b, 1aac4b, 1aac4f and 1aac4h showed only one allele compared to eight to ten alleles reported previously. The outcomes of this study along with prior reports (Xu *et al.*, 2013; Gladieux *et al.* 2008; Tenzeret *et al.*, 1999; Guérinet *et al.*, 2004; Padderet *et al.*, 2011) confirm the continuation of high variability in *Venturia inaequalis*. The distance between the Jammu region and the Kashmir region is approximately 400 km, so collected isolates are from geographically and topologically distinct regions. A high level of diversity was observed, which could be expected due to the climate differences.

It was observed that remarkable variability between and within *Venturia inaequalis* isolates obtained from dissimilar apple cultivars in a solo apple orchard. This was accredited to assortment pressure applied by diverse geographical locations and cultivars. Another possible explanation of variation in diversity is sexual recombination (Xu *et al.*, 2013). The isolates in this study shared a high percentage of identical alleles, indicating considerable gene flow among all isolates of *Venturia*

inaequalis populations in J&K. This was also observed in the magnitude of migration between the regions. The Kashmir region seems to have the highest migration rate towards it and the lowest away from it. Migration towards the Jammu region was the lowest indicating that this region is isolated, probably due to warmer winter temperatures. Overall, the migration results indicated the possible free movement of the pathogen between the regions. The present study undoubtedly showed that there is high diversity of *Venturia inaequalis* between the two regions and revealed that the larger part of variability existed within the individuals.

Structure analyses divided the isolates into two populations ($K = 2$) with a clear differentiation between the two apple growing regions. Pathogen distance seems to be the most significant factor in plunging gene flow because it explains 50% of the variation among the *Venturia inaequalis* isolates. Pair-wise F_{ST} values, ranging from 0.248 to 0.881 showed noteworthy demarcation between the subpopulations. This signified that the two groups are notably dissimilar from one another. This outcome is also validated by the principal coordinate analysis that distributed the isolates into two major populations and two isolates among them were different falling among admixture and as size is small these two can't be considered as a population.

Active natural defence related products in plants can be categorized into three broad groups: phytoalexins, phytoanticipins and signal molecules. Various phenylpropanoids display broad spectrum antimicrobial activity and are supposed to help the plant against microbial diseases (Shafi *et al.*, 2019; Vanetten *et al.*, 1994). The best-described phenylpropanoids derived phytoalexins are the isoflavans and isoflavanones. The prenylated isoflavones of lupin, which are synthesized during seedling development, are a good example of phytoanticipins (Gagnon *et al.*, 1995). Various reviews have potted the standards for the organization of compounds as phytoalexins or phytoanticipins, as well as providing widespread details on the distribution and biological activities of phenylpropanoid compounds involved in plant defence (Dixon, 2001; Dixon and Paiva, 1995; Grayer and Harborne, 1994).

Generally, with infection or pathogen attack phenolic content increases as a consequence of defense reaction towards infection. A reasonable answer for the greater or lesser level of resistance to scab, affirming that one type of resistance is possibly polygenic horizontal resistance in which there is a collective action of many

factors, *e.g.*, fungitoxic compounds delivers a modest contribution to overall resistance (Gessler *et al.*, 2006). Phenolics are undoubtedly not the only compounds that contribute to resistance of apples to scab, but as it could be seen from the results of present study, their synthesis is increased by the infection. The data presented herein indicates that phenolic compounds may contribute to the resistance of apple cultivars to scab. The synthesis of defense related phenolics such as flavonoids, hydroxycinnamic acid and dihydrochalcones is enhanced in response to pathogen attack and infection.

In Rosaceae family, the contribution of phenolics to limit fire blight is species specific and that pear cultivars that are resistant to fire blight were characterized by enhanced content of arbutin (hydroquinone O- β -D glucoside) while the sensitive ones had higher level of chlorogenic acid. In the current study, a significant increase in polyphenolic content was observed in *Venturia inaequalis* inoculated cultivar at different time points. The initial increase (1-DPI till 24-DPI) may be associated with defense process of susceptible cultivar. In *Venturia inaequalis*, inoculated susceptible cultivar polyphenols like ferulic acids, coumaric acid, apigenin, rutin*etc.* accumulated at a faster rate than in the non-inoculated (control) apple cultivar.

According to previous studies, it was hypothesized that infected leaves have a higher content of certain phenolic compounds compared with healthy leaves, but the content of chlorogenic acid, coumaric acid derivatives and flavanols was generally higher in the resistant than in the susceptible apple varieties (Picinelliet *al.*, 1995). However, our study suggests that there is a positive correlation between susceptible apple cultivar and accumulation of hydroxycinnamic acids in the relationship between *Malus $\times$ domestica* and *Venturia inaequalis*. The greatest share *i.e.*, the total content of hydroxycinnamic acids belongs to ferulic acid and the smallest share belongs to the p-coumaric acids. The scab infected susceptible cultivar post 24 days of infection had the highest content of total hydroxycinnamic acids at the first three sampling dates. The leaves scattered with numerous scab spots containing the highest amount of these acids. These results may be explained by the fact that the most intensive defense reactions like the synthesis of phenolic substances, occupy the borderline between the spot and the healthy tissue and the similar results were also obtained (Feucht, 1994; Mikulicet *al.*, 2007; Mikulicet *al.*, 2008; Mikulicet *al.*, 2009) in their previous studies.

The increase in all analyzed hydroxycinnamic acids after the infection may also be a consequence of the stimulated activity of *phenylalanine ammonia lyase* (PAL).

The dihydrochalcones and compounds within this group (phloridzin in particular) are the main polyphenols representing more than 90% of the extractable phenolic compounds in the leaves (Goschet *et al.*, 2009). Phloridzin and the corresponding aglycon, phloretin are often implicated in resistance to various diseases. The scab infected leaves had phloridzin content $39.24 \mu\text{g} \times \text{g}^{-1}\text{fw}$ after 24-days of post inoculation. In case of inoculation, the infected leaves accumulated more phloridzin from first day post inoculation. This suggests that with pathogen (*V. inaequalis*) attack phloridzin accumulated faster as a defense response in infected leaves. This finding was in agreement with the literature values (Leser and Treutter, 2005; Lattanzio *et al.*, 2006).

It can be concluded that in infected tissue, phloridzin is an active compound against the fungi attack, but its content does not always correlate with the degree of resistance of different apple cultivars (Goschet *et al.*, 2009). It was found that phloridzin levels increased in leaves of the *Venturia inaequalis* inoculated cultivar where after 24 hours of inoculation, there was approximately 6 fold increase in phloridzin content and highest level was found after 24 days, when symptoms were prominent, thus, infected leaves accumulated more phloridzin around the tissues. This indicates that not the overall levels of those phenolics, but the rate at which phloridzin is transformed is likely to play a role in resistance (Skłodowska *et al.*, 2018).

Flavonoids play a role in the resistance of apples to *V. inaequalis*. The ability to accumulate flavanols after infection in the tissue surrounding infected sites, is a differential property of susceptible and resistant cultivars (Treutter and Feucht, 1990a; 1990b). It became apparent that the infection with apple scab in susceptible cultivar caused higher synthesis of catechin, apigenin and rutin. In the non-inoculated cultivar, contents of catechin, apigenin and rutin were 1.3 to 2.8 times lower than in infected leaves. An increased synthesis of flavanols after the *Venturia inaequalis* attack *i.e.*, catechin, apigenin and rutin are probably involved in the defence reaction and their content in surrounding of infected tissue increased. Catechin accumulation after 24 hours was more as compared to apigenin and rutin. The same reports were produced (Treutter and Feucht, 1990a; Picinelli *et al.*, 1995; Mikulic *et al.*, 2009).

The pathogen induces up regulation of a F3'5 H gene, which is assumed to be responsible for 30-hydroxylation of flavonoids in Golden Delicious fits in the accumulation of catechins, often observed in infected apple leaves (Mayr *et al.* 1997; Leser and Treutter, 2005; Treutter and Feucht, 1990; Picinelliet *al.* 1995), but on the other hand accumulation of phenolic compounds in apple tissues is also influenced by the growing season apart from apple scab infection as reported by Mikulic *et al.* 2007. The accumulation of different phenolic compounds as well as the possible activation of the phenylpropanoid pathway which has been established by previous experiments (Treutter and Feucht, 1990a;1990b) may explain the broad and unspecific prevention of plant diseases such as apple scab.

The quantification of important phenolics and flavonoids of apple cultivars was carried out using HPLC technique as this is a valuable tool for compound identification and it can provide chromatographic fingerprints for identification and quality control of the product under investigation. Among chromatography techniques, HPLC is considered faster, quicker and cost effective for identification and quality control of plant-based products (Poudyaet *al.*, 2010).

PAL, C3H, F3H and CHS are the key branch-point enzymes regulating the metabolism of the phenylpropanoid pathway and the accumulation of phenylpropanoids (Dixon *et al.*, 2002 and Vrancken *et al.*, 2013). Our data presented that after inoculation, there was noteworthy increase in activities of PAL, C3H, CHS and F3H genes that accounts for the increased production of phenolic compounds and flavonoids. qRT-PCR results have demonstrated that PAL, CHS, C3H and F3H gene expression levels / gene fold change was found highest in 24 days post inoculation (103.20) among scab susceptible cultivar as compared to control. The results are in coherence with earlier studies conducted on apple, muskmelon, cherry and tomato fruit, showed that increase in PAL, C3H, F3H and CHS activities, enhanced the disease resistance against pathogens (Wei *et al.*, 2019).

Moreover, the up-regulation of the early flavonoid gene CHS resulted in an increase of phenylpropanoids. Among the early events related with plant reaction to pathogen attack, is the quick accumulation of phenolic compounds at the infection site eventually aimed at preventing the spread of the pathogen by isolating it at the primary entry point and by affecting biochemical processes that are vital for the

bacterium. The flavonoids are designated as having antibacterial, antitoxin, antiviral, antifungal activities and as being involved in structural defense (Treutter, 2005). The researchers found similar kind of activity of PAL in the poly e lysine (PL) treated apple fruits which peaked at 8 days after treatment, while PAL activity increased slowly in the control fruits from 0 to 8 days after treatment. PAL activity was peaked at 8 days after treatment in PL-treated fruits which was 1.66 times higher than the control. PL treatment increased the content of total phenolic compounds in apple fruits after 2 days and reached a peak on 8th day after treatment. The content of total phenolic compounds was 1.34 times and 1.32 times higher ($p < 0.05$) in PL-treated fruit compared to control at 8th and 10th day after treatment respectively. PL treatment induced the increase of flavonoids content from 2 to 10 days after treatment. Flavonoids content peaked at 8th day after treatment in both the PL-treated and control fruits which were 1.1 times higher ($p < 0.05$) in PL-treated fruits compared to the control. PL treatment significantly increased ($p < 0.05$) lignin content in fruits from 4th to 10th day after treatment. Lignin content in PL-treated fruit peaked at 8th day which was 1.3 times higher than the control (Yonghong *et al.*, 2018).

The amounts of phenolics and flavonoids determined in the present study before and after inoculation of *Venturia inaequalis* are in similar correlation as observed in earlier studies (Mikulic *et al.*, 2008). The results achieved represented an important contribution to understanding plant pathogen interaction and may be useful for further investigations. The content and type of phenolics / flavonoids compounds in apple leaves could act as important markers of enhanced levels of scab resistance against *Venturia inaequalis*. It was reported that pretreatment with p-coumaric acid (1 mM) alleviates the disease symptom caused by *Xanthomonas campestris* inoculation, with an accumulation of phenolic compounds delivered along the phenylpropanoid pathway by up regulating CHS and HCT gene expression in Chinese cabbage (Islam *et al.*, 2018). The results of present study demonstrated that scab infection caused a significant increase in hydroxycinnamic acids, catechin, epicatechin and phloridzin in infected tissue. The results achieved, represented an important contribution to understand plant-pathogen interaction and may be useful for further investigations. The content and type of phenolics / flavonoids compounds in apple leaves could act as important marker of enhanced levels of scab resistance against *Venturia inaequalis*. There seems a positive correlation between levels of gene expression of PAL, CHS,

C3H and F3H genes and production of different phenolics and flavonoids such as apigenin, p-coumaric acid, (+) - catechin, phloridzin, rutin and ferulic acid.

To conclude, the accumulation of different phenolic compounds as well as the possible activation of the phenylpropanoid pathway, which has been established by previous experiments (Treutter and Feucht, 1990a;1990b) may explain the broad and unspecific prevention of plant diseases such as apple scab. However, there still remains the question of whether the constitutive level of some phenolic compounds is really involved in resistance mechanisms or it represents only the capacity of the tissue to produce large amount of these compounds (Treutter, 2001).

For further studies, other specific phenolics as well as other compounds should be studied in more detail. In particular, the time scale in which they are synthesized could be of great importance as a mechanism of quick response to apple scab infection, which can lead to a higher degree of resistance. The mechanisms underlying the effects of sinapic acid, coumaric acid and ferulic acid on plant disease resistance still need to be characterized. The results of the present investigation gave a new insight into the response of apples, which are susceptible to the scab fungus and may help to understand mechanisms of basal defence and cultivar specific resistance. The comparison of non-inoculated and inoculated Golden Delicious, provided evidence of *Venturia inaequalis* induced genes in the susceptible cultivar. These genes may represent the basal defence response which is strongly inhibited or blocked by the pathogen.



Summary & Conclusion

CHAPTER-VI

SUMMARY AND CONCLUSION

Apple industry in India is responsible for generation of huge horticultural revenue and lakhs of people are associated with its cultivation and trade. The major states / union territories dealing with apple cultivation in India are Jammu and Kashmir, Himachal Pradesh, Uttarakhand and some parts of Arunachal Pradesh, Sikkim, Nagaland, Meghalaya. Among these, Jammu and Kashmir contribute highest production share with total annual production of approximately 17,26,834 metric tons, covering an area of 1,62,971 hectares. Despite of its economic importance, apple crop is frequently attacked by scab disease, which hampers its production with a loss of 30-35% per year. Therefore, in the present research work, an attempt was made to study genetic diversity of *Venturia inaequalis* across different geographical locations of J&K and effect of *Venturia inaequalis* on PAL biochemical pathway.

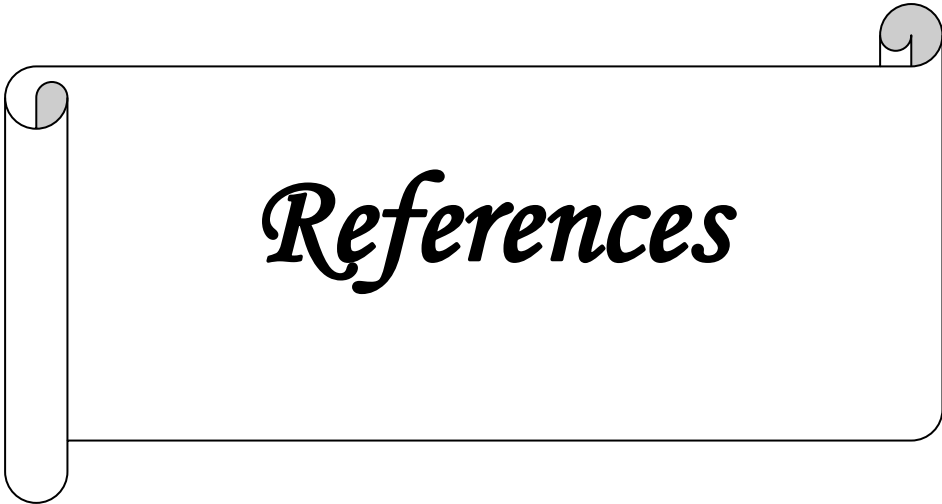
The current investigation has identified the existence of genetic diversity among strains of *Venturia inaequalis* collected from different regions of Jammu and Kashmir based on ITS and SSR approach. ITS based ribotyping of the 30 isolates showed sequence homology with strains / isolates, reported from different regions of the world where as intra sequence comparison has demonstrated variability among the isolates collected from Bhaderwah (Doda) and Padder (Kishtwar) regions. Thirty sequences were published in NCBI database. The collected isolates were grouped into two major populations (P1 & P2) with a small admixture (19 and 27). Significant levels of genetic diversity was observed based on SSR markers, reflecting existence of polymorphism among selected isolates of *Venturia inaequalis* based on mean genetic diversity indices like Nei and PIC (0.36 and 0.18 respectively). *Venturia inaequalis* collected from different apple growing regions in Jammu and Kashmir showed significant levels of genetic variation within the populations in the similar fashion as observed in other *Venturia inaequalis* population studies, conducted in Europe and elsewhere. Moreover, SSR molecular data demonstrated three major clusters (I, II and III) significantly different from each other, based on neighbor

joining clustering approach. AMOVA results showed 25% of variation among the subpopulations, 9% among individuals and 66% within individuals. Based on the structural analysis, thirty *Venturia inaequalis* isolates were grouped into two populations showing 34.138 ΔK peak values.

HPLC based analysis of major marker polyphenolic and flavonoid compounds (ferulic acid, coumaric acid, catechin, rutin and phloridzin) and spectrophotometric analysis of total phenolics, flavonoids, tannins was found significantly higher in *Venturia inaequalis* inoculated cultivar than in control. The results represented the basal or first line of defence response against the pathogen. Relative gene expression / fold change of PAL pathway genes was found in the order as PAL > CHS > C3H > F3H. *Venturia inaequalis* isolates studied in the present experiment, could act as a novel repository that can be utilized for screening and testing of newly developed hybrid apple cultivars against scab disease. The isolates from Bhaderwah and Padder regions can be tested in future, using differential host to confirm, if they could be source of new race of *Venturia inaequalis*. The collected *Venturia inaequalis* strains could be used in proper management of scab by devising targeted anti-fungal agents that would possibly lower the number of fungicides used, as well as the annual cost involved to curb scab menace. Variation in expression of PAL pathway genes upon infection by *Venturia inaequalis* explains the role of polyphenolic marker compounds in imparting disease resistance against scab, apart from other innate resistance mechanisms. Thorough investigation and knowledge of the production / accumulation of resistance related secondary metabolites can contribute to a better understanding of plant defence against *Venturia inaequalis*, which can lead to a higher degree of resistance.

Conclusively, genetic diversity results, from the present study, could help in constructive management of scab. Cisgenesis and modern breeding programmes could be used to introduce the transcription factors for up-regulation of PAL pathway genes, as an adjunct approach to reduce scab, apart from disease resistant 'R' genes in the development of new apple cultivars. Development of high-quality disease resistant

apple cultivars is the preferred long term mean of reducing fungicide use, in apples. Resistance breeding of new varieties that combine resistance genes from different sources is desirable. The use of polygenic cultivars should be increased, so that the fungus has smaller chances of adaptation. Thus, the interaction studies between the fungus and the host are of great importance. Further, continued research is required to identify more resistance gene.



