Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir

Asha Nabi
(2013-479-D)

Division of Plant Pathology

Faculty of Horticulture

Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir

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Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir

Asha Nabi
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Doctor of Philosophy in Plant Pathology

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Dedicated
to my beloved Parents

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Pathology

Certificate - I

This is to certify that the thesis entitled, **"Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir"** submitted in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology**, to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Asha Nabi (Regd. No. 2013-479-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

(Prof. Mushtaq Ahmad)
Chairman
Advisory Committee

Endorsed

Prof. & Head,
Division of Plant Pathology

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Pathology

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We, the members of the Advisory Committee of **Ms. Asha Nabi (Regd. No. 2013-479-D)**, a candidate for the degree of **Doctor of Philosophy in Plant Pathology** have gone through the manuscript of the thesis entitled, **"Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

Advisory Committee

Chairman

Prof. Mushtaq Ahmad,
Director Extension, SKUAST-Kashmir

Members

Dr. Mehraj-Ul-Din Shah,
Assistant Professor,
Division of Plant Pathology,

Dr. Bilal Ahmad Padder,
Assistant Professor,
Division of Plant Pathology

Dr. Gul Zafar,
Assoc. Director, Dryland Agriculture
Research Station, SKUAST-Kashmir

Dean's Nominee

Dr. Hafiza Ahsan,
Prof. & Head, Division of Food Science &
Technology, SKUAST-Kashmir

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Faculty of Horticulture, Division of Plant Pathology
-::o::-

Certificate - III

This is to certify that the thesis entitled, **"Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir"** submitted by **Ms. Asha Nabi (Regd. No. 2013-479-D)** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology** was examined and approved by the Advisory Committee and External Examiner on …….....

Chairman
Advisory Committee

External Examiner

Prof. & Head,
Division of Plant Pathology

Dean
Faculty of Horticulture, SKUAST-K

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Pathology

Name of the student	:	**Ms. Asha Nabi**
Registration No.	:	2013-479-D
Major Subject	:	Plant Pathology
Minor Subject	:	Genetics and Plant Breeding/ Biotechnology
Major Advisor	:	**Dr. Mushtaq Ahmad,** Professor, Plant Pathology & Director Extension, SKUAST-Kashmir
Title of the Thesis	:	**"Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. causing apple (*Malus* × *domestica*) scab in Kashmir "**

ABSTRACT

The present studies were carried out to discern the sensitivity of *Venturia inaequalis* populations to extensively used fungicides such as dodine, myclobutanil, hexaconazole, difenconazole and flusilazole in Kashmir valley, to descry the cross-resistance, and characterize the resistant and sensitive isolates at molecular level. Thirty baseline isolates of *V. inaequalis* were collected from an orchard in Baramulla district never exposed to fungicides and 200 other isolates from fungicide-exposed orchards of district Baramulla and Shopian. To establish the baseline sensitivity, thirty isolates were tested at seven concentrations *viz.,* 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, and 10.0 µg ml^{-1}, of different fungicides using mycelial growth assays. The mean ED_{50} values of dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole for baseline population were 0.14, 0.036, 0.17, 0.02 and 0.022 µg ml^{-1}, respectively, and accordingly discriminatory doses of 0.20, 0.04, 0.20, 0.03 and 0.03 µg ml^{-1} were selected for dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole, respectively. To monitor the shift in fungicide-exposed isolates, all the two hundred isolates were tested for their sensitivity at the respective discriminatory doses of different fungicides and the frequency distributions of their relative growth (RG) values were compared to that of the baseline isolates. A major shift towards reduced sensitivity was observed in myclobutanil followed by flusilazole. However, the

frequency distribution of RG values exhibited slight variation in case of dodine, hexaconazole and difenoconazole. The highest proportion of isolates (35.5%) exhibited 'resistant' response to myclobutanil followed by 19.5 per cent isolates resistant to flusilazole, whereas 0.5 per cent of the isolates were 'resistant' to dodine.The pathogen isolates 'resistant' and 'shifted' to different fungicides constituted the greater proportion of those orchards which either had prolonged fungicide usage or the respective orchardists did not follow the recommended schedule of fungicide application and employed more than six sprays in a single growing season. Fungicide-exposed pathogen isolates when assessed for cross-resistance revealed a high level of cross-resistance between myclobutanil and flusilazole (21% of the cross resistant isolates), providing an evidence of similar resistance mechanism(s) between them. Molecular analysis of selected 'resistant' and 'sensitive' isolates of *V. inaequalis* using 46 RAPD primers yielded a single locus difference between 'resistant' and 'sensitive' bulks with P16 and P17 primers, which needs further characterisation. However, genotyping with ISSR markers could not separate 'resistant' and 'sensitive' isolates in different clades, indicating that these markers are not suitable to discriminate sensitive isolates from the resistant ones.

Key words: Apple scab, Cross-resistance, DMIs, Dodine, Fungicide sensitivity, Fungicide resistance, Molecular characterisation, *Venturia inaequalis*,

Signature of Student
Dated_____

Signature of Major Advisor
Dated _____

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The most Beneficent and Merciful

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Chapter - 1

INTRODUCTION

Apple is a deciduous temperate fruit crop which belongs to family rosaceae and sub family pomoidae. In the Jammu and Kashmir state, it covers an area of 162.971 thousand hectares with an annual production of 17, 26, 834 metric tonnes and a productivity of 10.59 metric tonnes per hectare (Anonymous, 2016), and forms the backbone of the economy of the state.

Apple scab (*Venturia inaequalis* (Cke.) Wint.) is the major disease in all the apple growing regions of the world. The frequent epidemics of scab have been witnessed on almost all cultivars of apple in Kashmir since early seventies, inflicting heavy losses. As most of the commercial apple cultivars are susceptible to scab, orchardists mainly rely on foliar sprays with fungicides such as mancozeb, dodine, captan, ziram, hexaconazole, myclobutanil, difenoconazole, triadimephon, bitertanol, fenarimol, propioneb, flusilazole etc. for its management. At least six to eight applications of fungicides are recommended against this disease in Kashmir (Padder *et al.*, 2013), but orchardists often resort to indiscriminate use and post-symptom applications of site-specific fungicides. Earlier, orchardists used to manage the apple scab only with the help of protectants like mancozeb, captan, dodine etc. Subsequently, systemic fungicides *viz.,* benzimidazoles, were introduced to manage this disease effectively. However, reduced efficacy of carbendazim in the field was reported after few years (Basu Chaudary and Puttoo, 1984), without ascertaining whether or not the resistance in the pathogen populations was developed, which subsequently led to the withdrawal of this fungicide from the 'pesticide spray schedule for apple foliar diseases' in Kashmir. For the past two decades, ergosterol biosynthesis inhibitors (demethylation inhibitors in particular) like myclobutanil, hexaconazole, fenarimol, difenoconazole, flusilazole etc. are being increasingly used to manage major fungal diseases of apple. Of late, orchardists in different parts of Kashmir

valley have been complaining of inefficacy of one or the other of these fungicides against *V. inaequalis* in their orchards. Therefore, it is hypothesized that resistance to demethylation inhibitors (DMIs) might have developed in *V. inaequalis* populations in Kashmir.

Dodine and DMIs have encountered the problem of resistance build-up in target pathogens, mostly in developed countries. Dodine was first used to manage apple scab in the late 1950s in New York, but its use was discontinued after 10 years due to resistance development (Szkolnik and Gilpatrick, 1969). Resistance to dodine was then reported from other apple producing areas of the USA (Gilpatrick and Blowers, 1974; Jones and Walker, 1976; Yoder and Klos, 1976; Koller *et al.*, 1999), Canada (Ross and Newbery, 1977; McKay and MacNeill, 1979; Sholberg *et al.*, 1989), New Zealand (Bakker, 1999) and Poland (Meszka, and Bielenin, 2001). The first field report of *V. inaequalis* resistance to DMI fungicides was from Michigan in 1997 (Koller *et al.*, 1997) and afterwards, resistance to DMI fungicides has been documented from different areas *viz.,* New York, Michigan and Virginia in the USA, Quebec and Ontario in Canada, Switzerland and the United Kingdom (Koller *et al.*, 1997; Kunz *et al.*, 1997; Errampalli, 2004; Jobin and Carisse, 2007; Gao *et al.*, 2009). In Kashmir valley, sensitivity study on dodine has not been carried out to date, whereas, preliminary sensitivity studies of *V. inaequalis* populations (from few locations) to myclobutanil, difenconazole and fenarimol have been carried out few years back (Fatima, 2008; Kacho *et al.*, 2013). However, no information is available regarding the current status of sensitivity of *V. inaequalis* to different fungicides from the other apple growing districts of the Kashmir valley. Therefore, need arises to undertake comprehensive sensitivity studies of *V. inaequalis* to dodine and most commonly used DMIs.