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Appendices

Appendix-I

Venturia inaequalis isolate sms47 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504437.1

[FASTA](#) [Graphics](#)

Go to: ▼	
LOCUS	MK504437 491 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms47 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504437
VERSION	MK504437.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 491)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 491)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
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misc_RNA	

venturia inaequalis isolate sms41 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504435.1

[FASTA](#) [Graphics](#)

Go to: ▼	
LOCUS	MK504435 496 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms41 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504435
VERSION	MK504435.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 496)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 496)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
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source	1..496 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms41" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India: J&K" /collection_date="15-Mar-2018" <1..>496 /note="contains small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
misc_RNA	

Venturia inaequalis isolate sms7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504429.1

[FASTA](#) [Graphics](#)

Go to: ▼	
LOCUS	MK504429 476 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504429
VERSION	MK504429.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 476)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 476)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..476 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms7" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India: J&K" /collection_date="15-Mar-2018" <1..>476 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
misc_RNA	

Venturia inaequalis isolate sms46 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504436.1

[FASTA](#) [Graphics](#)

LOCUS	MK504436 481 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms46 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504436
VERSION	MK504436.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..481 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms46" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India: J&K" /collection_date="15-Mar-2018" <1..>481 /note="contains small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
misc_RNA	

Venturia inaequalis isolate sms30 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504434.1

[FASTA](#) [Graphics](#)

Go to: ▼	
LOCUS	MK504434 479 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms30 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504434
VERSION	MK504434.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 479)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 479)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..479 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms30" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India: J&K" /collection_date="15-Mar-2018" <1..>479 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
misc_RNA	

Venturia inaequalis isolate sms6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504428.1

[FASTA](#) [Graphics](#)

LOCUS	MK504428 312 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504428
VERSION	MK504428.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	Venturia inaequalis Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 312)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 312)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..312 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms6" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India: J&K" /collection_date="15-Mar-2018" <1..>312 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
misc_RNA	

Venturia inaequalis isolate sms17 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MKS04431.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MKS04431	481 bp	DNA	linear	PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms17 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.				
ACCESSION	MK504431				
VERSION	MK504431.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 481)				
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	DNA Barcoding of Venturia inaequalis				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 481)				
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..481 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms17" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018" c1..>481 /note="contains small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, and internal				
misc RNA					
GenBank	Send to:				

Venturia inaequalis isolate Nalhti_Baderwah_J&K internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK583539.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MKS83539	466 bp	DNA	linear	PLN 20-MAR-2019
DEFINITION	Venturia inaequalis isolate Nalhti_Baderwah_J&K internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.				
ACCESSION	MK583539				
VERSION	MK583539.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 466)				
AUTHORS	Mansoor,S.S., Sharma,V., Ahmed,N., Mir,J.I., Nabi,S.U., Jan,S. and Masoodi,K.Z.				
TITLE	DNA Barcoding of Venturia inaequalis				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 466)				
AUTHORS	Mansoor,S.S., Sharma,V., Ahmed,N., Mir,J.I., Nabi,S.U., Jan,S. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (02-MAR-2019) Biotechnology, SKUAST Shalimar Kashmir, Shalimar, Srinagar, Jammu and Kashmir 190027, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..466 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Nalhti_Baderwah_J&K" /isolation_source="Apple_tree_J&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="27-Apr-2018"				

Venturia inaequalis isolate Faisal Abad_Kishtwar internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532036.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MKS32036	482 bp	DNA	linear	PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Faisal Abad_Kishtwar internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.				
ACCESSION	MK532036				
VERSION	MK532036.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 482)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 482)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..482 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Faisal Abad_Kishtwar" /isolation_source="Apple_trees_J&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018"				

Venturia inaequalis isolate sms15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MKS04430.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MKS04430	429 bp	DNA	linear	PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.				
ACCESSION	MK504430				
VERSION	MK504430.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 429)				
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	DNA Barcoding of Venturia inaequalis				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 429)				
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..429 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="sms15" /isolation_source="apple orchid" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018" c1..>429 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"				
misc RNA					
ORIGIN					

Venturia inaequalis isolate Tund_Kishtwar_J&K internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532037.1

[FASTA](#) [Graphics](#)

LOCUS	MKS32037	483 bp	DNA	linear	PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Tund_Kishtwar_J&K internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.				
ACCESSION	MK532037				
VERSION	MK532037.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 483)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 483)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..483 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Tund_Kishtwar_J&K" /isolation_source="Apple_trees_J&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018" c1..>483 /note="contains internal transcribed spacer 1. 5.8S				
misc RNA					

Venturia inaequalis isolate Faisal Abad_Kishtwar internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532036.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MKS32036	482 bp	DNA	linear	PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Faisal Abad_Kishtwar internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.				
ACCESSION	MK532036				
VERSION	MK532036.1				
KEYWORDS	.				
SOURCE	Venturia inaequalis				
ORGANISM	Venturia inaequalis				
	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.				
REFERENCE	1 (bases 1 to 482)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 482)				
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.				
TITLE	Direct Submission				
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
FEATURES	Location/Qualifiers				
source	1..482 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Faisal Abad_Kishtwar" /isolation_source="Apple_trees_J&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018"				

Venturia inaequalis isolate Uri_Baramulla internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532035.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK532035 481 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Uri_Baramulla internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK532035
VERSION	MK532035.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..481 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Uri_Baramulla" /isolation_source="Apple_trees_3&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018"

Venturia inaequalis isolate Padder_Kishtwar2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532034.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK532034 481 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Padder_Kishtwar2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK532034
VERSION	MK532034.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 481)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..481 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Padder_Kishtwar2" /isolation_source="Apple_trees_3&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018" /c1..>481

[misc_RNA](#)

GenBank =

Send to =

Venturia inaequalis isolate Baderwah_J&K1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532031.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK532031 484 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Baderwah_3&K1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK532031
VERSION	MK532031.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 484)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 484)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..484 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Baderwah_3&K1" /isolation_source="Apple_trees_3&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India"

Venturia inaequalis isolate Padder_Kishtwar1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK532033.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK532033 473 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Padder_Kishtwar1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK532033
VERSION	MK532033.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 473)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 473)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..473 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Padder_Kishtwar1" /isolation_source="Apple_trees_3&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India" /collection_date="25-Apr-2018"

Venturia inaequalis isolate Baderwah_J&K2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK532032.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK532032 482 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate Baderwah_3&K2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK532032
VERSION	MK532032.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 482)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Venturia inaequalis isolates collected from different hotspots of Jammu and Kashmir
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 482)
AUTHORS	Mansoor,S.S., Ahmed,N., Mir,M.A., Jan,S., Nissar,M., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (19-FEB-2019) K_LAB PLANT BIOTECHNOLOGY, SKUASTK, Shalimar, Srinagar, Jammu and Kashmir 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..482 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="Baderwah_3&K2" /isolation_source="Apple_trees_3&K" /specimen_voucher="Mansoor_Khalid" /db_xref="taxon:5825" /country="India"

Venturia inaequalis isolate SMS31 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK359032.1

[FASTA](#) [Graphics](#)

Go to:	
LOCUS	MK359032 448 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS31 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK359032
VERSION	MK359032.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 448)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 448)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, 3&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..448 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS31" /isolation_source="Bandipora_3&K" /specimen_voucher="Mansoor-Khalid31" /db_xref="taxon:5825"

Venturia inaequalis isolate SMS23 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK359031.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359031 446 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS23 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK359031
VERSION	MK359031.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 446)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 446)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..446 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS23" /isolation_source="Matan Anantnag,J&K" /specimen_voucher="Mansoor-Khalid23" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018" <1..446 /note="contains internal transcribed spacer 1, 5.8S
misc_RNA	

Venturia inaequalis isolate SMS15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK359029.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359029 472 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK359029
VERSION	MK359029.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 472)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 472)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..472 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS15" /isolation_source="Naidkhai Bandipora,J&K" /specimen_voucher="Mansoor-Khalid15" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018" <1..472 /note="contains internal transcribed spacer 1, 5.8S
misc_RNA	

Venturia inaequalis isolate SMS11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MK359027.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359027 479 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK359027
VERSION	MK359027.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 479)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 479)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..479 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS11" /isolation_source="Kupwara,J&K" /specimen_voucher="Mansoor-Khalid11" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018"
misc_RNA	

Venturia inaequalis isolate SMS19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK359030.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359030 474 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK359030
VERSION	MK359030.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 474)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 474)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..474 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS19" /isolation_source="Pranu Baderwah,J&K" /specimen_voucher="Mansoor-Khalid19" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018" <1..474 /note="contains internal transcribed spacer 1, 5.8S
misc_RNA	

Venturia inaequalis isolate SMS12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and large subunit ribosomal RNA gene, partial sequence

GenBank: MK359028.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359028 481 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.
ACCESSION	MK359028
VERSION	MK359028.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 481)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 481)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..481 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS12" /isolation_source="Pulwama,J&K" /specimen_voucher="Mansoor-Khalid12" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018"
misc_RNA	

Venturia inaequalis isolate SMS5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK359026.1

[FASTA](#) [Graphics](#)

Go to: [v]	
LOCUS	MK359026 472 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK359026
VERSION	MK359026.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 472)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 472)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumira,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..472 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS5" /isolation_source="Beervia budgan,J&K" /specimen_voucher="Mansoor-Khalid5" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018"
misc_RNA	

Venturia inaequalis isolate SMS2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK359025.1

[FASTA](#) [Graphics](#)

Go to: ⌵	
LOCUS	MK359025 469 bp DNA linear PLN 10-JAN-2019
DEFINITION	Venturia inaequalis isolate SMS2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK359025
VERSION	MK359025.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 469)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumra,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 469)
AUTHORS	Mansoor Shafi,S., Ahmed,N., Mir,M.A., Sharma,V., Dar,N.A., Dar,Z.A., Ammarah,H., Andrabi,S.A.H., Tanzeel,B., Sumra,J., Nabi,S.U., Wani,S.M., Nehvi,F.A., Mir,J.I. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (04-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..469 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS2" /isolation_source="Baderwah,J&K" /specimen_voucher="Mansoor-Khalid2" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018"
misc_RNA	<1..>469 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"

Venturia inaequalis isolate SMS4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK478887.1

[FASTA](#) [Graphics](#)

Go to: ⌵	
LOCUS	MK478887 441 bp DNA linear PLN 09-FEB-2019
DEFINITION	Venturia inaequalis isolate SMS4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK478887
VERSION	MK478887.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 441)
AUTHORS	Mansoor,S.S., Vikas,S., Ahmed,N., Sweeta,M. and Masoodi,K.Z.
TITLE	Apple Pathogens
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 441)
AUTHORS	Mansoor,S.S., Vikas,S., Ahmed,N., Sweeta,M. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (03-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..441 /organism="Venturia inaequalis" /mol_type="genomic DNA" /isolate="SMS4" /isolation_source="Appletrees,Shopian_Kashmir" /specimen_voucher="Mansoor_khalid" /db_xref="taxon:5825" /country="India" /collection_date="15-Mar-2018"
misc_RNA	<1..>441 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"
361616N	

Venturia inaequalis isolate sms20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK504432.1

[FASTA](#) [Graphics](#)

Go to: ⌵	
LOCUS	MK504432 432 bp DNA linear PLN 02-APR-2019
DEFINITION	Venturia inaequalis isolate sms20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK504432
VERSION	MK504432.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 432)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 432)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
FEATURES	Location/Qualifiers
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misc_RNA	<1..>432 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2"

Venturia inaequalis isolate SMS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK478885.1

[FASTA](#) [Graphics](#)

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LOCUS	MK478885 334 bp DNA linear PLN 09-FEB-2019
DEFINITION	Venturia inaequalis isolate SMS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
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VERSION	MK478885.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 334)
AUTHORS	Mansoor,S.S., Vikas,S., Ahmed,N., Sweeta,M. and Masoodi,K.Z.
TITLE	Apple Pathogens
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 334)
AUTHORS	Mansoor,S.S., Vikas,S., Ahmed,N., Sweeta,M. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (03-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
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Venturia inaequalis isolate SMS13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MK367580.1

[FASTA](#) [Graphics](#)

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DEFINITION	Venturia inaequalis isolate SMS13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION	MK367580
VERSION	MK367580.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 410)
AUTHORS	Mansoor,S.S., Sharma,V., Ahmad,N., Moni,G., Guleria,S. and Masoodi,K.Z.
TITLE	Barcode of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 410)
AUTHORS	Mansoor,S.S., Sharma,V., Ahmad,N., Moni,G., Guleria,S. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (05-JAN-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
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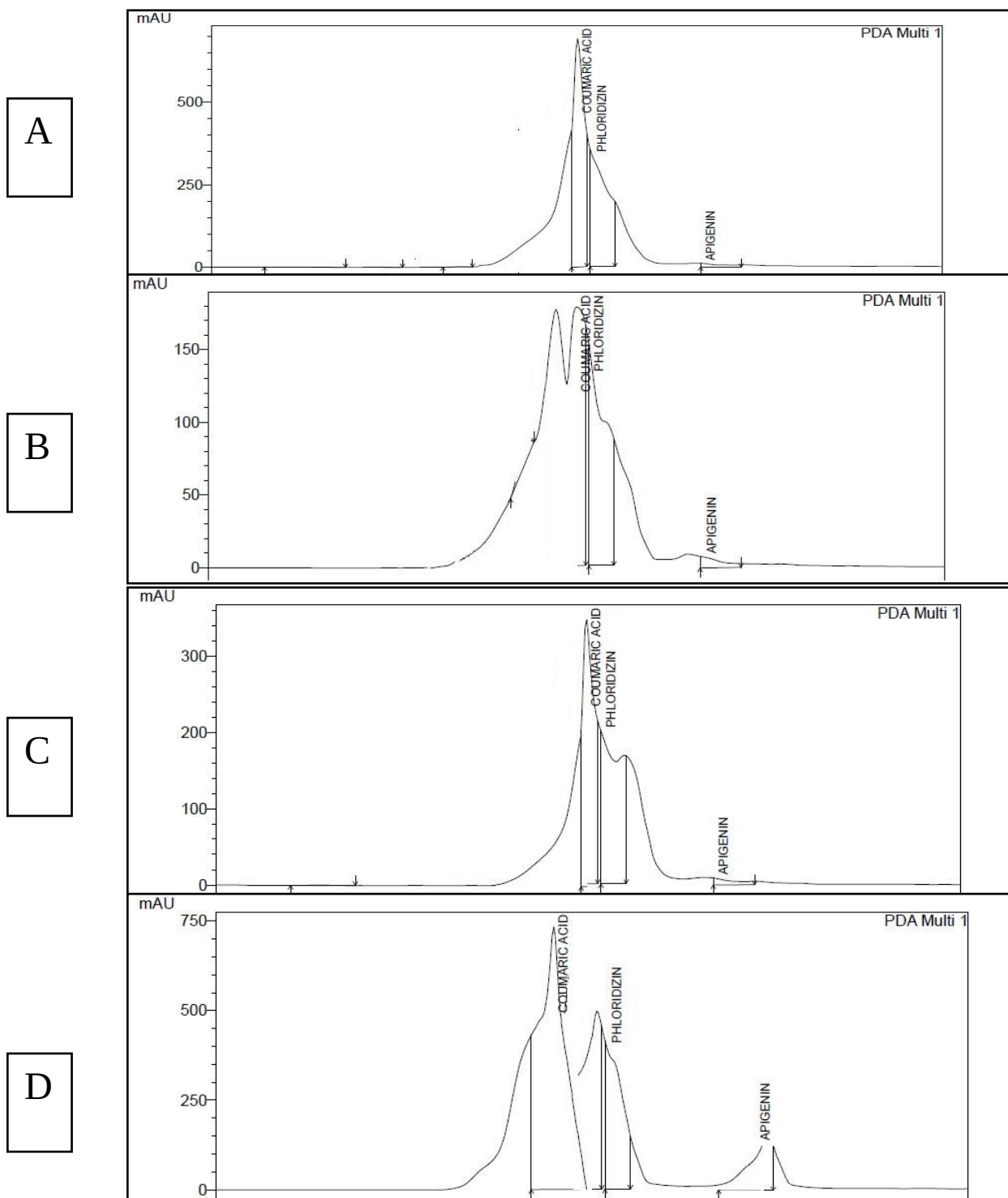
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[FASTA](#) [Graphics](#)

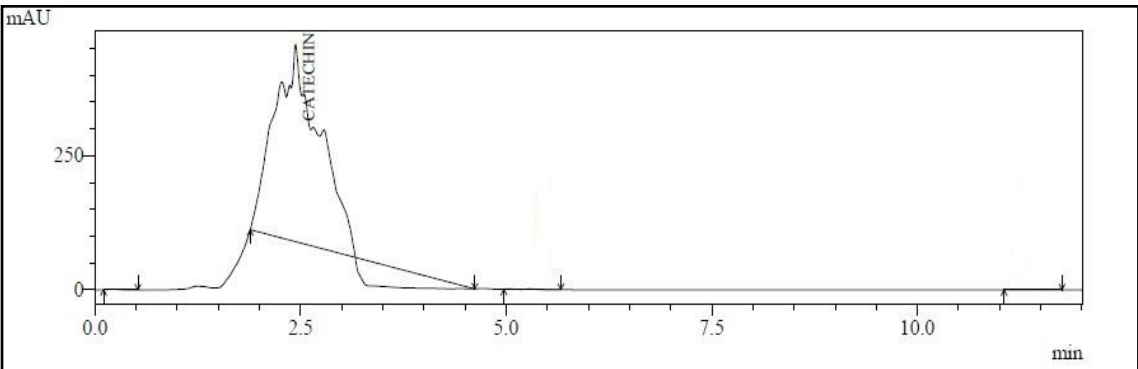
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ACCESSION	MK504433
VERSION	MK504433.1
KEYWORDS	.
SOURCE	Venturia inaequalis
ORGANISM	<i>Venturia inaequalis</i> Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Venturiales; Venturiaceae; Venturia.
REFERENCE	1 (bases 1 to 459)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	DNA Barcoding of Venturia inaequalis
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 459)
AUTHORS	Mansoor,S.S., Ahmed,N., Sharma,V., Mir,J.I., Nabi,S.U. and Masoodi,K.Z.
TITLE	Direct Submission
JOURNAL	Submitted (09-FEB-2019) Plant Biotechnology, SKUAST-Kashmir, Shalimar, Srinagar, J&K 190025, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
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Appendix-II

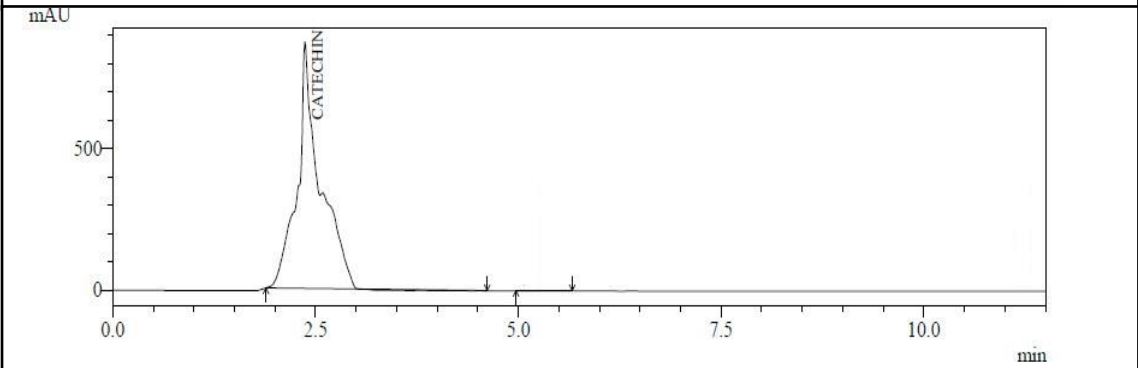
HPLC Chromatograms of water inoculated (control) A: Before inoculation, B: 1 day post inoculation, C: 7 days post inoculation and D: 24 days post inoculation



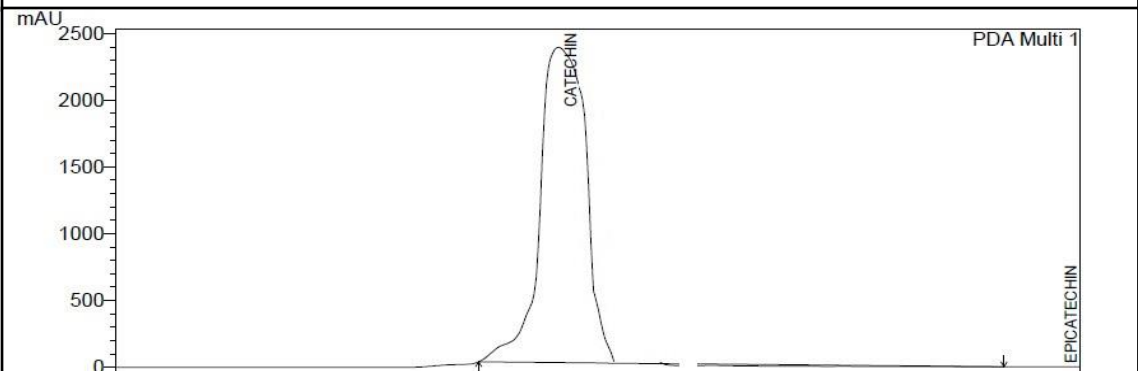
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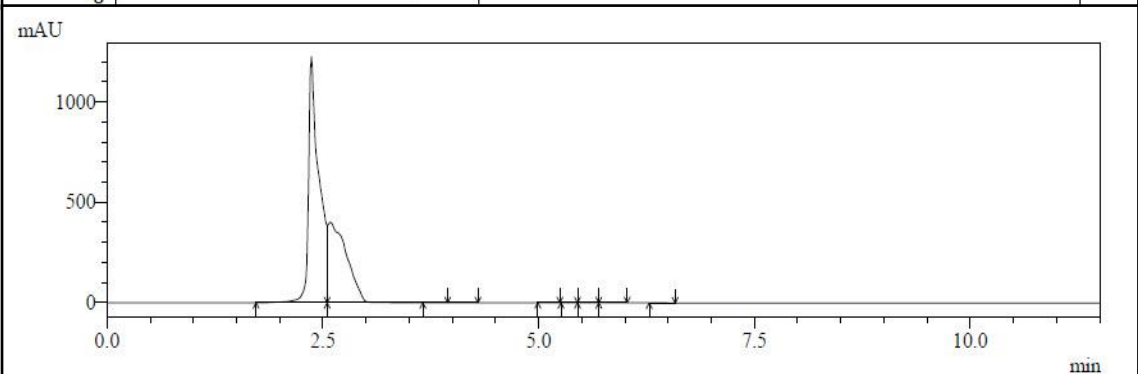
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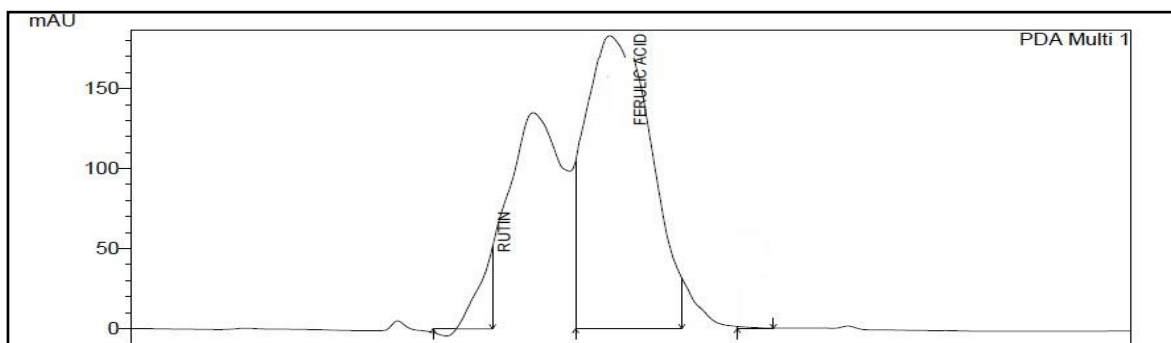
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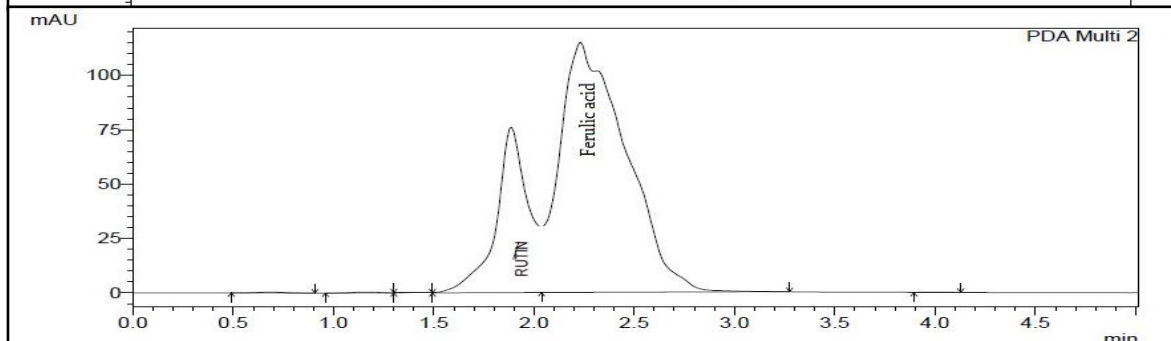
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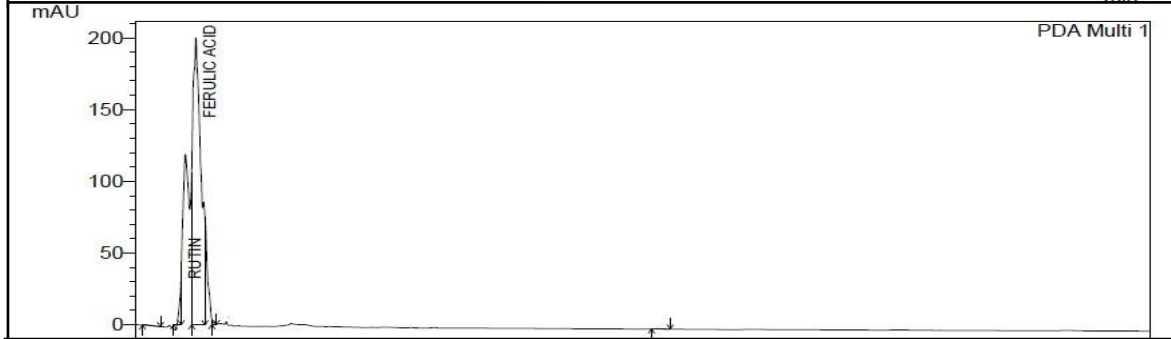
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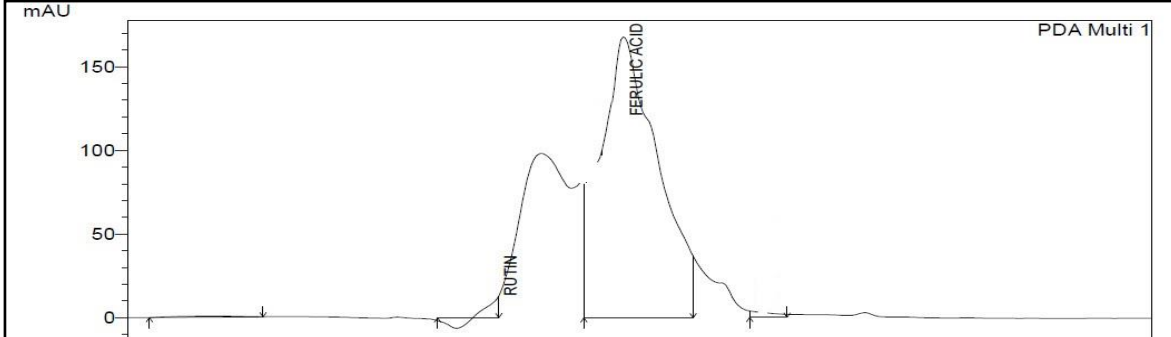
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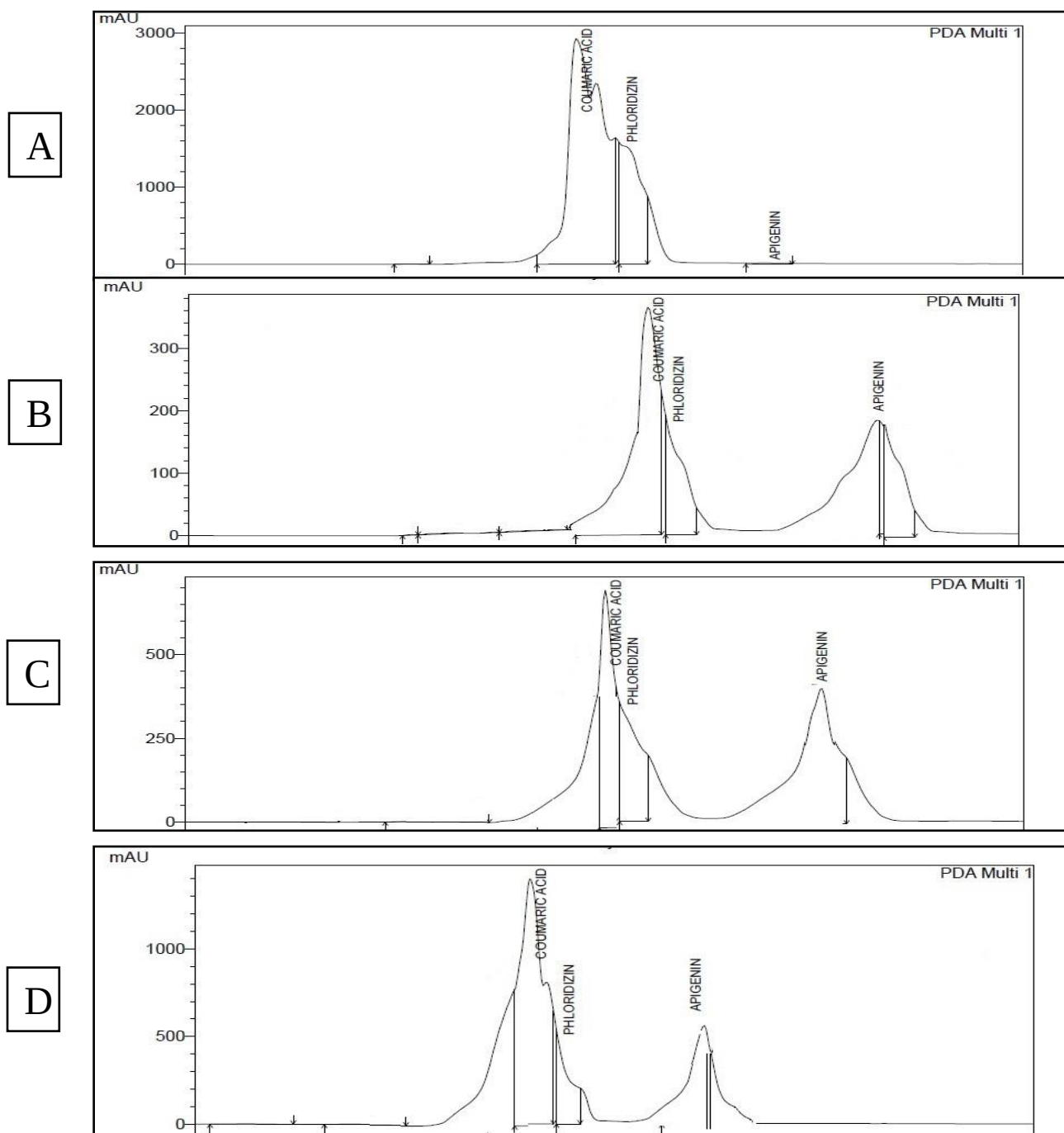
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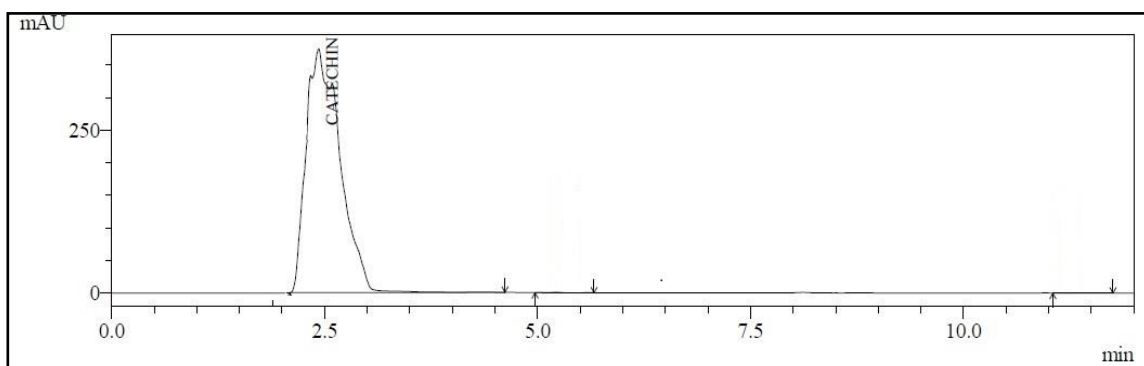
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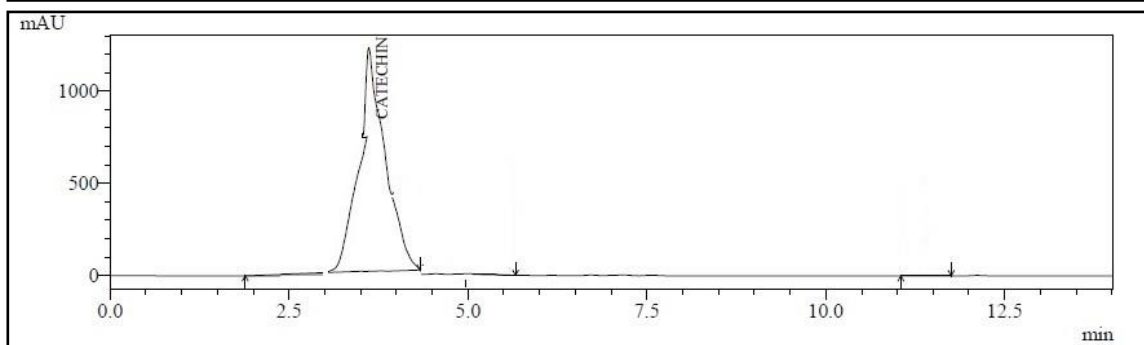
HPLC Chromatograms of *Venturia inaequalis* inoculated (Treated) A: Before inoculation, B: 1 day post inoculation, C: 7 days post inoculation and D: 24 days post inoculation



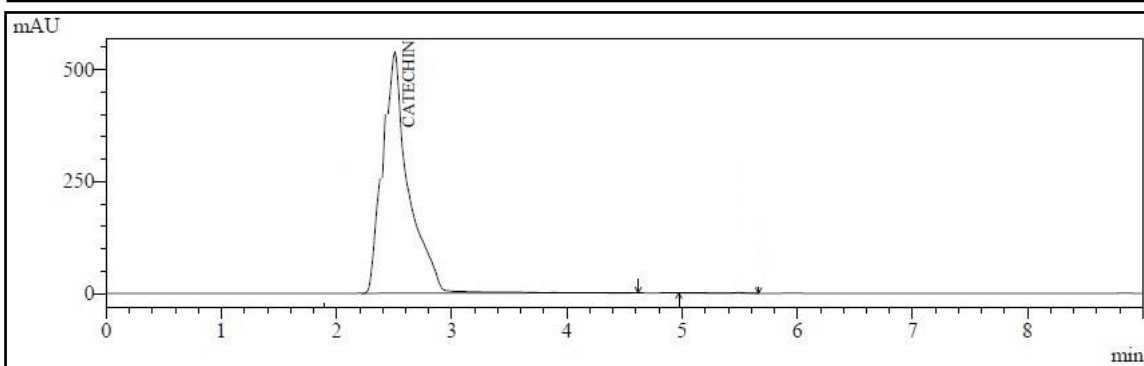
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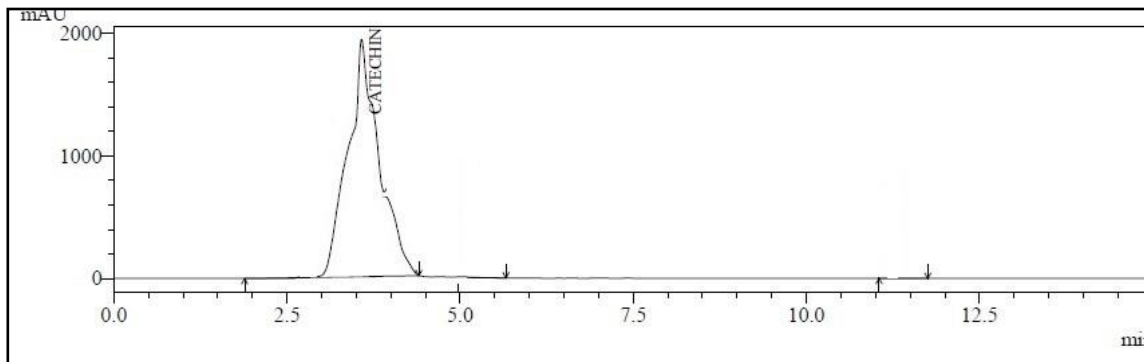
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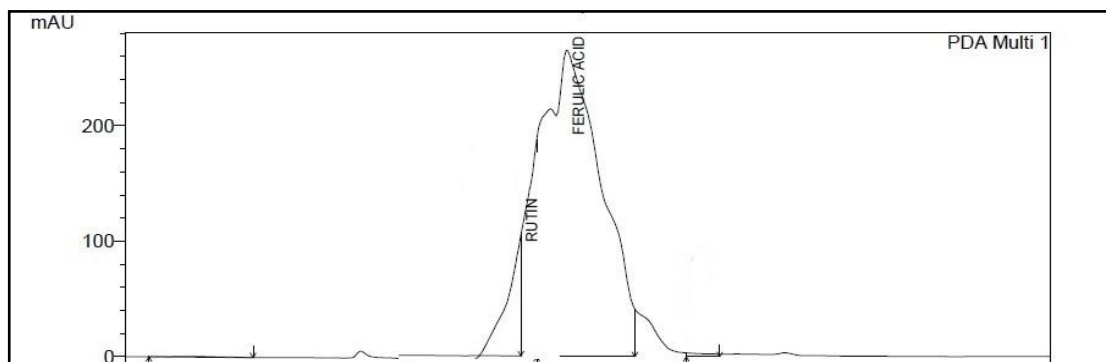
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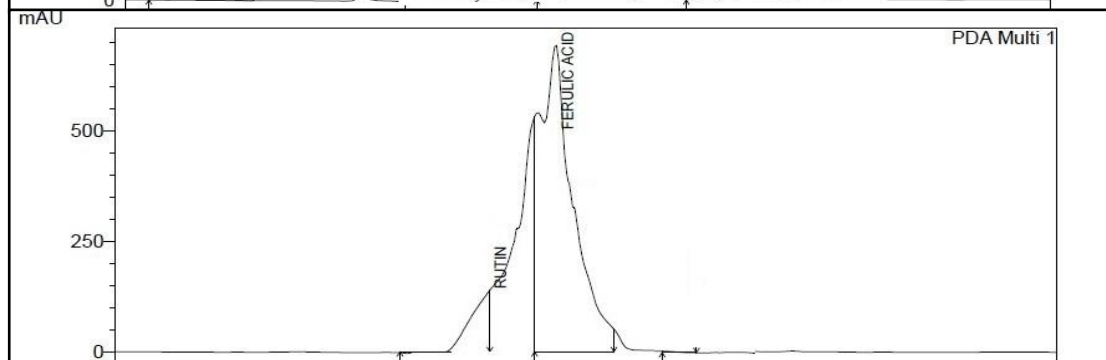
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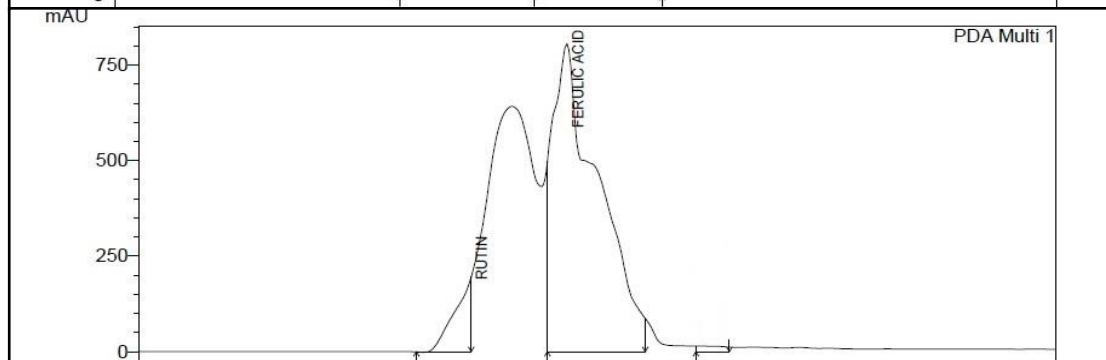
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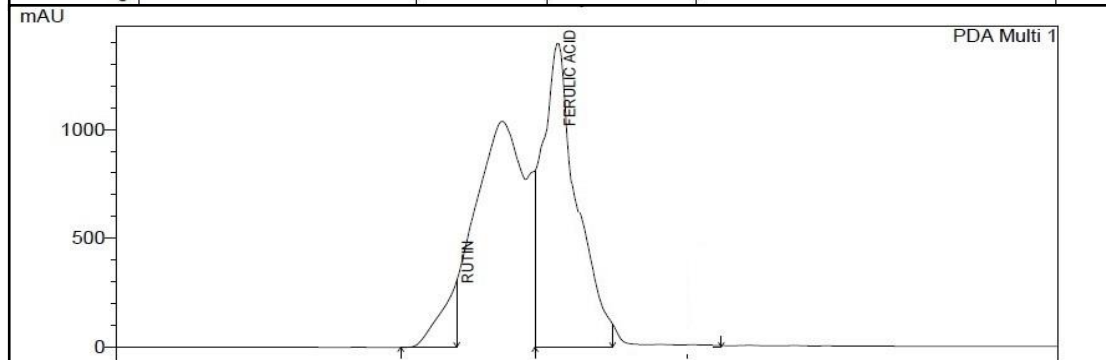
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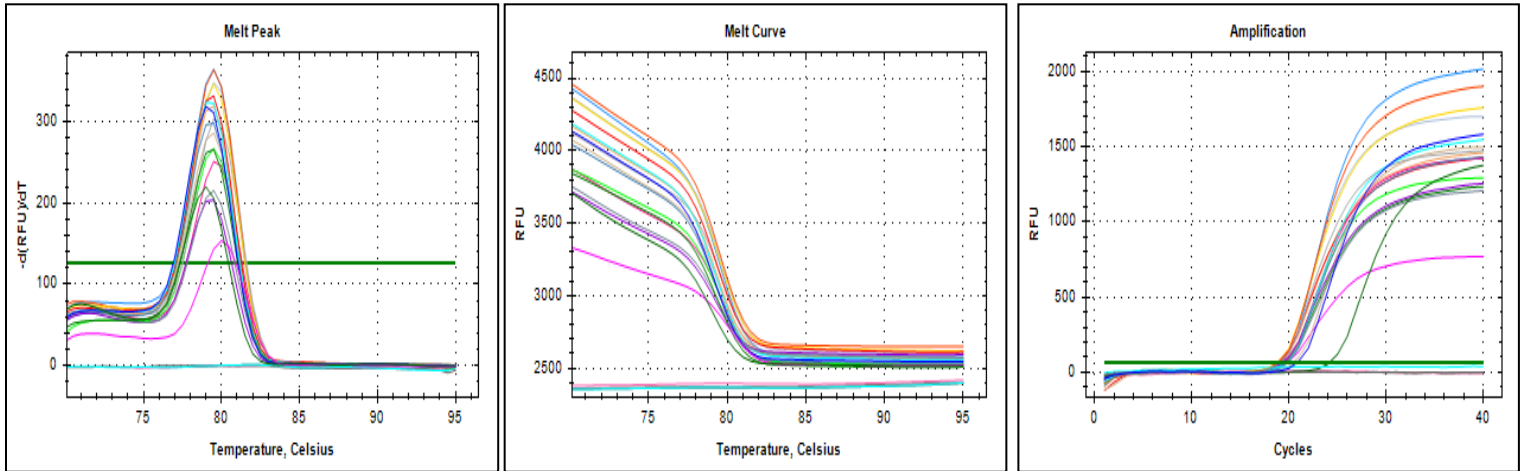
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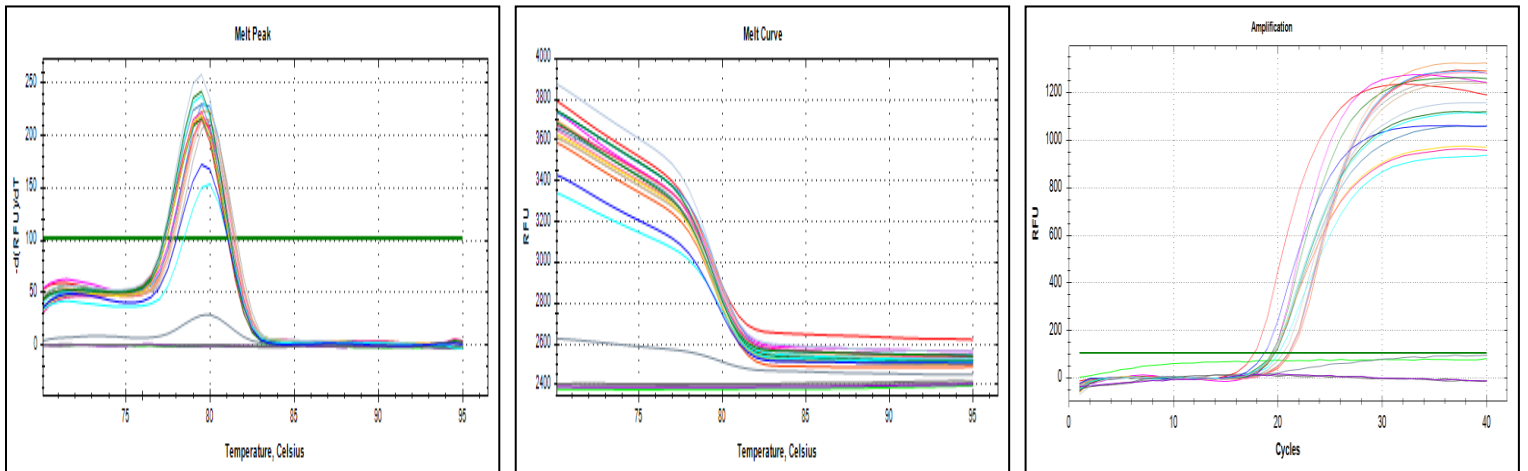
Appendix-III