Different methods based on pathogen bio-sensitivity such as effects on spore germination, germ tube length and pathogen morphology and radial mycelial growth are being used for determining resistance to different fungicide

fungicides (Koller *et al*., 1991; Smith *et al*., 1991). However, radial growth assays are more commonly used and described as best fit method for assessment of DMI sensitivity by Fungicide Resistance Action Committee (FRAC, 2006). Nowadays, there has been substantial development in the field of molecular biology and the techniques using molecular markers help to discern the resistant and sensitive strains in the pathogen populations. Therefore, molecular characterization of resistant and sensitive strains is equally important for their quick detection.

Resistance across different chemical classes (cross-resiatance) is a serious concern as it limits growers' options for fungicides for control of pathogens. Information on this aspect is important for devising anti-resistance strategies while attempting at controlling the diseases through fungicidal sprays. Since, DMIs have a similar mode of action, *V. inaequalis* is likely to develop resistance against most of the DMIs. However, cross resistance across DMIs doesn't seem to be universal but varies from one pathogen to another (Hisang *et al.*, 1997; Karaoglandis and Thanassoulopoulos, 2003; Mavroedi and Shaw, 2005). Moreover, cross resistance between the chemicals having different modes of action (dodine and DMIs) has also been documented (Koller and Wilcox, 1999; Koller and Wilcox, 2001), indicating therefore that mechanisms of resistance development in dodine and DMIs may not be entirely independent. To understand these complex patterns, studies on cross-resistance across different fungicides is also important.

In view of devising resistance management strategies for different fungicides, the present study was undertaken to monitor the sensitivities in *V. inaequalis* populations to various fungicides in major apple growing regions of Kashmir with the following objectives:

1. To monitor the shift in sensitivity of populations of *V. inaequalis* to extensively used fungicides such as dodine, myclobutanil, hexaconazole, difenconazole and flusilazole in Kashmir;

2. To characterize the pathogen strains showing reduced sensitivity to fungicides for cross resistance; and
3. To characterize the selected fungicide-resistant and sensitive strains of *V. inaequalis* using molecular markers.

Chapter - 2

REVIEW OF LITERATURE

Fungicides are essential for disease free and quality production of crops with viable economic returns. Prior to 1970, most fungicides used were multi-site inhibitors that have lowest risk of resistance development. However, fungicide resistance has become a more common phenomenon with the expanded use of modern classes of site-specific fungicides (Koller, 1990). Practical resistance occurs when the levels of fungicide resistance are great enough to limit the effectiveness of disease control in the field (Proffer *et al.*, 2006).

In case of *Venturia inaequalis*, resistance first developed to dodine, a highly selective fungicide primarily used for the control of apple scab (Jones, 1981; Koller *et al.*, 1999). Although mechanism of dodine resistance has not been elucidated, resistance development is typical of polygenic resistance, with resistant phenotypes comprising the least sensitive part of continuous distribution of isolate sensitivities in baseline populations (Koller *et al.*, 1999). Dodine resistance was counteracted by the introduction of benzimidazole fungicides, leading to second round of resistance development (Gilpatrick, 1982; Jones, 1981). Resistance to benzimidazoles is qualitative in nature, caused by mutational amino acid exchanges within the β-tubulin site responsible for benzimidazole binding (Koenraadt *et al*., 1992). The benzimidazoles were subsequently replaced by the class of Sterol demethylation inhibitor (DMI) fungicides. Resistance to DMIs developed in a pattern typical for polygenic resistance (Koller *et al.*, 1997). Although the mode of action of DMI fungicides is well understood (Koller, 1992), the research on comprehensive characterization of the multiple mechanisms involved in DMI resistance is still in a progressive stage (Delye *et al.*, 1997; Joseph-Horne *et al.*, 1997; Schnabel and Jones, 2001).