Melt curve, melt peak and amplification curve of A. *PAL*, B. *CHS*

A

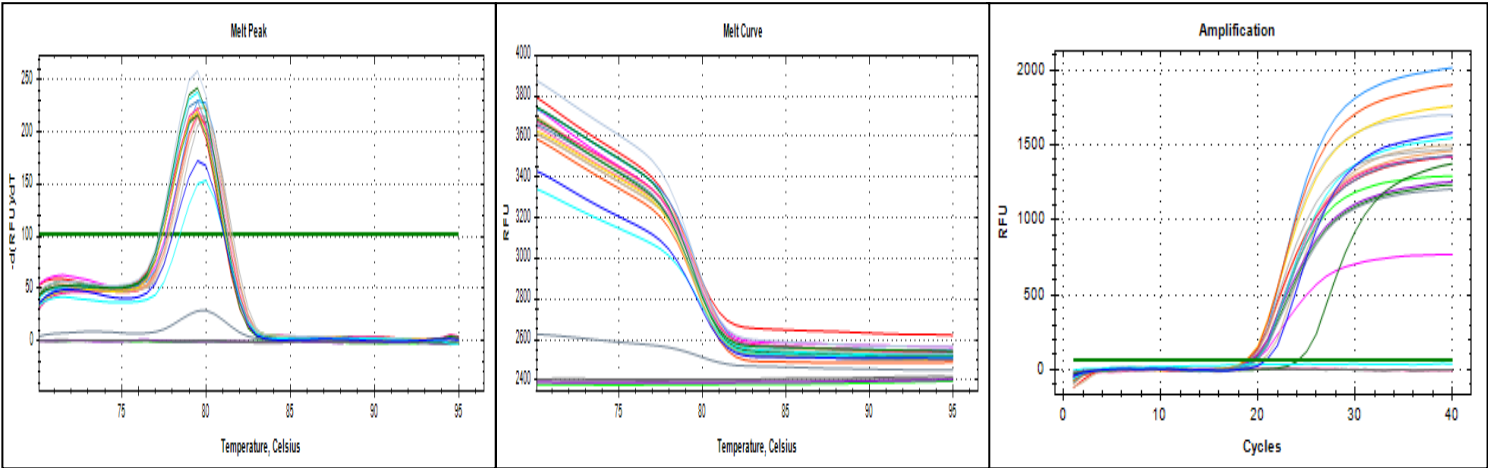


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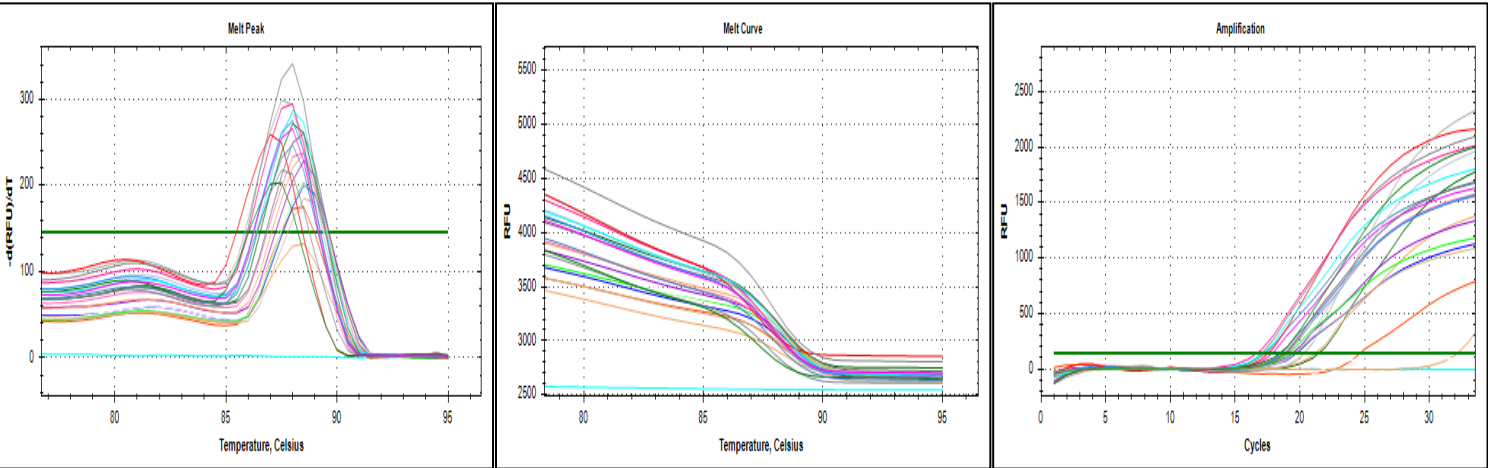


Melt curve, melt peak and amplification curve of *C.C3H* and *D. F3H* genes

C



D



A horizontal scroll graphic with a light gray background and a thin black border. The scroll is partially unrolled, with the top and bottom edges showing a slight curve. The word "VITA" is written in the center of the scroll in a black, elegant, cursive script. The scroll is positioned in the middle of the page, with a large amount of white space above and below it.

VITA

VITA

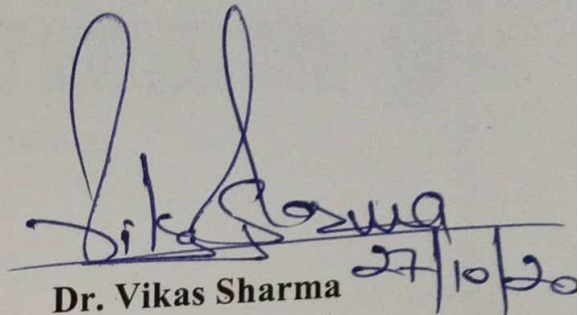
Name of the Student	:	Sheikh Mansoor Shafi
Father's Name	:	Mohammad Shafi Sheikh
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E mail	:	mansoorshafi21@gmail.com
Permanent address	:	House No. 86, Gadoora, Ganderbal, Kashmir-191131, J&K

EDUCATIONAL QUALIFICATION

Bachelor Degree	:	BSc. Biochemistry
University and year of award	:	University of Kashmir, 2010
Percentage of marks (OGPA)	:	54%
Master's degree	:	MSc. Biochemistry
University and year of award	:	HNBGU Uttarakhand, 2014
Percentage of marks (OGPA)	:	7.8/10.0
Ph. D Degree	:	Ph.D Biochemistry
University and year of award	:	SKUAST-Jammu, 2020
OGPA	:	7.64/10.0
Title of Ph.D thesis	:	DNA barcoding of different <i>Venturia inaequalis</i> strains and their effect on PAL biochemical pathway of <i>Malus domestica</i> Borkh

CERTIFICATE – IV

Certified that all the necessary corrections as suggested by the External Examiner and the Advisory Committee have been duly incorporated in the thesis entitled “DNA barcoding of different *Venturia inaequalis* strains and their effect on PAL biochemical pathway of *Malus domestica* Borkh” submitted by Mr. Sheikh Mansoor Shafi, Registration No. J-16-D-09-BS



Dr. Vikas Sharma
Major Advisor and Chairman
Advisory Committee

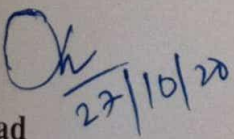
Place: Jammu

Date

27/10/20

Head

Division of Biochemistry, FBSc.





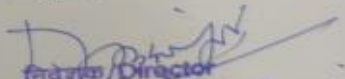
ICAR-CENTRAL INSTITUTE OF TEMPERATE HORTICULTURE

Old Air Field, Post Office Rangreth-191132, Srinagar, J&K (India)



CERTIFICATE

This is to certify that Mr. Sheikh Mansoor Shafi, Ph. D. Student, Division of Bio-Chemistry, SKUAST-J, Chatha has successfully completed part of his Ph.D. research work w.e.f. 01.03.2018 to 01.08.2018 on "Diversity analysis of *Venturia inaequalis* and Bio-chemical analysis "under guidance of **Dr. Javid Iqbal Mir** (Sr. Scientist), Department of Plant Biotechnology, ICAR-Central Institute of Temperate Horticulture, Srinagar, J & K.


निदेशक / Director
केन्द्रीय शीतोष्ण फल-संस्थान
Central Institute of Horticulture
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Srinagar (J&K)

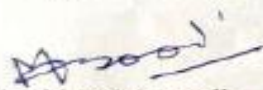


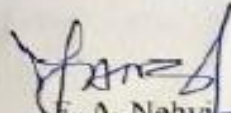
Division of Plant Biotechnology
Shalimar, Srinagar 190025; Tel: 0194-2262642

No: AU/CPB/2019/601
Date: 13-11-2019

To Whom it May Concern

This is to certify that vide No. AU/DR/PhD/2869-75, dated 5-9-2017. Mr. Sheikh Mansoor Shafi, Ph. D. Student of Division of Biochemistry, Faculty of Basic Sciences, SKUAST-Jammu, has conducted part of his Ph.D research work w.e.f. 01.09.2018 till 13.11.2019 under supervision of Dr. Khalid Z. Masoodi, Assistant Professor at Transcriptomics Laboratory, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir-Shalimar, Srinagar, J&K.


Dr. Khalid Z. Masoodi
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SKUAST-Kashmir, Shalimar, Srinagar


F. A. Nehvi
Prof. & Head
Division of Plant Biotechnology
SKUAST



PUBLICATIONS

(from Ph.D research work.)

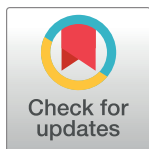
RESEARCH ARTICLE

Elucidating genetic variability and population structure in *Venturia inaequalis* associated with apple scab disease using SSR markers

Sheikh Mansoor¹, Nazeer Ahmed², Vikas Sharma¹, Sumira Jan³, Sajad Un Nabi³, Javid I. Mir³, Mudasir A. Mir², Khalid Z. Masoodi^{2*}

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Abstract

Apple scab caused by *Venturia inaequalis* Cooke (Wint.) is one of the important diseases of trade and industrial significance in apple. In present study variability studies in pathogen isolates were studied, which is one of the most important factors for devising management studies of scab disease in apple. Genetic diversity of 30 *Venturia inaequalis* isolates from 12 districts of two geographical distinct regions of Jammu and Kashmir was calculated based on the allele frequencies of 28 SSR markers and the internal transcribed spacer (ITS) region of the ribosomal DNA. The ITS based characterized sequences were submitted to NCBI GenBank and accession numbers were sanctioned. Dendrogram showed that all the accessions formed 2 main clusters with various degree of sub clustering within the clusters. Analysis based on SSR study reveals that the heterozygosity ranged from 0.0 and 0.5, with an average value of 0.39. The expected heterozygosity or gene diversity (H_e) ranged from 0.0 to 0.50 with an average of 0.40. The F_{st} value ranges from 0 to 0.6 with an average of 0.194. Diversity within each population (H_s) values ranging from 0.26 to 0.33. Average differentiation among populations (G_{ST}) was 0.11 and populations were isolated by significant distance ($r^2 = 0.50$, $P < 0.01$). From the AMOVA analysis, 25% of variation was observed among population, 9% among individuals and 66% within individuals observed in the population. Structure analysis grouped isolates into two populations. Principle coordinate analysis explained variation of 36.6% in population 1, 14.30% in population 2 and 13.10% in population 3 (Admixture) with 64.07% as overall cumulative percentage of variation. This indicates that extensive short-distance gene flow occurs in Kashmir region that dispersal over longer distances also appears to occur frequently enough to prevent differentiation due to genetic drift. Also it is evident that Jammu and Kashmir most likely has *V. inaequalis* subpopulations linked to diverse climatic conditions of the Jammu region compared to the mountainous inland Kashmir region. The results of present study would help to understand the genetic diversity of *V. inaequalis* from Jammu and Kashmir that would lead in the development of more effective management strategies and development of new resistant cultivars through marker-assisted selection.

OPEN ACCESS

Citation: Mansoor S, Ahmed N, Sharma V, Jan S, Nabi SU, Mir JI, et al. (2019) Elucidating genetic variability and population structure in *Venturia inaequalis* associated with apple scab disease using SSR markers. PLoS ONE 14(11): e0224300. <https://doi.org/10.1371/journal.pone.0224300>

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Data Availability Statement: Sequences available in NCBI Genbank with accession numbers <https://www.ncbi.nlm.nih.gov/nucleotide/> MK478885.1 MK359025.1 MK504436.1 MK478887.1 MK359026.1 MK504428.1 MK504429.1 MK359032.1 MK530205.1 MK359027.1 MK359028.1 MK367580.1 MK504434.1 MK359029.1 MK504430.1 MK504437.1 MK504431.1 MK504435.1 MK359031.1 MK359030.1 MK583539.1 MK504432.1 MK504433.1 MK532037.1 MK532036.1

MK532035.1 MK532034.1 MK532033.1
MK532032.1 MK532031.1

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Competing interests: The authors have declared that no competing interests exist.

Introduction

Apple (*Malus × domestica* Borkh.) is most widely and commercially cultivated species in the genus *Malus* throughout temperate regions of world [1]. It is susceptible to number of diseases incited by fungi, bacteria, viruses, viroids and phytoplasmas [2]. Scab is very severe and among one of the mainsignificant diseasesall through the world in terms of economic losses in temperate regions with cool and moist climate during spring [3]. It ranks number one disease in terms of yield loss, which poses potential threat to apple industry [4]. This disease imposes a severe threat in commercial apple growing regions, due to premature fruit drop and unmarketable diseased fruits and results in losses up to 70% [5] or even complete crop loss is possible if prophylactic steps are not taken in the orchard for its management [6]. Scab disease is caused by *Venturia inaequalis* Cooke (Wint.) which is an ascomycetous heterothallic and hemibiotrophic fungus. Earlier, this genus was included in the family *Venturiaceae*, order *Pleosporales*, according to its “Pleospora-type centrum and bitunicate asci” [7]. on the other hand, recent molecular phylogenetic analyses of Dothideomycetes, using both nuclear and mitochondrial gene regions, have indicated that the family *Venturiaceae* forms a well-supported monophyletic group separate from the *Pleosporales* [8, 9]. Thus, Zhang recently reordered *Venturiaceae* into *Venturiales*. It has a broad geographic dimension and is found in almost all apples growing areas. The fungus exists in two states i.e., saprophytic (sexual state *Venturia inaequalis* (Cke) and parasitic (asexual state *Spilocaea pomi* Fr) [3]. It overwinters as pseudothecia in regions with severe winter, whereas, conidia in dormant buds in regions with moderate winter [10]. In early spring, when temperature and moisture are suitable, Ascospores start maturing and are released forcibly in air [11].

One sexual and multiple asexual cycles, of this pathogen annually causes noteworthy variations in *Venturia inaequalis* population [12]. Recombination takes place by sexual reproduction which ultimately leads to high variation and diversity in fungi and also changes population genetic structure [13]. In devising the management strategies against the disease, important factor that is taken into contemplation is variation within pathogen population [14]. Detailed investigations about pathogens variation and population genetic structure in different geographical regions are required, which reflects the history as well as evolutionary potential of the pathogen [15] and also give an idea about centers of origin of this pathogen [16]. In wide range of organisms the ribosomal rRNA genes sequence investigation and the internal transcribed spacer (ITS) region is used as new tool in phylogenetic relationship studies [17]. As rRNA repeat develop slower as a result it is very handy for studying secluded related organisms [18]. Besides sequence divergence in ITS sequences, polymerase chain reaction (PCR) amplification length polymorphism in nuclear rDNA due to intron insertion has also been used to assess the extent of genetic variability within populations [19]. As per reports ITS based analysis is the best was to identify sub species than rbcL and matK [20]. Keeping in view the above background information, detailed investigations on the molecular characterization, genetic diversity and population structure of the *Venturia inaequalis* in numerous apple growing districts of Jammu and Kashmir was carried out.

Materials and methods

Collection of isolates

Samples were collected from 12 apple growing districts of Jammu and Kashmir India during the year 2017–18 as shown in Table 1. The locations sites from where samples were collected are provided in Fig 1. Diseases samples including only apple leaves with scab symptoms were collected from May to September 2018–19. Most of the cultivars were Red Delicious and

Table 1. Samples collected from two regions of Jammu and Kashmir with sample code, geographic location, latitude, longitude and accession number provided by Genbank from various apple growing areas.

S.No	Sample Code	Location	District	Latitude	Longitude	Accession number
Kashmir region isolates						
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2	M2	Bankoot	Bandipora	34.420°N	74.650°E	MK359025.1
3	M3	Char-i-sharief	Budgam	33.862°N	74.766°E	MK504436.1
4	M4	Syedpora	Shopian	33.72°N	74.83°E	MK478887.1
5	M5	Beerva	Budgam	34° 001°N	74.5953°E	MK359026.1
6	M6	Chadoora	Budgam	33.802°N	75.100°E	MK504428.1
7	M7	Batpora	Srinagar	34.936°N	74.464°E	MK504429.1
8	M8	Hajin	Bandipora	34.09°N	74.79°E	MK359032.1
9	M9	Yaripora	Kulgam	33.7°N	75.0°E	MK530205.1
10	M10	Handwara	Kupwara	34.40°N	74.28°E	MK359027.1
11	M11	Kakapora	Pulwama	33.88°N	74.92°E	MK359028.1
12	M12	Wakura	Ganderbal	34.05°N	74.47°E	MK367580.1
13	M13	Gutlibagh	Ganderbal	34.09°N	74.09°E	MK504434.1
14	M14	Naidkhai	Bandipora	34.09°N	74.79°E	MK359029.1
15	M15	Gantmulla	Baramulla	34.086°N	74.033°E	MK504430.1
16	M16	Pinjura	Shopian	33.72°N	74.83°E	MK504437.1
17	M17	Pattan	Baramulla	34.85°N	74.37°E	MK504431.1
18	M18	Sogam	Budgam	34.020°N	74.780°E	MK504435.1
19	M19	Mattan	Anantnag	33.701°N	75.285°E	MK359031.1
25	M25	Uri	Baramula	34.080°N	74.05°E	MK532035.1
Jammu region isolates						
20	M20	PranuBaderwah	Doda	32.58° N	75.538° E.	MK359030.1
21	M21	NalthiBaderwah	Doda	32.93°N	75.712°E	MK583539.1
22	M22	Doda	Doda	33.13°N	75.57°E	MK504432.1
23	M23	Kishtwar	Kishtwar	33.32°N	75.77°E	MK504433.1
24	M24	Tund	Kishtwar	33.32°N	75.77°E	MK532037.1
26	M26	Faisal Abad	Kishtwar	33.32°N	75.77°E	MK532036.1
27	M27	Padder	Kishtwar	33.13°N	75.09°E	MK532034.1
28	M28	Padder	Kishtwar	33.13°N	75.220°E	MK532033.1
29	M29	KotiBaderwah	Doda	33.145°N	75.547°E	MK532032.1
30	M30	Baderwah	Doda	33.13°N	75.57°E	MK532031.1

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Golden Delicious as these are most cultivated apple varieties grown in Jammu & Kashmir. Sampling was carried out from trees having at least two to three scab lesions on leaf. The samples were collected as part of thesis work and due permissions from farmers of the various orchards. The present study did not involve any endangered or protected species of the region.

Isolation, purification and identification of fungal cultures

The fungus from the infected samples was isolated and purified using monoconidial method by streaking out spores on plates containing 2% water agar, pure fungal cultures were obtained by transferring single germinated conidium on potato dextrose agar containing antibacterial chloromphenicol (50 µg/ ml) to avoid bacterial contaminations[21]. Total 30 cultures were identified by comparing with available literature[22] and maintained for further studies. The spores were also verified using compound microscope (Olympus) at different resolutions 4x to 40x.

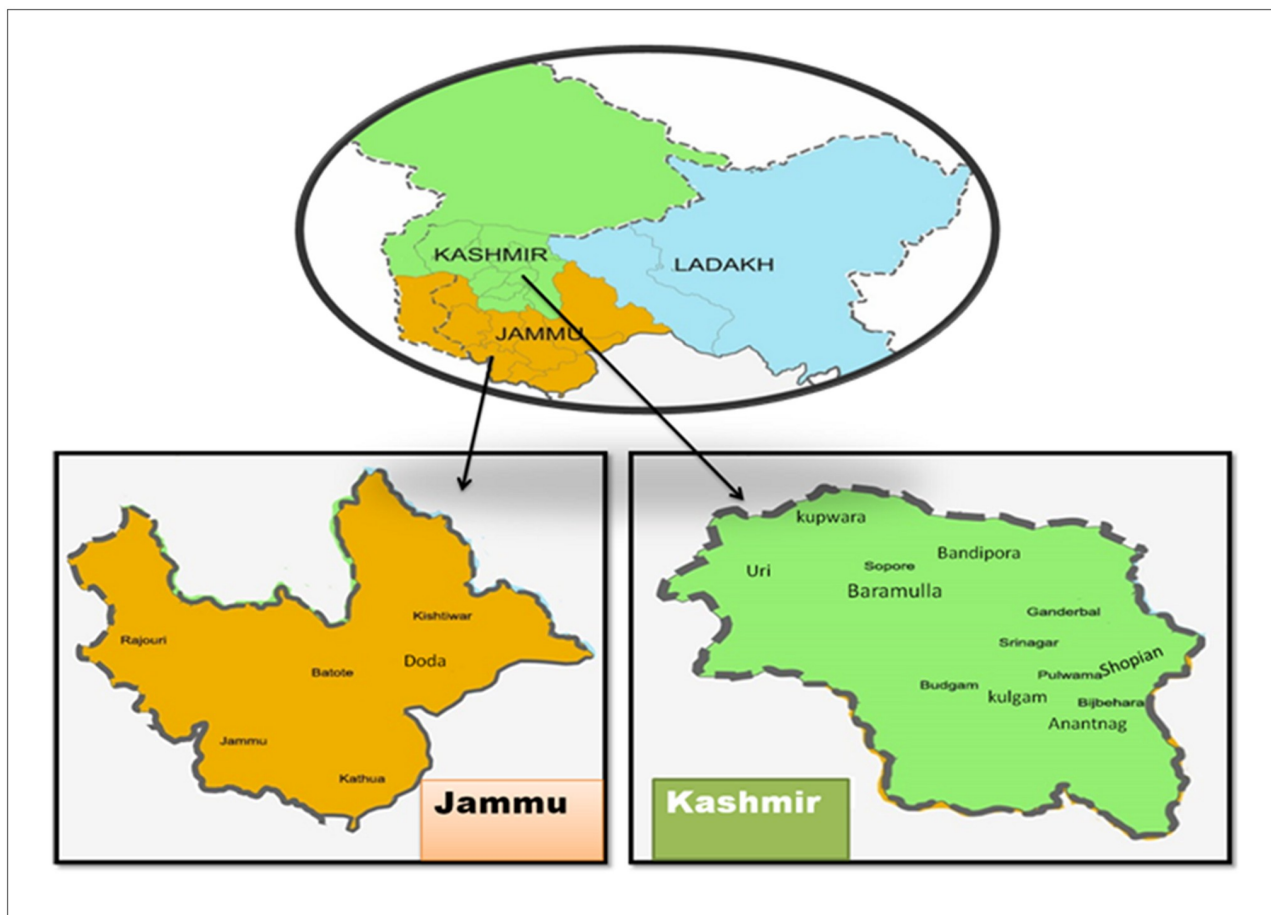


Fig 1. Two apple growing regions of Jammu and Kashmir and areas from where sample collection was carried out.

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DNA extraction

Cultured fungal isolates in 100ml of potato dextrose broth was kept in incubator-shaker at 19°C for about 25–30 days under continuous dark. The mycelia harvested was blotted dry between the tissue layers and immediately frozen in liquid nitrogen. After freeze-drying, DNA was extracted using Fungal DNA isolation kit (GCC Biotech India Pvt. Ltd). The DNA was quantitatively and qualitatively checked using a Nanodropspectrophotometer (Themoscientific) and was further diluted to a working concentration of 30ng/μl and stored at -20°C for further use.

PCR amplifications

ITS rDNA amplification. All 30 isolates were amplified using polymerase chain reaction (PCR) in a thermal cycler (Takara Japan) using 30ng of genomic DNA in a final volume of 25 μl per reaction. The universal ITS primers with ITS 1 as forward and ITS4 as reverse primer were used for PCR amplification (White et al., 1991). The PCR was performed in a 0.2-ml tube containing 0.5μM forward and reverse primer, 200 μM each dNTP, 1 unit kappa Taq polymerase and 1ul of genomic DNA in a 10xkappa buffer and 5 mM MgCl₂. The PCR was normalized after repetitive cycles till optimal amplification was achieved and consists of 35 cycles involving initial denaturation step at 94°C for 5 min, followed by 94°C for 30 s, annealing at

53°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 15 min [23]. The PCR products were electrophoresed in 1% agarose gel in 0.5 X Tris-Borate-EDTA buffers (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA and pH 8.5) at 110 V. For estimating amplicon size, 100 bp DNA molecular ladder was used (ABgene, UK) and electrophoresis was done for 1 hour [24]. The fragments were observed under UV lamp in gel-documentation (Bio Rad, Gel Doc XR system 170–8170).

SSR amplification. For diversity and structure analysis of selected fungal samples, 28 published SSR primer pairs were used Table 2 [11, 13]. Conditions for PCR were initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation step at 94°C for 30 s, 45 s of annealing at 50–60°C, 1 min of extension at 72°C, and a final extension of 15 min at 72°C which was performed in a 10 µl final volume containing 2 µl of 10X PCR buffer, 3 mM MgCl₂, 0.5 mM dNTP, 0.5 µl of *Taq* DNA polymerase (kappa), 1 µM of each primer and 1 µl DNA template [23]. The amplified PCR products were resolved in 2.5% agarose gel at 110 V for 3 h. The bands amplified in different isolates using SSR primers in gel were visualized using a Gel documentation system (Bio Rad, Gel Doc XR system 170–8170).

Sequencing, nucleotide alignment and phylogenetic analysis. Amplified PCR products were sequenced at Agri Genome Labs (Infopark Road, Kakkanad, Kerala, India). Primers for the sequencing PCR product were the same as for the PCR amplification. The sequences of PCR products were assembled using DNA Baser V.4 program to produce complete contig. These were further aligned using CLUSTAL W method of Bio-Edit software and aligned sequences were deposited in NCBI GenBank. A database search of homologous sequences was performed by BLAST analysis at NCBI (<http://ncbi.nlm.nih.gov/BLAST>). The sequence generated from the present study and reference strain sequences retrieved from GenBank were used to construct phylogeny by neighbour joining method with 1000 replications for each bootstrap value using MEGA 7.0 software version [25]. The other species of *Venturia pirina* and *nashicola* were also included in phylogeny to separate *Venturia inaequalis* from these species. For validation of results, an out group non-fungal pathogen *Pseudomonas syringae* was selected.

Statistical analysis. Analysis was carried out using POPGENE for gene frequency, allele number, effective allele number, polymorphic loci, gene diversity, Shannon index, gene flow, genetic distance. The GenAlEx version 6.5 for distance-based analysis like AMOVA (Analysis of molecular variance), and PCoA. (Principle coordinate analysis) [26, 27]. The scoring was done as base pair scoring and binary scoring in which bands were scored as '1' (for presence) and '0' (absence) [28]. Index of association *r_d* statistics was applied to examine associations of alleles among different loci [29, 30], which is a comprehensive measure of multilocus linkage disequilibrium [30]. DARwin software version 5.0.158 was used in phylogenetic analysis [31]. Population structure and individual clustering (K) was done by means of Structure software ver. 2.3.4 [32], ΔK method [33] was applied to best estimate K, and was computed using Structure Harvester ver. 0.56.3 [34, 35].

Results

Morphological identification

Identification based on morphological characters from fungal culture (Fig 2a) revealed that the conidia are single-celled, uninucleate and narrower at one end than the other (Fig 2b). In mass, conidia appear brown or olive, but they are lighter when viewed individually under the microscope. Conidia ranges from 6 to 12 µm wide and 12 to 22 µm long and are produced by specialized short hyphae called conidiophore. The characters observed were similar to those described by [22] for *Venturia inaequalis*.

Table 2. List of 28 SSR primers with forward (F) and reverse(R) sequence, allele size and annealing temperature.

Locus	Primer sequence 5'-3'	Allele Size (bp)	Annealing Temp.
<i>Vitc1/2</i>	F:CTTACCTCTCACTTGCTAAC R:GTTCTGACAAGACTGTGTTG	173–241	58
<i>Vitc1/82</i>	F:ACTGTCTCTAGGCGAAAG R:ACTTGAAGCTCGCTAAG	227–247	58
<i>Vitc1/130</i>	F:GATTGGTGACGCATGTGT R:GCTGGAGATTGCGTAGAC	132–152	58
<i>Vitc2/D</i>	F:GCTCCTTCTGGGTAAGA R:CTCTACATCTCATCCCATC	184–278	58
<i>Viaggt8/1</i>	F:GTGCGGAATATCCAAGTA R:CCAGACTCCTCTACTACAACC	188–196	58
<i>Vica9/152</i>	F:GCACCTGCTCTGTCTATCTC R:AAGGTTCAAGGCACTGGAG	167–191	58
<i>Vitca7/P</i>	F:GAATACTTCCAAGTGCACAG R:GTGACGCGGATATGGTAC	192–224	58
<i>Vitg11/70</i>	F:GAAGAGGTTGGAGTGGTTG R:GAACCGAATCTGTACAGGAC	184–196	60
<i>Vica9/134</i>	F:ATACAGGAGTGAACAGCAGG R:ATACTGCTCACTTGCGTGC	228–236	60
<i>Vigt10/95</i>	F:AGGTGTTGCTGTCTTGAG R:CGATAGTGTCTTTCCAATCC	134–169	58
<i>Vicag8/42</i>	F:TGTCAGCCACGCTAGAAG R:CACCGACGAATCATGC	196–232	60
<i>Vigt8/146</i>	F:TGGAGAGAAGAGAAGAGTGG R:GGCAACATCCAATAATCG	128–134	60
<i>Vitc2/16</i>	F:ACATTGACGAAGACGAGC R:TACAATTGAGGCGTGTCC	147–165	58
<i>Viga3/Z</i>	F:ACGCCCTCCTTACTTCTTG R:CCCTCGTATTACGTTCTC	87–97	58
<i>Viga7/116</i>	F:GCCTGGTTGTGGATCTGTC R:ATCCTGCTACATCGACCTTC	159–173	60
<i>Vitg9/99</i>	F:CCGTGTCGAGACCTAATATC R:TGGCTCTTCGTAAGTCCTTC	155–167	58
<i>Vica9/X</i>	F:TCGCGCATCACTATCTACAC R:AGACAGGAATGTGGTGAAG	225–239	58
<i>Vica10/154</i>	F:CCTCCTTCCTATTACTCTCG R:CTGAAGCGAACCTATGTCC	108–172	58
<i>ViaacS10</i>	F:ATTCCAAGCCTTACAACACCC R:TCCACTTCACCCATCGTTC	180–186	58
<i>Vigt10/ε</i>	F:GCAGTGCAGGAATAGTAAGG R:GCTGTGATACCAGAGAACGA	171–173	60
<i>Vitg9/129</i>	F:CTAATTCAACTCGCTGCGTC R:TTTCAGCCAGCTAACCTAGG	267–285	58
<i>l1c1a</i>	F:TCGAGATCCTCAAACCTCCTT R:TTTAACTGTGCGGCCTG	109–187	54
<i>l1c1b</i>	F:CGATTGGGGATATGAAGACTT R:TTAGTAATCAAATCGCACCCA	149–210	54
<i>l1c1g</i>	F:TCACTCAACAATACAGTTTCTTAG R:TTTCACGGTAGCGATAGGAG	111–185	57
<i>laac3b</i>	F:AGCGCTAGGTCGTGAAATC R:TTTCTGAAGTGTGTGGGACAT	118–174	55
<i>laac4b</i>	F:GGTGAGGAGGGAGACGAG R:CATCAGCCCCCTATCAAAC	166–177	58
<i>laac4f</i>	F:CTTGACAGACCACAGCGAC R:CTGACTGAGAGTGGCATCG	96–116	58
<i>laac4h</i>	F:TCGTTTCATCGTTCTGTTTTTCG R:AATAGTGCGTACCCATATATCCA	198–201	56

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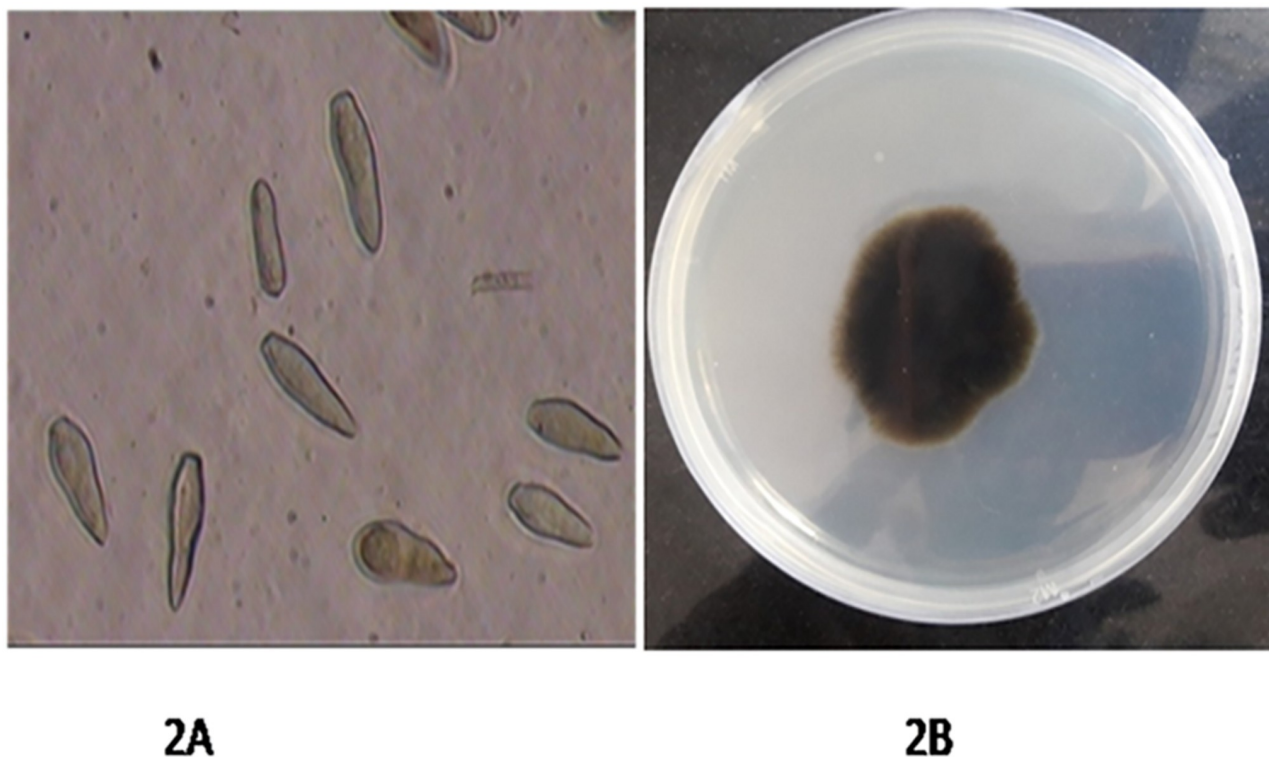


Fig 2. The spores verified under compound microscope (Olympus) at 10x to 40x. 2A. Conidia observed under 40 x resolutions. 2B. Pure culture of *Venturia inaequalis*.

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Molecular characterization

The ITS based primers amplified ~550bp amplicon products after sequencing were run for BLASTn and all obtained sequences showed 96%-98% sequence homology with *Venturia inaequalis* GenBank submitted sequences. Sequences were submitted to NCBI GenBank and accession numbers were received (Table 1). Phylogenetic analysis revealed that our isolates clustered along with other submitted *Venturia inaequalis* isolates in GenBank. The sequences of other isolates *Venturia pirina* and *Venturia nashicola* formed separate subclusters. *Pseudomonas syringae* formed a different cluster (outgroup) in phylogeny (Fig 3). During present study the molecular characterization using ITS ribotyping of 30 isolates collected from two regions of J&K showed sequence homology with isolates reported from different regions of worlds particularly Iran (Khe1) and (MG2), South Africa (KELB2), India (Vi22) and Netherland (CBS).