2.1 Baseline sensitivities of fungicides

The baseline sensitivity assessment is a pre-requisite for detection and quantification of fungicide resistance development in pathogenic fungi.

Smith *et al.* (1991) conducted baseline sensitivity studies on flusilazole and found that the mean ED_{50} values were 0.006 , 0.01 and 0.07 μg ml^{-1}, respectively, for unexposed orchard, an orchard that was left un managed for 15 years and an orchard with prolonged exposure to DMI fungicides. They also reported that a sample size of 50 is sufficient to detect the difference of 1.6 times the mean ED_{50} value. The sample size greater than 50 didn't greatly improve the precision of the test. In the United States, a set of monoconidial *V. inaequalis* isolates was used to study the baseline sensitivities to flusilazole and myclobutanil and the mean ED_{50} values were 0.008 and 0.07 μg ml^{-1}, respectively. The resistance factors were determined to be 15 and 9 for flusilazole and myclobutanil, respectively. A wider sensitivity distribution was observed in case of flusilazole; therefore, the development of practical resistance in this case could be less abrupt. Further discriminatory doses of 0.01 and 0.1 μg ml^{-1} were also calculated for flusilazole and myclobutanil, respectively (Koller *et al.*, 1991). Koller *et al.* (1997) compared ED_{50} and corresponding relative growth (RG) values of baseline population isolates for fenarimol and myclobutanil by regression analysis and found that both measures of DMI sensitivities were highly correlated, inferring therefore that the RG values were quantitative measures of isolate sensitivities. Kunz *et al.* (1997) determined the *V. inaequalis* sensitivity by microscopical evaluation of conidiophore development for untreated populations and found that ED_{50} values were 0.3 and 0.09 mg/l for flusilazole and difenoconazole, respectively. Furthermore, a strong non-linear correlation (R = 0.96) was found between the resistance factors and the sum of all DMI treatments. Sensitivity distributions of *V. inaequalis* isolates determined for a wild type population to dodine revealed that ED_{50} values for the most- and least-sensitive baseline isolates were 0.024 and 1.20 μg ml^{-1}, respectively, separated by a factor of 50. However,

the mean baseline sensitivity (RG = 41) in terms of ED_{50} value was 0.17 μg ml^{-1} (Koller *et al.*, 1999). Errampalli (2004) conducted studies on baseline sensitivity to myclobutanil on wild-type (not exposed to fungicides) population of *V. inaequalis*, collected from two crab apple trees and found that ED_{50} values ranged between 0.005 and 0.485 μg $ml^{-1}$with a mean ED_{50} value of 0.074 μg $ml^{-1}$of myclobutanil. A discriminatory dose of 0.1 μg $ml^{-1}$of myclobutanil was derived from a concentration close to the mean ED_{50} value. *V. inaequalis* population that was not exposed to myclobutanil had a baseline sensitivity (mean ED_{50}) of 0.064 μg ml^{-1} and showed a log normal distribution (Jobin and Carissse, 2007). Stevic *et al.* (2010) reported that ED_{50} values of *V. inaequalis* isolates ranged from 0.005 to 0.148 and 0.016 to 0.362 μg $ml^{-1}$for flusilazole and difenoconazole, respectively. Xu *et al.* (2010) reported that baseline sensitivity of *V. inaequalis* in terms of ED_{50} values for fenbuconazole ranged from 0.004 to 0.550 μg ml^{-1}, and for myclobutanil it ranged from 0.028 to 1.017 μg ml^{-1}. While studying the sensitivity of wild Chilean populations to different fungicides, discriminatory doses of 0.04, 1.0, 0.6, and 0.2 μg ml^{-1} of difenoconazole, fenarimol, mancozeb, and pyrimethanil, respectively, were proposed (Jose-Luis-Henriquez *et al.*, 2011). The ED_{50} value for the baseline population in Kashmir valley was found to be 0.0135 μg ml^{-1} for hexaconazole and 0.0352 μg ml^{-1} for fenarimol and hence, single discriminatory dose of 0.02 and 0.05 μg $ml^{-1}$for hexaconazole and fenarimol, respectively was determined (Kacho *et al.*, 2013). Villani *et al.* (2015) worked out ED_{50} value of 0.002 μg ml^{-1} for difenoconazole against 44 baseline isolates of *V. inaequalis*. These values were considerably lower than previously described ED_{50} values.