SSR genotyping and genetic diversity analysis

In total 30 *Venturia inaequalis* isolates were genotyped using 28 SSR markers. The results obtained through POPGENE software analysis for genetic diversity parameters are presented in (Table 3). The number of alleles per marker varied from 1 to 15 with an average of 4 per locus detecting the highest number of 15 alleles from Vitc1/2 and Vitc1/130 in the set of 30 isolates. The mean PIC value was found to be 0.073 with minimum value of 0.014 (Vitc1/130) and maximum of 0.195 (Vitg11/70). The average heterozygosity (Ave_Het) ranged between 0.0 and 0.5, with an average of 0.39. The expected heterozygosity or gene diversity (He) ranged from 0.0 to 0.50 with an average of 0.40. The effective number of alleles (Ne) ranged from 1.0

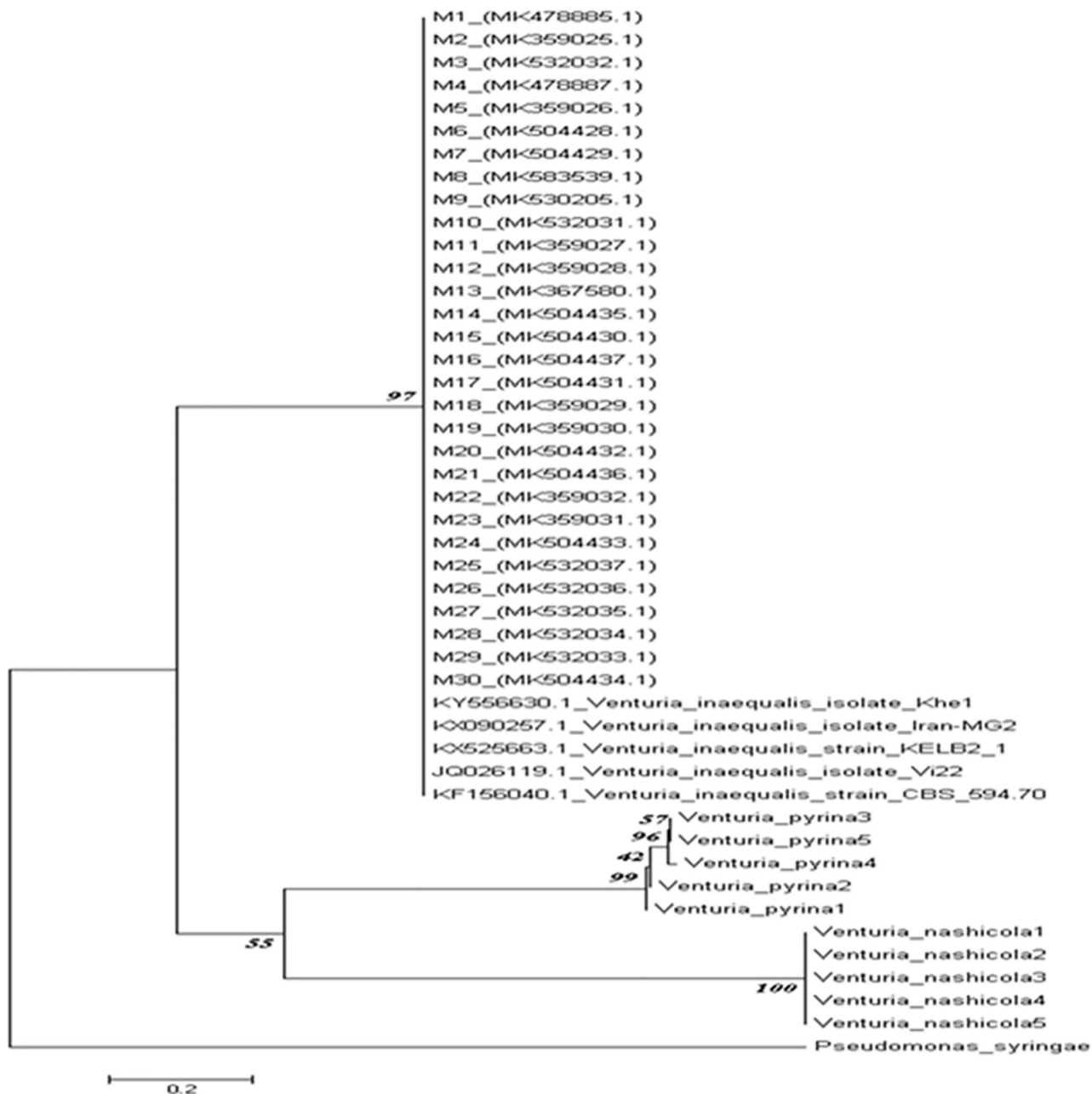


Fig 3. Phylogenetic relationship of *Venturia inaequalis* using the internaltranscribed spacer (ITS) region gene nucleotide sequence alignment with Bootstrap values supporting the branches are shown at nodes; branch lengths are proportional to divergence.

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to 2.0 with an average of 1.74. Shannon index (I) an estimate of diversity ranged from 0 to 0.6 with an average of 0.57. The Fst value ranges from 0 to 0.6 with an average of 0.194.

Cluster analysis

The cluster analysis of 30 isolates revealed a high genotypic diversity within *Venturia inaequalis* populations. Three major clusters I, II, III were obtained using neighbour joining method in Darwin 5.0 software using SSR scoring data. The cluster I accommodated 11 isolates (M20

Table 3. Summary of the genetic analysis of *Venturia inaequalis* isolates with 28 SSR loci.

Locus	Ne	I	Ob_He	Exp_Ho	Exp_He	Nei	Obs_Hom	Ave_Het	Pic	Fst
Vitc1/2	1.25	0.36	0.23	0.79	0.20	0.20	0.76	0.20	0.20	0.15
Vitc1/82	1.18	0.28	0.16	0.84	0.15	0.15	0.83	0.15	0.15	0.08
Vitc1/130	1.92	0.67	0.66	0.51	0.48	0.48	0.33	0.48	0.10	0.32
Vitc2/D	1.38	0.45	0.33	0.71	0.28	0.27	0.66	0.27	0.25	0.21
Viaggt8/1	1.99	0.69	0.83	0.49	0.50	0.49	0.16	0.49	0.16	0.64
Vica9/152	1.99	0.69	0.86	0.49	0.50	0.49	0.13	0.49	0.32	0.07
Vitcca7/P	2.00	0.69	1.00	0.49	0.50	0.50	0.00	0.50	0.52	0.00
Vitg11/70	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.20	0.12
Vica9/134	1.94	0.67	0.76	0.50	0.49	0.48	0.23	0.48	0.16	0.00
Vitgt10/95	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.07	0.24
Vicacg8/42	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.16	0.00
Vitg8/146	1.94	0.67	0.43	0.50	0.49	0.48	0.56	0.48	0.16	0.00
Vitc2/16	1.98	0.68	0.63	0.49	0.50	0.49	0.36	0.49	0.16	0.15
Viga3/Z	1.14	0.24	0.13	0.87	0.12	0.12	0.86	0.12	0.16	0.00
Viga7/116	1.96	0.68	0.86	0.50	0.49	0.49	0.13	0.49	0.16	0.00
Vitg9/99	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.16	0.00
Vica9/X	1.94	0.67	0.83	0.50	0.49	0.48	0.16	0.48	0.16	0.04
Vica10/154	2.00	0.69	1.00	0.49	0.54	0.50	0.00	0.50	0.16	0.00
ViaacS10	1.96	0.68	0.86	0.50	0.48	0.49	0.13	0.49	0.30	0.00
Vitg10/ε	1.99	0.69	0.96	0.49	0.50	0.49	0.03	0.49	0.10	0.00
Vitg9/129	1.64	0.57	0.53	0.60	0.39	0.39	0.46	0.39	0.25	0.00
Itc1a	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.15	0.00
Itc1b	2.00	0.69	1.00	0.49	0.50	0.50	0.00	0.50	0.12	0.00
Itc1g	1.30	0.39	0.26	0.76	0.23	0.23	0.73	0.23	0.10	0.00
Iaac3b	1.76	0.62	0.63	0.55	0.44	0.43	0.36	0.43	0.13	0.00
Iaac4b	1.86	0.65	0.73	0.52	0.47	0.46	0.26	0.46	0.15	0.00
Iaac4f	1.06	0.14	0.06	0.93	0.06	0.06	0.93	0.06	0.10	0.18
Iaac4h	1.96	0.68	0.73	0.50	0.49	0.49	0.26	0.49	0.15	0.00
Mean	1.74	0.57	0.64	0.59	0.40	0.39	0.35	0.39	0.18	0.19
Standard deviation	0.34	0.19	0.31	0.15	0.15	0.15	0.31	0.15	0.08	0.03

(Exp_Ho) Expected homozygosity (Exp_he) heterozygosity were computed using Levene (1949), (Nei) Nei's (1973) N_a = Observed number of alleles, N_e = Effective number of alleles [Kimura and Crow (1964)], I = Shannon's Information index [Lewontin (1972)], (Ob_He) observed heterozygosity, (Ob_Ho) homozygosity, F_{st} = genetic differentiation.

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to M30), cluster II contained 18 isolates (M1 to M18) and Cluster III included only 1 isolate (M19). Both The Cluster I and II were further subclustered into two subclusters (Fig 4). The isolates could be grouped into separate clusters on the basis of geographical distribution as shown in Table 1.

Population structure

Structure analysis revealed that isolates of *V. inaequalis* collected from different places Jammu and Kashmir were grouped into two major populations. The assumed values of probable sub-populations (K) were ascertained by choosing higher ΔK value, with respect to the number of clusters inferred by Structure [33]. As per the Evanno table output, (S1 Table) the $K = 2$ was observed to be the best due to high ΔK peak value of 34.6 among the assumed K (Fig 5). Isolates from Jammu region having same latitude in J&K geographical map were grouped as

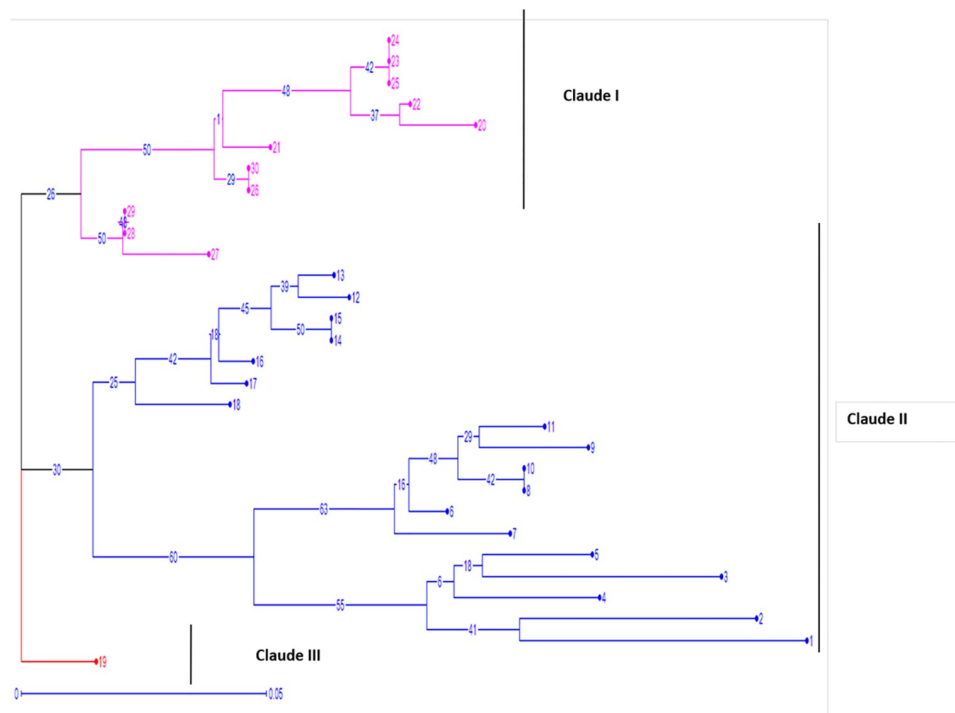


Fig 4. The cluster analysis (dendrogram) of 30 isolates revealing diversity within *Venturia inaequalis* populations. The bootstrap values are given on the nodes.

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subpopulation 1. Similarly, isolates from Kashmir region having same latitude in J&K geographical map were grouped subpopulation 2 with two admixtures (M19 & M27) (Fig 6). Moreover, STRUCTURE analysis grouped 2 individuals (6.6% of the total isolates) with a Q admixture proportion to the second cluster with the probability of 0.2 and 0.8, suggesting a substantial level of gene flow between the two clusters. Population 1 contains isolates from 1–18, while as population 2 comprised of isolates from 20–26, 28, 29 & 30. Two isolates (i.e. 19 & 27) fall as admixture minimally.

Analysis of molecular variance (AMOVA)

The two populations along with admixture isolates generated from structure analysis were analyzed for genetic variation among and within populations using AMOVA (Table 4). However, in population 3 sample sizes is less than 5 which cannot be considered as a population. From the analysis, 25% of variation was observed among population, 9% among individuals and 66% within individuals observed in the population. Wright's F statistic was estimated to determine deviation of Hardy-Weinberg expectation in the population. The F_{is} for all the 28 marker loci was 0.126, while F_{it} was 0.343 across the clusters. Pair wise F_{st} values showed significant differentiation among all the pairs of sub-populations ranging from 0.248 to 0.881 suggesting that all the three groups were significantly different from each other. The F_{st} values and their distribution pattern show clear differentiation of sub populations from each other. This result was also validated by the principal coordinate analysis (PCoA), (Fig 7) where co-ordinate 1 and 2 explained variation of 36.6% in population 1, 14.30% in population 2 and 13.10% in population 3 and overall cumulative percentage of variation of 64.07%.

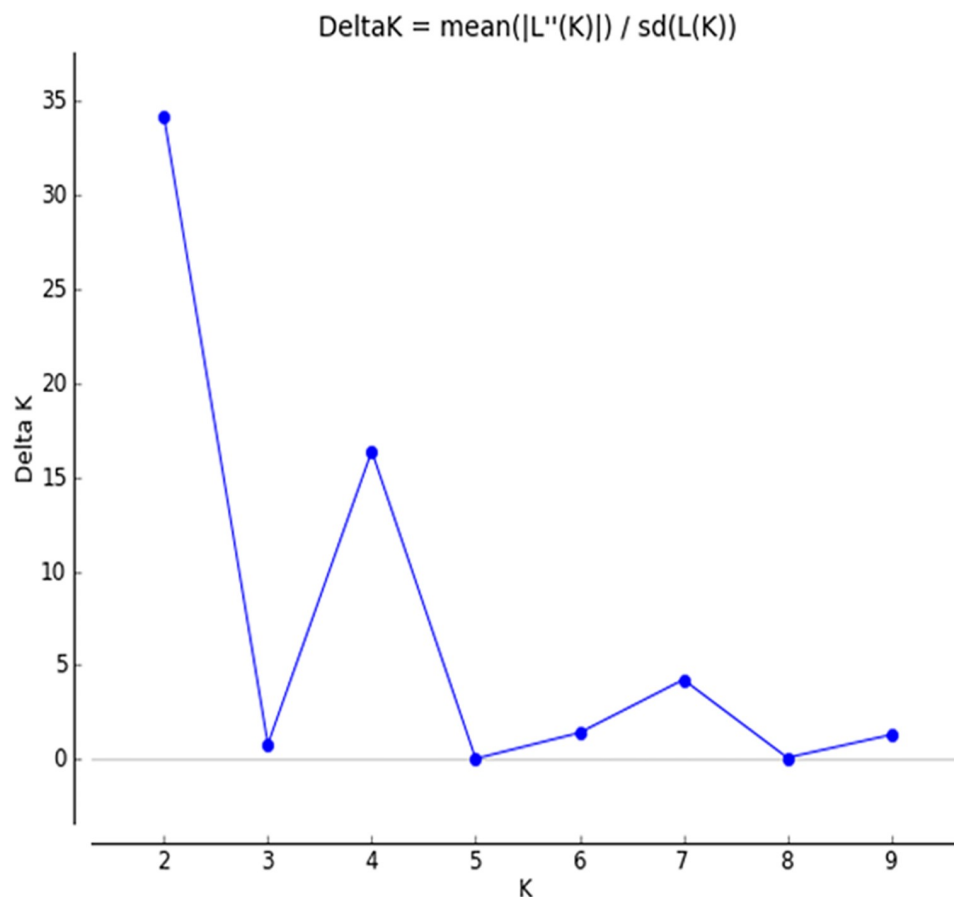


Fig 5. ΔK peak value of 34.6 among the assumed K.

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Discussion

Apples are grown in high altitude areas of India particularly in J&K[36]. It is the primary cultivated crop in J&K because though the climate conditions are distinct (sub-temperate to true

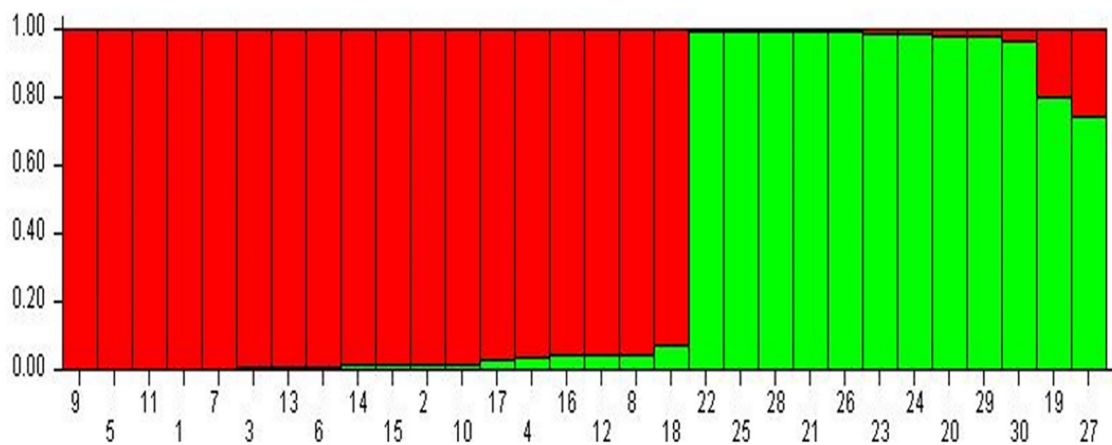


Fig 6. Population distribution using STRUCTURE analysis software, the isolates were grouped into two major populations with a small admixture (19 and 27 only).

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Table 4. Genetic variation among and within populations using AMOVA.

Source	Df	SS	MS	Est. Var.	%
Among Pops	2	21.720	10.860	0.554	25%
Among Indiv	27	50.980	1.888	0.211	9%
Within Indiv	30	44.000	1.467	1.467	66%
Total	59	116.700		2.231	100%
F-Statistics	Value	P			
F_{st}	0.248	0.001			
F_{is}	0.126	0.040			
F_{it}	0.343	0.001			

Df-Degree of freedom, SS Sum of squares, MS Mean sum of squares, Est. Var-Estimated variance

<https://doi.org/10.1371/journal.pone.0224300.t004>

temperate), they are very suitable for the cultivation of apples[37]. This crop suffers huge losses both quantitatively and qualitatively due to frequent epidemics of scab disease [23, 38], which is caused by *V. inaequalis*. The molecular characterization elucidates the genetic diversity among the isolates and for better resistance against any pathogen, the diversity must be known and accordingly resistant varieties can be developed. Hence in present study the molecular characterization of *V. inaequalis* was undertaken in order to provide better management strategies for this disease in the form of resistance and cisgenic breeding approaches.

We used ITS ribotyping of 30 isolates collected from two distinct regions. The noncoding ribosomal DNA ITS sequences doesn't change more rapidly than the coding sequences and

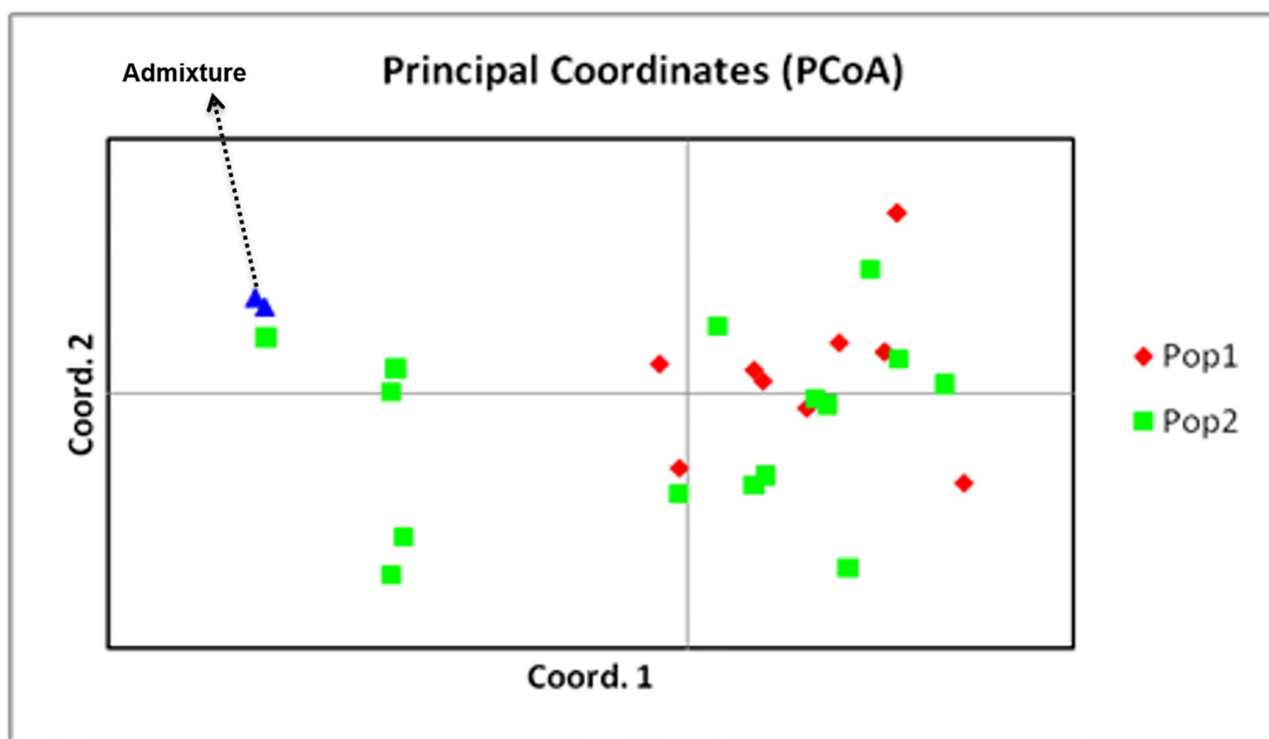


Fig 7. Principal coordinate analysis showing the clustering of two *Venturia inaequalis* populations and admixture population from different apple growing regions in the Jammu and Kashmir with population 1 (36.6%), population 2 (14.30%) and admixture (13.10%).

<https://doi.org/10.1371/journal.pone.0224300.g007>

may diverge between species and populations [18]. Analyzing ITS regions has become one of the primary methods for identification and characterization of a fungal strain or species [39, 40]. We observed sequence homology with isolates reported from different regions of the world particularly Iran, South Africa, Netherlands, and Canada. As expected, after phylogenetic analysis of 45 *Venturia* sequences (35 *V. inaequalis*, 5 *V. pirina*, and 5 *V. nashicola*), two clades emerged: one with all the *V. inaequalis* sequences and another with the *V. pirina* and *V. nashicola* sequences that further separated into two subclades. Bilal *et al* [21] had similar results. The current study identified taxonomic relationships or differences between 30 *V. inaequalis* isolates, which can help to identify characteristics such as resistance or susceptibility toward a particular anti-fungal agent.

Microsatellites or SSRs are very useful markers for population genetics analysis because of their high specificity, polymorphism, and reproducibility. These are major advantages of using SSR markers over Random Amplification of Polymorphic DNA. The SSR markers used in this study were highly variable and results generally corresponded with previous population genetics studies conducted in Europe. However, in this study the markers 1tc1a, 1tc1b, 1tc1g, 1aac3b, 1aac4b, 1aac4f, and 1aac4h showed only one allele compared to eight to ten alleles reported previously [41, 42]. The outcomes of this study along with prior reports [4, 11, 13, 21] confirm the continuation of high variability in *V. inaequalis*.

The distance between the Jammu region and the Kashmir region where we collected samples is approximately 400 km and allowed us to collect *V. inaequalis* isolates from geographically and topologically distinct regions. We observed a high level of diversity, which could be expected due to the climate differences. Xu *et al* [43] also observed remarkable variability between and within *V. inaequalis* isolates obtained from dissimilar apple cultivars in a solo apple orchard. This was credited to assortment pressure applied by diverse cultivars. Another possible explanation of variation in diversity is sexual recombination.

The isolates in this study shared a high percentage of identical alleles, indicating considerable gene flow among all isolates of *V. inaequalis* populations in J&K. As most of the apple cultivation area in Jammu and Kashmir is dominated by single cultivar Red Delicious and no resistant variety is under cultivation yet, so there is no selection pressure on pathogen to bring some change, hence this could be the reason having high percentage of identical alleles among *V. inaequalis* populations. The movement of the *V. inaequalis* from one place to other can be through planting material, high speed winds, but the planting material was one of the most important factors of introduction of *V. inaequalis* to India. This was also observed in the magnitude of migration between the regions. The Kashmir region seems to have the highest migration rate towards it and the lowest away from it. Migration towards the Jammu region was the lowest, indicating that this region is isolated, probably due to warmer winter temperatures. Overall the migration results indicated the possible free movement of the pathogen between the regions. The present study undoubtedly shows that there is high diversity of *V. inaequalis* in the Kashmir valley and reveals that the larger part of variability existed within the individuals.

Structure analyses divided the isolates into two populations ($K = 2$) with a clear differentiation between the two apple-growing regions. Pathogen distance seems to be the most significant factor in steep gene flow because it explains 50% of the variation among the *V. inaequalis* isolates. Pair-wise F_{st} values, ranging from 0.248 to 0.881 showed noteworthy demarcation between the subpopulations. This signifies that the two groups are notably dissimilar from one another. This outcome is also validated by the principal coordinate analysis, which distributed the isolates into two major populations with one admixture (two isolates). The admixture is of small size, hence can't be considered as a population.

Conclusion

The genetic variation and population structure of scab causing *V. inaequalis* from different apple growing regions in Jammu and Kashmir shows significant levels of genetic variation within the populations in the similar fashion as observed in other *V. inaequalis* population's studies conducted in Europe and elsewhere. Results indicated that gene flow between regions is occurring and has significant implications for the apple industry if fungicide resistant strains move between regions. Based on ITS sequencing, a database can be maintained to list out the sequence-based isolation of various fungal strains or species. In order to control such menace caused by scab, proper prediction and forecasting systems are need of the hour to prevent apple scab disease well in advance and also understanding the host pathogen interaction, which can provide new insights for effective management of this disease. Cisgenesis can be one of the approaches for introgression of resistance gene through biotechnological intervention under control of its own regulatory sequences from same species or related species which can also maintain the original cultivar characteristics.

Supporting information

S1 Table.
(DOCX)

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Original article

Quantification of polyphenolic compounds and relative gene expression studies of phenylpropanoid pathway in apple (*Malus domestica* Borkh) in response to *Venturia inaequalis* infection

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ABSTRACT

Apple (*Malus domestica* Borkh) is rich in phenolic compounds, which may enhance resistance to scab disease caused by *Venturia inaequalis*. In present study, apple cv. Golden Delicious was used for estimation of total phenols, flavonoids and quantification of six individual phenolic compounds. between control vs inoculated samples at different inoculation stages. The relative gene expression of phenylalanine ammonia lyase, chalcone synthase and flavanone 3 hydroxylase increased and polyphenolic compounds were constitutively upregulated at different post-inoculation stages. Data suggest that synthesis and accumulation of polyphenols is closely related with disease resistance against *Venturia inaequalis*. This study may play a vital role in understanding and finding out the governing mechanisms of scab resistance.

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1. Introduction

Apple scab caused by *Venturia inaequalis*, a filamentous fungus, is one of the most detrimental fungal pathogens that remain a major threat to apple production worldwide (Mansoor et al., 2019). It is among the most noteworthy threats to apple growing areas situated in the regions where climate is cool (MacHardy, 1996). Causal agent of apple scab *Venturia inaequalis* (Cke) belongs to subdivision Ascomycota, class Loculoascomycetes, order Pleosporales and family Venturiaceae and genus *Venturia*. Saprophytic (sexual state *Venturia inaequalis* (Cke) and parasitic (asexual state *Spilocaea Pomi* Fr) are the two states of this fungus. Damage caused by this pathogen are developed in between the cuticle and

the cell wall of epidermal layers (MacHardy, 1996; Mansoor et al., 2019).

Phenolic compounds are vital factors which determine the sensory and nutritious quality of apple. These compounds have been suggested to be involved in plant defense against various biotic and abiotic stresses. Various different type of phenolic compounds has been reported to be present in apple tissues (Fünfgelder et al., 1993; Bazzi et al., 2003). Plant phenols arising from phenylpropanoid pathway is the secondary metabolites of several classes of structurally diverse products. They play a significant role in pigmentation, growth and especially resistance to pathogens (Lattanzio et al., 2006). These phenols deposits inside the cell wall and acts as first line of defense, thus prevents the fungal penetration and infection (Schwalb and Feucht, 1999). Polyphenols possess antibacterial, antiviral and antifungal properties (Martini et al., 2009). Flavonoids present in plant tissues are reported to play crucial role in plant resistance e.g phloretin found in apple fruits and leaves confer resistance against *Venturia inaequalis* (Treutter, 2005). Large number of fungi including *Venturia inaequalis* hydrolyses phloridzin in to phloretin thus induces defense response. Other compounds such as hydroxycinnamic acids also play a defensive role in apple against *V. inaequalis* among which

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flavonols (epicatechin, procyanidin B1, catechin) and dihydrochalcones play a key role (Slatnar et al., 2012). Phenolic acids, are reported to impact the healing by increasing lignification of damaged areas and their concentrations may increase at site of infection after pathogen attack (Michalek et al., 1998).

Keeping in view, the objective of this study was to determine the concentrations of different polyphenolic compounds at different time periods of infection as well to analyze the relative gene expression.

2. Materials and method

2.1. Plant material and inoculation

One-year old plants of apple cv. Golden Delicious grafted on M9 rootstock were kept in growth chamber where temperature and humidity were maintained (18 °C temperature and humidity (Fig. 1). The experimentation was carried out at Plant Biotechnology, ICAR-Central Institute of Temperate Horticulture Srinagar.

2.1.1. Preparation of inoculation load

Conidia of *Venturia inaequalis* pure culture were used for inoculation in a concentration of 5×10^5 spores/ml measured with the help of Neuberg hemacytometer.

2.1.2. Inoculation conditions and sample collection

The inoculation of plants was carried out by spraying the leaves with the spore solution, the plants were continuously irrigated. In addition, non-inoculated (control) plants were mock-sprayed with water under the same conditions. Pooled samples consisting of six to eight leaves from three to four plants were taken pre-inoculation, 1-day, 7-days and 24-days post inoculation, frozen in liquid nitrogen, and stored at -80°C for gene expression analyses. Leaves from 24 dpi having highest percentage of infected area were used for relative gene expression studies.

2.2. Preparation of extracts

2.2.1. Extraction procedure

The Fragmentation of leaves was carried out in a mortar-pestle and were immediately frozen with liquid nitrogen (1:2w/v) in order to avoid the oxidation of the phenolic compounds (Guyot et al., 2002). Homogenization and final extraction of freeze-dried

material without midrib of leaves was carried out by method given by Shafi et al. (2019).

2.2.2. Determination of total polyphenolic content (TPC)

Total phenol contents were determined by the modified Folin-Ciocalteu method (Omoruyi et al., 2012). Absorbance was then measured at 765 nm using spectrophotometer. The results were expressed as mg of gallic acid equivalents/100 g fw.

2.2.3. Determination of total flavonoid content

Total flavonoid content was determined using the method of Chang et al. (2002). The absorbance was then measured at 415 nm using spectrophotometer. Results are expressed in terms of catechin equivalent (mg/100 g fw).

2.3. High performance liquid chromatography (HPLC) analysis

The HPLC analysis of samples was carried out in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps, degasser coupled to a photo-diode-array detector and injection valve with a 20 μL loop. An injection volume of 20 μL , a flow rate of 1.0 ml min^{-1} with 1 h of run time was used for separation process. Thrice the analysis was carried out for each sample. Quercetin was detected at 262 nm and the retention time was 1.915 mins. Quercetin, catechin and rutin standard were obtained from Sigma Aldrich. Chromatographic separations were performed on C18 (250 mm $\times$ 4.6 mm), 5 μm column using a solvent system in gradient mode followed by isocratic run as represented in Table 1. The filtration of mobile phase was done through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and was subjected to 40mins ultrasonication. Instrument control, data acquisition and data processing were done by using Class WP software (version 6.1) from Shimadzu. Quantitative determinations were made by taking into account the respective peak areas of standards at particular retention time versus concentration and expressed in $\mu\text{g/g}$ of apple leaves (Shafi et al., 2019).

2.4. RNA isolation

Prior to RNA isolation, microfuge tubes, tips, tip boxes, mortar and pestle, spatula, forceps were DEPC (Diethyl pyrocarbonate) treated (0.1%) overnight and then autoclaved followed by drying in hot air oven. Total RNA was extracted from frozen leaf samples of inoculated apple cultivars before inoculation and 1-day, 7-days



Fig. 1. One year-old plants grafted on rootstock M9 grown in a greenhouse and shifted to growth chamber where temperature and humidity was maintained.

Table 1

Mobile phase used for determining standard peak area at particular retention time for quantitative determination.

Compound	Mobile Phase	Gradient	Lambda max
Catechin	Solvent A–	3–9% B (0–5 min)	280 nm
Phloridzin	2.5% Acetic acid	16–36.4% B (15–33 min)	326 nm
Coumaric acid	Solvent B–	100% B (5 min)	254 nm
Apigenin	Aceto-nitrile		280 nm
Rutin			254 nm
Ferulic acid			322 nm

and 24 days post inoculation using cetyltrimethylammonium bromide (CTAB) method (Asif et al., 2006).

2.4.1. First-strand cDNA synthesis, validation and PCR amplification

First-strand cDNA was synthesized from isolated RNA using oligo-dT and Superscript II (Invitrogen) in a reaction volume of total 20 µL. cDNA synthesis confirmation was determined using GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Guerriero et al., 2012) and cDNA (50 ng) was further capitalized for PCR amplification

2.4.2. Relative gene expression studies

The relative gene expression was performed according to manufactures instructions (BIORAD, Californian, USA). The PCR conditions were same as specified (95 °C for 3 min, 40 cycles each at 95 °C for 30 s and 50–62 °C for 30 s). The reference gene used was actin to calculate relative gene expression of candidate PAL pathway genes. Actin gene is constitutively expressed in most of plant tissues, thus is commonly useful internal real-time PCR endogenous base line control in plants (Sukno et al., 2007). All the reactions were carried out in three technical triplicates and no-template controls (NTC). The quantification of target PAL pathway genes was determined relative to actin using the $\Delta\Delta C_t$ method and expressed as $2^{-\Delta\Delta C_t}$ for graphic representation (Livak and Schmittgen, 2001). Relative gene expressions were determined according to the Livak and Schmittgen (2001) formula.

$$\text{Relative Gene Expression} = 2^{-\Delta\Delta C_t}, \text{ where } \Delta\Delta C_t \\ = [\Delta]C_{t\text{sample}} - [\Delta]C_{t\text{reference}}$$

$[\Delta]C_{t\text{sample}}$ – C_t value for any sample normalized to the endogenous housekeeping gene

$[\Delta]C_{t\text{reference}}$ – C_t value for the reference sample normalized to the endogenous housekeeping gene

3. Results

3.1. Estimation of total phenols and flavonoids using spectrophotometer

The *Venturia inaequalis* inoculated cultivar has demonstrated statistically differential levels of total polyphenolic content ranging between 0.205 and 2.395 mg GAE/100 g fw. There was a 4-fold increase in total phenolics post 1 day (24-hours) of inoculation and from 1 day to 7 days content increased by 1.3 times while as from pre-inoculation to post 24 day of inoculation phenolic content increased 12-fold. In control, no such increase in the phenolic levels at different stages post treatment was found, the highest level in control was found 24 days post inoculation as 1.871 mg GAE/100 g fw (Fig. 2). Total Flavonoids content was again highest in inoculated cultivar, where the content was found to be 4.89 mg CE/100 g fw at 24-day post inoculation. Before inoculation phenolic content was 0.15 mg CE/100 g fw, followed by 1.74, 2.70 & 4.89 mg CE/100 g fw and highest content reached at 24-DPI when symptoms in susceptible were clear visible. Pre-inoculation

to post 1 day (24 h) the flavonoid content increased by 11.6 times and from 24 h to 7 days 1.6-time increase was found. While as from 7 day to 24 day there was 1.8-fold increase in flavonoid content. After 24- days post inoculation the flavonoid content was 1.03 mg CE/100 g fw (Fig. 2).

3.2. HPLC based quantification of different phenolic compounds

In present investigation, the following phenolic compounds were quantified in the leaves of inoculated apple cultivars: apigenin, p-coumaric acid, (+) - catechin, phloridzin, rutin and ferulic acid.

The current investigation has revealed that among selected apple cultivar, the highest ferulic acid content was showed by inoculated apple cultivar ranging between 0.50 and 23.94 µg/g fw with significant increase at each post treatment advanced stage. Pre-inoculation to post 1 day (24 h) in *Venturia inaequalis* treated cultivar showed 16-fold increase in ferulic acid level, while as in control (water sprayed) 1.3-fold increase was observed. *Venturia inaequalis* treated cultivar showed highest ferulic acid levels as compared to control in which lower levels of ferulic acid were found at each advance stage post treatment (Fig. 3).

Coumaric acid content was observed to be 1.15 µg/g fw before *Venturia inaequalis* inoculation and after 1 day (24 h) the content reached to 7.67 µg/g fw which shows a 1.6-fold increase in its content. While as in other stages a 2- fold increase was observed. In case of control this content remained in constant range till 7-day 1.02 µg/g fw to 1.91 µg/g fw. While as after 24-days the content reached up to 5.22 µg/g fw. Thus a 2-fold increase was observed from 7 to 24 days post inoculation in control (Fig. 3). In susceptible treated with *Venturia inaequalis* both of the hydroxycinnamic acids rapidly increased with time period.

Within dihydrochalcones phloridzin content ranged from 2.06 µg/g fw to 39.24 µg/g fw in *Venturia inaequalis* treated cultivar and in control the content was in range of 1.83 µg/g fw to 3.18 µg/g fw. In case of control phloridzin increased 1.2-fold while as in *Venturia inaequalis* treated the increase way 5.7-fold after 1-day (24 h) of inoculation.

Flavanol catechin production showed drastic increases in *Venturia inaequalis* treated cultivar the content of catechin raised by 16-fold, apigenin 2.4-fold and rutin 2.7- fold after 1-day (24 h) of inoculation. While as, in control catechin level increased by 1.7-fold, apigenin 1.4-fold and rutin by 1.7-fold after 1-day (24 h) of inoculation. After 24 days of post inoculation the content of catechin was found highest in *Venturia inaequalis* treated cultivar 33.64 µg/g at this point of time infection was severe and spots were prominent. Isoflavonoid apigenin content before inoculation was 2.45 µg/g fw which increased after inoculation and had a peak value of 11.87 µg/g fw and 18.89 µg/g fw at 7-days and 24-day respectively. Rutin content increased from 2.70 µg/g fw (BI) to 31.31 µg/g fw (24-Days PI) in *Venturia inaequalis* treated cultivar. In control, all the three flavonoids showed a small increase from pre-inoculation stage to 24- days post inoculation stage (Fig. 3). The phenolic contents overall showed a marked increase in *Venturia inaequalis* treated susceptible cultivar than in control. In control, phenolic content either remained constant or increased only marginally along with time period.

3.3. RNA isolation and cDNA preparation

The total RNA was isolated from each of the mentioned samples, the intactness, size and quality of RNA extracted was checked on 1.5% agarose gel electrophoresis (Fig. 4A). The total RNA isolated was quantified having concentration between 200 and 717.2 ng/µL. cDNA synthesis confirmation was seen based on GAPDH amplification at 180 bp region (Fig. 4B).

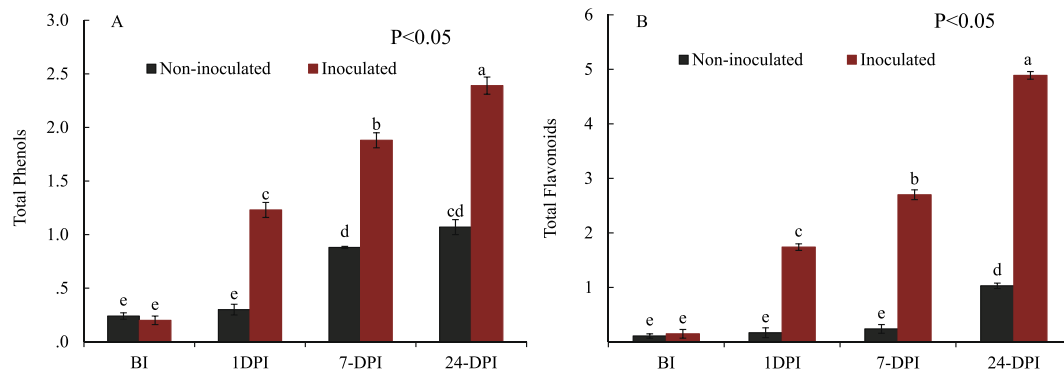


Fig. 2. Effect of *Venturia inaequalis* on total phenolics and flavonoids of apple. Total phenols (mg GAE equivalent (gallic acid)/100 g fw) and flavonoids (mg CE equivalent (catechin)/100 g fw) were measured at different time-points (BI, 1, 7 and 24 Days) of pathogen inoculation and sterile water treatment (as the control). Data are shown as mean $\pm$ SD (Standard Deviation) of three replicates from one independent experiment.

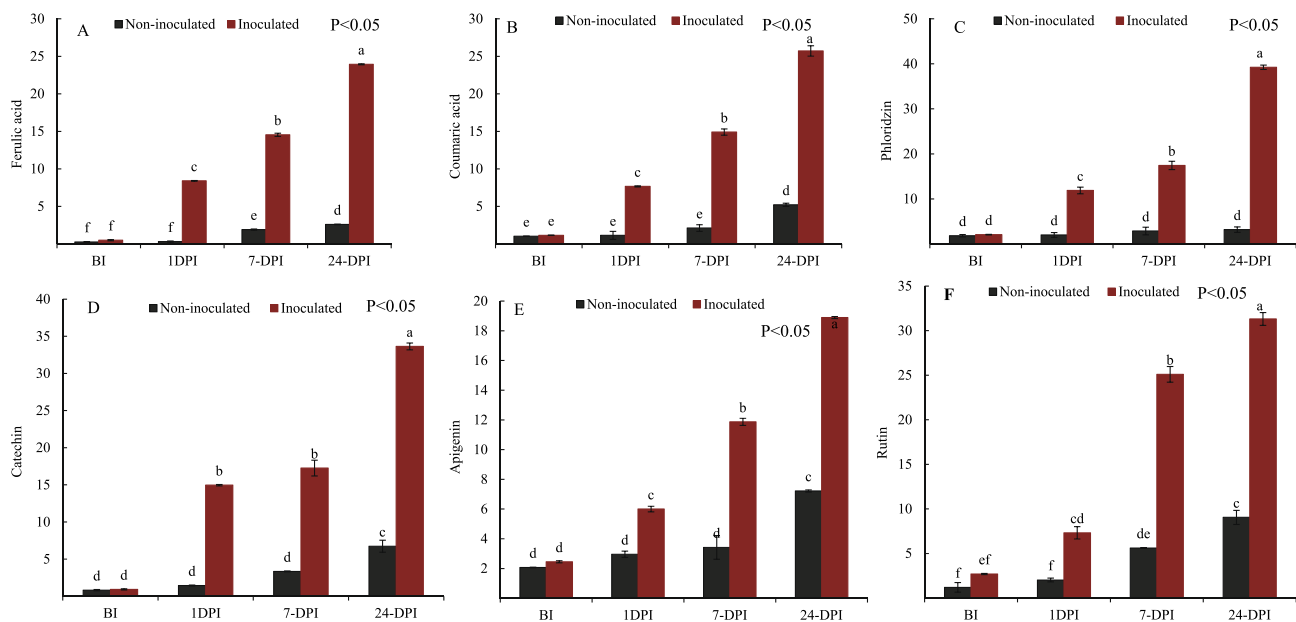


Fig. 3. Effect of *Venturia inaequalis* on ($\mu\text{g/g fw}$) ferulic acid, coumaric acid, catechin, apigenin, rutin and phloridzin content which were measured at different time-points (BI, 1, 7 and 24 Days) of pathogen inoculation and sterile water treatment (as the control). Data are shown as mean $\pm$ SD (Standard Deviation) of three replicates from one independent experiment.