2.2 Sensitivity of populations exposed to fungicides

2.2.1 Dodine

Dodine was first used to manage apple scab in the late 1950s in New York, but its use was discontinued after 10 years due to resistance development (Szkolnik and Gilpatrick, 1969). Initially, resistance to dodine was reported from

Lake Ontario fruit belt and in the Geneva area of New York State. Resistance to dodine was then reported from other apple producing areas of the USA (Gilpatrick and Blowers, 1974; Jones and Walker, 1976; Yoder and Klos, 1976; Koller *et al.*, 1999), Canada (Ross and Newbery, 1977; McKay and MacNeill, 1979; Sholberg *et al.*, 1989), New Zealand (Bakker, 1999), and Poland (Meszka, and Bielenin, 2001).

A seasonal change in sensitivity to dodine was observed in *V. inaequalis* population in New York State and the least sensitive segment in the population was found to increase towards late summer (Koller *et al.*, 1995). Koller *et al.* (1999) evaluated the RG values of *V. inaequalis* at a discriminatory dose of 0.2 µg ml^{-1} and found that the isolates with RG values >90 had increased from a baseline level of 0.9 per cent to >30 per cent and, therefore, were rated resistant to dodine. For two orchards with confirmed cases of previous dodine resistance, frequencies of resistant isolates had declined to 11 per cent and 14 per cent after dodine use was discontinued for 13 and 4 years, respectively, but sensitivities had not returned to baseline levels suggesting that practical dodine resistance could recur rapidly if dodine usage is resumed. Broniarek-Niemiec and Bielenin (2008) reported a high level of resistance (above 50% of resistant forms) to dodine in 7 orchards which varied from 30.1 to 49.7 per cent in 9 out of 64 monitored orchards. However, low level of dodine resistance (below 18.9 % of resistant forms) was found in the remaining 48 orchards. In Poland, the resistant isolates of *V. inaequalis* were also prevalent in orchards where dodine was rarely or not used during the last few years (Meszka *et al.*, 2008). Sensitivity to dodine in Canada was determined by monitoring the growth of *V. inaequalis* isolates on culture medium amended with different concentrations of dodine, and ED_{50} values of wild type and fungicide exposed orchard populations were 0.525 and 1.735 µg ml^{-1}, respectively. The isolates classified as dodine-sensitive ($ED_{50} < 1.0$ µg/ml) and dodine-resistant ($ED_{50} \geq 1.0$ µg/ml) were in the proportion of 25.6 % and 31.4 %, respectively. It was also clarified that the resistance persisted upto 30 years

after discontinuing the use of dodine in these orchards (Carisse and Jobin, 2010). However, Cox *et al.* (2010) reported that *V. inaequalis* populations resistant to dodine were found to be sensitive after discontinued use of dodine for more than 20 years. Cox (2011) reported that the fungicide could potentially be used for control of early season apple scab after its resistance in New York State. Chapman *et al.* (2011) reported that *V. inaequalis* isolates resistant to dodine comprised only a small percentage (5.2 %) of the total population; hence, recommendations were put forth by extension specialists to use dodine only in orchards with low levels of resistance. Beresford *et al.* (2012) reported that there was high sensitivity to dodine in all the surveyed orchards (mean ED_{50} of 0.24 mg/l) in Hawke's Bay, New Zealand and no evidence of resistance development was observed due to limited applications (only three per season) of the fungicide. Dodine resistance was not a problem in New Zealand orchards and there was no correlation between frequency of resistant isolates and dodine usage while surveying 41 orchards in four major apple growing regions of New Zealand (Beresford *et al.,* 2013).