3.4. Relative gene expression of PAL, CHS and F3H genes

Relative levels of gene expression of polyphenolics biosynthetic genes i.e. PAL, CHS, C3H and F3H among apple cultivars, each in triplicates ($n = 3$) was detected by their mean Ct values. Sharp amplification, melt peaks and melting curves were observed post completion of qRT-PCR. The sharp amplification peaks rose up once cycle number reached near 20 cycles corresponding to relative fluorescence units (RFU) indicating correctness of PCR conditions (Fig. S1). Using Ct values of samples as well as housekeeping gene ACTIN, the relative gene expression levels of each sample was determined through $\Delta\Delta C_T$ approach. The relative gene expression of PAL gene with respect to ACTIN among scab susceptible *Venturia inaequalis* treated apple cultivars was found statistically significantly higher as compared to control. Among susceptible cultivar, there was significant increase in the PAL expression levels i.e. 1.83 ± 0.25 pre-inoculation and 14.08 ± 0.39 post 1-day (24 h) of inoculation, which showed a 7.8-fold increase. While as in control no such

significant change was found. The level of gene expression in control was in 1.11 ± 0.15 to 3.36 ± 0.13 range which demonstrated lower insignificant expression of PAL gene. Therefore, PAL gene expression levels were found highest in *Venturia inaequalis* treated after 24 days post inoculation 32.51 ± 0.83 , thus indicating the highest PAL expression levels observed 24 days post inoculation (Fig. 5).

Another important gene in the form of CHS was found up-regulated in *Venturia inaequalis* treated cultivar at different stages i.e. i.e. 4.11 ± 0.55 (1DPI), 10.1 ± 0.42 (7-Days PI) and 16.67 ± 0.82 (24 days PI). From pre-inoculation to post 1-day inoculation in *Venturia inaequalis* treated cultivar there was a 23-fold increase in gene expression level. While as from 1-day to 7-day 2.5-fold and 7-day to 24-day 1.7-fold increase in CHS expression level and the lowest insignificant relative gene expression was found in control samples (Fig. 5). The moderate levels of F3H gene expression was observed in *Venturia inaequalis* treated apple cultivar at different stages, which ranged from 0.04 to 6.52 from pre-inoculation to post 24-day inoculation. Before inoculation to post

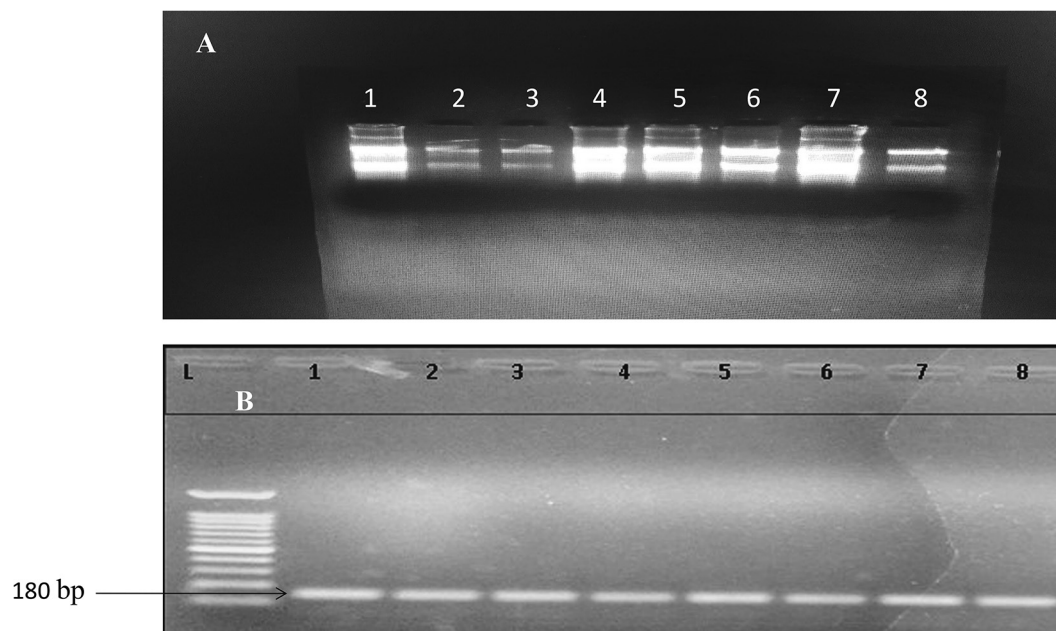


Fig. 4. RNA extracted at different stages after inoculation. (A) (1) Before inoculation (Treated), (2) Before inoculation (Control), (3) One day post inoculation (Treated), (4) One day post inoculation (Control), (5) 7- day post inoculation (Treated), (6) 7- day post inoculation (control), (7) 24-days post inoculation (Treated) and (8) 24- days post inoculation (control) and (B) cDNA confirmation of isolated RNA from leaves of apple cultivars using GAPDH primer (Band at 181 bp). L indicates marker DNA ladder starting from 100 bp.

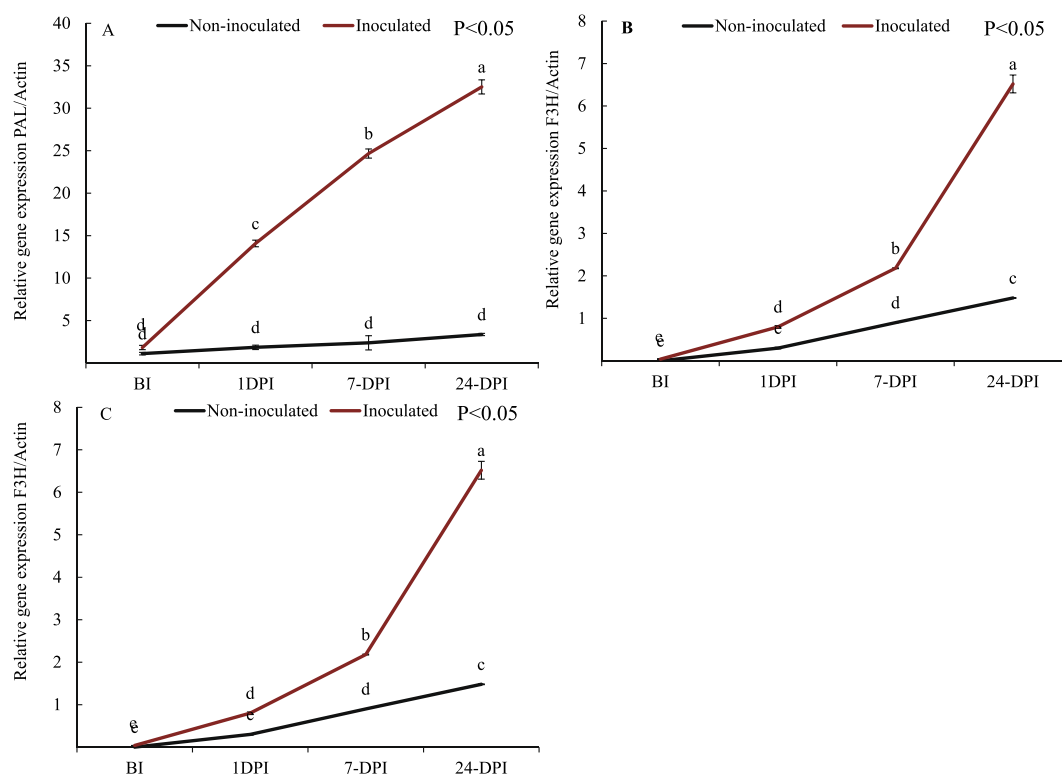


Fig. 5. Gene Expression and Heatmap for (A) *PAL* (B) *CHS* and (C) *F3H* genes representing relative levels of gene expression at different stages before and post inoculation events when compared to corresponding house-keeping gene *ACTIN*. Where NI- Non inoculated, I- inoculated, BI-Before inoculation and DPI- Days Post inoculation. Each point represents the mean of three replicates and was expressed as mean $\pm$ SD (Standard deviation). Asterisks (*) indicate significant differences ($P < 0.05$) between *Venturia inaequalis* treatment (inoculated) and control (Non-inoculated).

1-day *F3H* was highly upregulated i.e. a 20- fold increase while as no such upregulation were observed in control. The heatmap was constructed indicating differential gene expression levels of all

genes i.e. *PAL*, *F3H*, *CHS*. Different color codes of heatmap demonstrate diverse gene expression levels (Fig. 6). e.g. reddish (Highest expression)

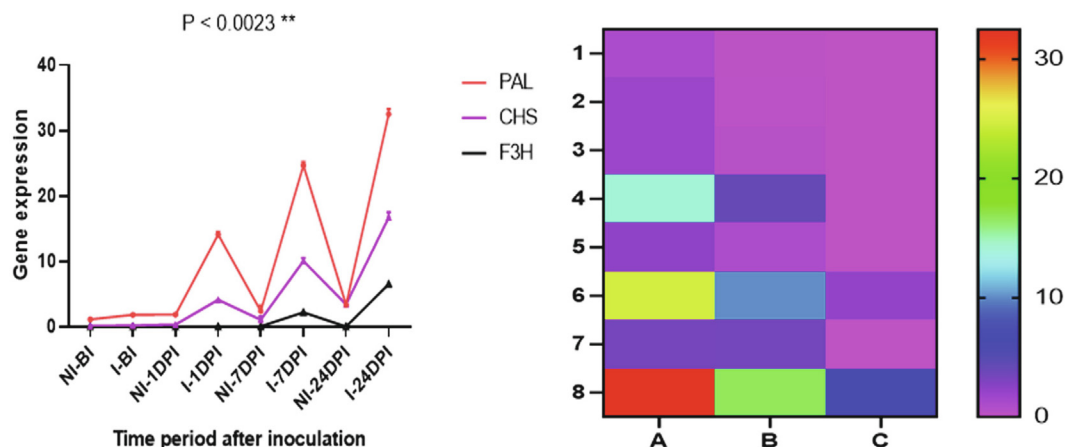


Fig. 6. Expression levels of all genes and heatmap indicating differential gene expression levels of PAL, F3H, CHS, color codes represent different gene expressions.

4. Discussion

Phenylpropanoids display large antimicrobial activities and are thus assumed to help the plant act against various microbial diseases (VanEtten et al., 1994). The secondary metabolites play a role as antimicrobial in plant defense systems (Yang et al., 2018). Role of various phytoalexins or phytoanticipins have been potted in previous studies as providing widespread facts on the biological activities of phenylpropanoid compounds entangled in plant defence (Dixon and Paiva, 1995; Dixon, 2001; Tsao et al., 2003; Shafi et al., 2019). Dihydrochalcones have been found to accumulate in scab infected neighboring tissues of leaves and fruits compared to that of healthy apple peel (Slatnar et al., 2016).

Generally polyphenolic content increases with infection or attack of any pathogen. Phenols certainly are not only component responsible for contributing to resistance of apples towards scab but, as it can be observed from results that with increase in time period after pathogen treatment their synthesis increased. The production and distribution of defensive phenolics such as flavonoids, hydroxycinnamic acid and dihydrochalcones is enhanced in response to pathogen attack and infection. In the current study, phenolics were at the higher level in leaves after inoculation, this significant increase in polyphenols was observed in *Venturia inaequalis* inoculated cultivars at a different time point. The higher level of increase may be associated with defense process in order to avoid further spread of infection. In inoculated cultivar ferulic acid and coumaric acid accumulate at a faster rate in the *Venturia inaequalis* treated apple cultivar. According to previous studies, it was hypothesized that with infection the leaves accumulated more phenolic compounds compared with healthy leaves. The maximum part from the total content among observed hydroxycinnamic acids belonged to p-coumaric acid and the minimum part belonged to ferulic acid. These results may be explained in relation to the previous studies where the increased production of hydrocinnamic acid with infection can contribute as an outcome of the enhanced activity of phenylalanine ammonia lyase (PAL) (Petkovšek et al., 2008, 2009).

Phloridzin is commonly anticipated for playing a defensive role against various kinds of pathogens as well as it is involved in resistance to various diseases. Ratio of flavanol/phloridzin are mostly debated with respect to resistance against apple scab (Picinelli et al., 1995; Petkovšek et al., 2008). In our study, the scab infected leaves had phloridzin content 39.246 µg/g after 24-days of post inoculation. In case of inoculated the infected leaves accumulated more phloridzin from first day post inoculation. This suggests that with pathogen (*Venturia inaequalis*) attack phloridzin accumulated

faster as a defense response in infected leaves, this result was in concord with results observed by Leser and Treutter (2005); Lattanzio et al. (2006). Another study reveals that exogenous. Epigallocatechin-3-Gallate (EGCG), application increased plant resistance to TMV as revealed by significantly decreased transcript levels of TMV-coat protein (CP) in tomato leaves and results suggest that EGCG maintains a delicate balance between ROS signaling and ROS scavenging to enhance tomato resistance to TMV (Zhang et al., 2020). As a result, it can be concluded that phloridzin is an active compound which is synthesized in response to fungi attack but the degree of resistance it will provide cannot correlated with different kind of apple cultivars (Gosch et al., 2009). We found that phloridzin levels increased in leaves of the *Venturia inaequalis* inoculated leaves where after 24 h of inoculation there was approximately 6 fold increase in phloridzin content and highest level was found after 24 days when symptoms were prominent, thus infected leaves accumulated higher phloridzin around the tissues, which specifies it is not the production of these phenolics only but the degree at which phloridzin is transformed may also likely play a role in resistance (Skłodowska et al., 2018). Flavonoids are reported to play a role in the disease resistance of apples to *Venturia inaequalis*. In treated cultivar, there was higher synthesis of catechin, apigenin and rutin on the entire infected leaf whereas in the control observed catechin, apigenin and rutin were 1.3 to 2.8 times lower than in infected leaves. Treutter and Feucht (1990b); Picinelli et al. (1995); Petkovšek et al. (2009) had reported same kind of results where after the *Venturia inaequalis* attack flavonoids production was enhanced. Catechin accumulation after 24 h was more comparatively to apigenin and rutin.

Phenylpropanoid pathway is the main secondary metabolic pathway which is responsible for production of defence related compounds. The rate limiting enzyme and the key regulator of this pathway is PAL which catalyzes conversion of phenylalanine to *trans*-cinnamic acid, then producing the precursors for another metabolite/compound biosynthesis. Phenolic and flavonoid are the compounds antifungal compounds that get accumulated after the attack of any pathogen attack and act by inhibiting the spore germination and mycelia growth (Mayr et al., 1997). It has been observed that with the pathogen attack there is an up-regulation of genes that are responsible for production and accumulation of different defense related flavonoids compounds (Treutter and Feucht, 1990a; Mayr et al., 1993, 1997; Picinelli et al., 1995; Leser and Treutter, 2005). This activation of the genes of PAL pathway and synthesis of polyphenolic compounds may explain the broad and unspecific role of these compounds in defense to disease like apple scab. Plants produce a number of metabolites through

secondary metabolic pathways, which function mostly in plant defense responses to biotic and abiotic stresses. Flavonoids and phenols are the major groups of secondary metabolites that accumulate in response to pathogen attacks. [Ahammed et al. \(2020\)](#) found that the application of melatonin and/or inoculation with arbuscular mycorrhizal fungi AMF increased the content of phenols and flavonoids, which might enhance defense against *Fusarium wilt* in cucumber ([Ahammed et al., 2020](#)).

PAL, is main and F3H and CHS are among the key branch-point enzymes that help in controlling the regulation of the PAL pathway ([Dixon et al., 2002](#); [Vrancken et al., 2013](#)). Our data presented that post inoculation there was noteworthy increase in activities of PAL, CHS and F3H genes, that accounted for the enhanced production of phenolic and flavonoid compounds. qRT-PCR results have demonstrated that PAL, CHS and F3H gene expression levels / gene fold change was found constitutively increasing post inoculation days in *Venturia inaequalis* infected cultivar. The results observed in our study are in coherence with previous studies on apple, muskmelon, cherry and tomato fruit that revealed that with up regulation in gene expression levels of PAL, F3H and CHS the disease resistance also tends to get increased against pathogens ([Liu et al., 2014](#); [Wei et al., 2019](#)). The observed early defense response of plant towards pathogen attack is quick accumulation of polyphenolic compounds ([Treutter, 2005](#)). Activation of the phenylpropanoid pathway contributes to plant defense against pathogenic fungi. Therefore, they analyzed the activity of PAL, the first key enzyme in the phenyl propanoid pathway. Time-course analysis of PAL activity revealed that inoculation with *C. gloeosporioides* caused an initial decline in the early few hours; however, PAL activity, then, gradually increased with post-inoculation time period. Expression analysis of genes involved in phenylpropanoid pathway showed that both exogenous EBR and *C. gloeosporioides* inoculation increased transcript levels of PAL, C4H, and 4CL; however, combined treatment with EBR and *C. gloeosporioides* resulted in a greater increase ([Zhang et al., 2018](#)). This was also observed in our results that after 24 h of inoculation the gene expression levels as well as production of polyphenols were much higher compared to control. This might be a strategy aimed to overcome pathogen and the spread of infection. It has been designated that these compounds possess antimicrobial potential which help the plant for structural defence against pathogen.

Similar kind of results that we observed were also reported in the poly L lysine treated apple fruits by [Ge et al. \(2018\)](#) where after one-week time the activity of PAL peaked in comparison to control which showed slow activity of PAL from 0 to 8 days. In the present study, it was observed that three enzymes (i.e., PAL, CHS, F3H) are involved in apple responses to *Venturia inaequalis* disease. These enzymes catalyze key reactions in the phenylpropanoid pathway, an increase in the activity of these enzymes upregulates the biosynthesis of phenolic acid, chalcones and flavonoids. There seems a positive correlation between levels of gene expression of PAL, CHS and F3H genes and production of different phenolics and flavonoids such as apigenin, p-coumaric acid, (+) - catechin, phloridzin, rutin, and ferulic acid. The amounts of phenolic acids and flavonoids were determined in the present study before and after inoculation of *Venturia inaequalis* and similar correlation has been observed in earlier studies as well ([Petkovšek et al., 2008](#)). Another study conducted by [Xu et al. \(2019\)](#) where fulvic acid treatment extremely enhanced production of polyphenolic compounds by increasing the activities of PAL, C4H and 4CL.

Our results are in consistent with previous studies in muskmelon, cherry, Chinese cabbage and tomato that displayed enhanced activity of PAL enzymes ([Liu et al., 2014](#); [Islam et al., 2018](#); [Skłodowska et al., 2018](#); [Wei et al., 2019](#)). The up-regulation of PAL, CHS and F3H genes in *Venturia inaequalis* treated apple cultivar

resulted in an enhanced production of phenylpropanoid compounds.

These results can provide new insights related to defense response of apple cultivar which may help to understand mechanisms of basal defense. The comparison of treated and non-treated Golden Delicious apple plants provided evidence that *Venturia inaequalis* up regulated genes in the inoculated cultivar. Our results demonstrate that scab infection caused a significant increase in hydroxycinnamic acids, catechin, epicatechin and phloridzin in infected tissue. The results observed signify a vital contribution to understand plant-pathogen interaction and may be useful for further investigations.

5. Conclusions

Our results are in support of the previous studies and hypothesis conducted on different fruits that phenolic compounds are involved in defense response and might play an important role in disease resistance and the mechanism of offering this resistance is directly linked to the host-pathogen interaction which involves different genes. Apart from PAL genes and secondary metabolites like phenols and flavonoids still pathogen overcomes the resistance in susceptible cultivar. So, detailed investigation on mechanism and time period of action of all PAL pathway genes, secondary metabolites and resistance genes may through a better light in understanding the plant pathogen interaction and defense related events as well. By overexpressing the genes related to polyphenols and introgressing them using cisgenic or intragenic approach can be used as an adjunct approach to reduce scab.

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Declaration of Competing Interest

No conflict of interest exists neither in the conduct of this research nor in preparation of this manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.09.007>.

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