2.2.2 Demethylation Inhibitors (DMIs)

Sterol-demethylation inhibiting fungicides (DMIs) are systemic protectants with curative action against fungal pathogens (Russell, 2005). Sterol inhibitors specifically target C-14α-demethylation of 24 methylenedi-hydrolanosterol and disrupt fungal sterol biosynthesis. Overuse of DMIs in some countries has led to selection pressure and subsequent establishment of *V. inaequalis* strains that are less sensitive to DMIs (Stanis and Jones, 1985; Hildbrand *et al.*, 1989; Braun and McRae, 1992; Koller *et al.*, 1997; Jobin and Carisse, 2007). Resistance to DMIs is believed to be quantitative (Smith *et al.*, 1991); consequently, the loss of sensitivity by the pathogen tends to be gradual. In South Africa, Schwabe and Rifst (1982) observed that the tolerance level in *V. inaequalis* populations to DMI's increased with frequent exposure to DMI fungicides in that the excessive use of DMIs should be avoided, especially in orchards where the pathogen is showing reduced sensitivity. Different isolates of

V. inaequalis with reduced DMI sensitivities were identified as early as 1985 (Stanis and Jones, 1985) but unsatisfactory control of apple scab with increased frequencies of *V. inaequalis* resistant phenotypes was reported from a research orchard sprayed with DMI fungicides intensively for more than 10 years (Hildebrand *et al.,* 1989; Braun and McRae, 1992). Reduced disease control was observed 6 years after introduction of DMI fungicides in Nova Scotia, Canada (Hildebrand *et al.,* 1989).

The first field report of *V. inaequalis* resistance to DMI fungicides was from Michigan in 1997 (Koller *et al.*, 1997) and afterwards, resistance to DMI fungicides has been documented from different areas *viz.,* New York, Michigan and Virginia in the USA, Quebec and Ontario in Canada, Switzerland and the United Kingdom (Koller *et al.*, 1997; Kunz *et al.*, 1997; Errampalli, 2004; Jobin and Carisse, 2007; Gao *et al.*, 2009). The researchers Koller *et al.* (1997) suggested that the resistance to myclobutanil may be a diagnostic tool to evaluate resistance to other DMIs. Sensitivities to fenarimol and myclobutanil were determined by a sensitivity test based on mycelial relative growth (RG) at a discriminatory dose and it was found that frequencies of isolates with RG values >80 increased more than 20-folds over baseline levels and such isolates were rated as resistant. However, *V. inaequalis* populations showed no shift towards resistance to DMI's in an orchard with a history of 12 years of continuous usage of DMI fungicides, due to the reason that mixture of DMIs with other protectants were always used in such orchards (Smith *et al.*, 1991)

DMI resistance has also been reported in many fungal plant pathogens such as *Aspergillus nidulans* (Del-Sorbo *et al.*, 1997), *Uncinula necator* (Delye *et al.*, 1997), *Erysiphe graminis* (Delye *et al.*, 1998), *Sclerotinia homeocarpa* (Hsiang *et al.*, 1997), *Cercospora beticola* (Karaoglanidis and Thanassoulopoulos, 2003), and *Blumeriella jaapii* (Wyand and Brown, 2005).

Al-Arab and Abou-Jawdah (1997) collected 230 isolates of *V. inaequalis* from 23 orchards in Lebanon to evaluate the resistance of benzimidazoles and

ergosterol biosynthesis inhibitors/ DMIs and observed that ED_{50} values for fenarimol ranged from 0.002-0.052 μg ml^{-1} and for two new ESBIs bromocunazol and fenbuconazol it ranged from 0.0089-0.025 and 0.008-0.039 μg ml^{-1}, respectively. They further observed that all the isolates were inhibited by fenarimol at 0.25 μg ml^{-1}, whereas majority of isolates were not inhibited by benomyl even at 50 μg ml^{-1} concentration. Hence, resistance to benzimidazoles in Lebanon was widespread whereas resistance to ESBIs was not detected.

Kunz *et al.* (1997) carried out sensitivity studies of *V. inaequalis* in Germany and found that intrinsic activities of fungicides against *V. inaequalis*, as indicated by baseline sensitivities varied considerably with difenoconazole having the highest intrinsic activity followed by flusilazole, fenarimol, tebuconazole and pyrifenox. Populations with DMI history showed significant resistance to flusilazole. A strong nonlinear correlation (R = 0.96) was found between resistance factors and sum of all DMI treatments of 3 years before taking the sample and it was concluded that resistance can be expected in all apple orchards in which more than two DMI treatments per season have been applied. Kunz *et al.* (1998) reported that ESBI's and anilinopyrimidines were effective in combating apple scab and suggested that their application should be limited and active agents should be alternated to minimize the risk of fungicide resistance development.

Errampalli (2004) carried out sensitivity studies in *V. inaequalis* populations collected from four geographical locations of Ontario towards myclobutanil. He observed that mean ED_{50} values were 0.114, 0.074, 0.076, 0.071 and 0.581 μg ml^{-1} in commercial orchards 1, 2, 3, 4 and in an experimental orchard 5, respectively. Mean ED_{50} values of commercial orchards and that of baseline population didn't differ significantly, whereas, experimental orchard (orchard 5) that was treated with myclobutanil showed tolerance to fungicide. Koller *et al.* (2004) collected *V. inaequalis* populations from a commercial orchard and an experimental orchard in New York and assessed their responses to DMI treatments (fenarimol and myclobutanil) as well as Quinone outside

inhibitors (Kresoxim-methyl and trifloxystrobin). They reported decline in sensitivities to DMI's and QoI's in both the orchard populations, but isolate sensitivities to DMI's and QoI's were not correlated. The mean ED_{50} values for the population of *V. inaequalis* from commercial orchards (2.600 µg ml^{-1}) was significantly ($P<0.05$) higher than the mean ED_{50} values of the baseline population (0.064 µg ml^{-1}), suggesting that the use of myclobutanil and DMIs should be discontinued or significantly reduced before field resistance is reached in Quebec province, Canada (Jobin and Carisse, 2007). The frequency of myclobutanil resistant isolates of *V. inaequalis* collected from treated trees during the sampling year in Virginia was higher than those collected from un-treated trees. There was no influence of collection date on fungicide resistance in pathogen population, suggesting that resistance assays can be conducted at any time during the year (Marine *et al.,* 2007). Gao *et al.* (2009) reported that the reduced sensitivity to myclobutanil was positively related to the number of DMI applications and ED_{50} value of *V. inaequalis* population exposed to DMI fungicides was higher (1.852 µg ml^{-1}) than that of baseline population (0.292 µg ml^{-1}).

Pfeufer (2010) evaluated the sensitivity of *V. inaequalis* isolates collected from Pennsylvania to DMI fungicides *viz*., myclobutanil, fenbuconazole and difenoconazole. The resistance to myclobutanil and fenbuconazole was widespread in Pennsylvania apple orchards with incidences of 64 and 24 per cent, respectively, based on a discriminatory dose of 0.5 µg ml^{-1}. Thus, it was concluded that myclobutanil is no longer an effective compound for control of apple scab in most of the Pennsylvania orchards.

Sensitivity studies in *V. inaequalis* to various fungicides in commercial orchards in Chile revealed reduced sensitivity to difenoconazole, fenarimol and mancozeb with resistance factors of 4.7, 5.8, and 2.1, respectively (Jose-Luis-Henriquez *et al.*, 2011). Chapman *et al.* (2011) documented the occurrence of *V. inaequalis* isolates with reduced sensitivity to one or more of the four major

curative fungicide classes used to manage apple scab in Michigan and Indiana, wherein 57 per cent of isolates were found resistant to myclobutanil only. No fitness penalty was observed to be associated with the fungicide resistance; fungicide-resistant or shifted isolates were as fit as sensitive isolates and were, thus, equally capable of surviving and persisting in an orchard. Beresford *et al.* (2012) reported that sensitivity of *V. inaequalis* to myclobutanil and penconazole was lower in orchards where DMI usage exceeded resistance management guidelines. Beresford *et al.* (2013) found reduced sensitivity in *V. inaequalis* to DMIs, particularly for myclobutanil in 41 surveyed orchards in New Zealand and reported that the frequency of resistant isolates was higher for myclobutanil than either penconazole or dodine in most of the orchards. In plant inoculation assays, flusilazole and difenoconazole gave signiicantly better disease control of resistant isolates than myclobutanil, penconazole or fenbuconazole at standard field rates. Palani and Lalithakumari (1999) reported that the mutant strains of *V. inaequalis* showed a high degree of resistance to penconazole and low uptake of penconazole due to energy-dependent efflux, which was found to be the mechanism underlying penconazole resistance in the mutant strains. Villani *et al.* (2015) sampled 141 commercial apple orchards from north eastern United States and found that field resistance to myclobutanil is present in populations of *V. inaequalis* in majority of the orchards but none of the orchards revealed field resistance to difenoconazole.

2.3 Cross resistance

Cross-resistance is the resistance to two or more fungicides conferred by the same genetic factor (Georgopoulos, 1977). Positive cross resistance has been found among some DMI's in various pathogens such as *Sclerotinia homoecarpa* (Hisang *et al.*, 1997), *Cladosporium caryigenum* (Reynolds *et al.*, 1997) and *Cercospora beticola* (Karaoglandis and Thanassoulopoulos, 2003). However, no cross resistance has been observed among DMIs in *Monilinia oxycocci* (McManus *et al.*, 1999) and *Mycosphaerella graminicola* (Mavroedi and Shaw, 2005). *V. inaequalis* populations developed resistance consecutively to dodine,

benzimidazoles, and DMI fungicides (Jones, 1981; Gilpatrick, 1982; Koller *et al.*, 1991; Jones, 1995; Koller *et al.*, 1997). Although DMI fungicides belong to different chemical families, they have a common mode of action on *V. inaequalis* and may lead to cross-resistance in its populations (Koller, 1988). However, a comprehensive characterization of multiple mechanisms involved in DMI resistance is also emerging at present (De Waard *et al.*, 1996; De Waard, 1997; Delye *et al.*, 1997). Previously, no cross resistance was found between dodine, benzimidazoles and DMI's. Benzimidazoles effectively controlled apple scab in orchards where dodine resistant strains were present (Jones, 1981), and DMIs effectively controlled scab in orchards with dodine and benzimidazole resistance (Wilcox *et al.*, 1992). However, the mechanisms of resistance in dodine and DMI fungicides might not be entirely independent (Koller and Wilcox, 1999). Penconazole resistant laboratory mutants of *V. inaequalis* exhibited cross resistance to DMI fungicides like triadimefon, difenoconazole, myclobutanil and fenarimol and negative cross resistance to mancozeb (Palani and Lalithakumari, 1999). Koller and Wilcox (1999) found cross resistance in *V. inaequalis* populations for myclobutanil and fenarimol. Although, myclobutanil had not been applied in the orchard, frequency of myclobutanil-resistant isolates was as high as that of fenarimol resistant isolates. Furthermore, frequency of dodine-resistant isolates was significantly higher in fenarimol-resistant sub-population than the fenarimol-sensitive subpopulation, suggesting that resistance of *V. inaequalis* to DMIs and dodine may not be an entirely independent trait. Kunz *et al.* (1997) reported the existence of cross-resistance among populations of *V. inaequalis* to flusilazole and the newly introduced DMI fungicides difenoconazole, tebuconazole and pyrifenox.

In the United States, *Venturia inaequalis* populations have progressed through three consecutive rounds of fungicide resistance development, first to dodine, then benzimidazoles, and most recently to the sterol demethylation inhibitors (DMIs). Beresford *et al.* (2012) reported high level of cross resistance

between myclobutanil and penconazole but there was no significant correlation between myclobutanil and dodine.

In cross sensitivity studies, a significant correlation ($r = 0.318$, $P < 0.002$) was found between the 93 isolates tested for myclobutanil and flusilazole sensitivity (Jobin and Carissse, 2007). Xu *et al.* (2010) reported that sensitivities to myclobutanil and fenbuconazole were highly correlated; however the strength of correlation may vary with the origin of isolates. Pfeufer and Ngugi (2012) studied cross resistance to three DMIs *viz.,* myclobutanil, fenbuconazole and Difenoconazole, among 498 *V. inaequalis* isolates and found that 22 per cent of isolates were resistant to both myclobutanil and fenbuconazole, whereas only two isolates (0.40%) were resistant to all three active ingredients. Therefore, it was suggested that at least two mechanisms confer resistance to DMI fungicides, one determining resistance to myclobutanil and fenbuconazole and an additional mechanism that confers resistance to all the three fungicides.

2.4 Molecular mechanism of development resistance to DMI fungicides

The molecular basis of development of resistance to the DMI fungicides has been extensively studied in various fungal plant pathogens including *V. inaequalis*, and different mechanisms have been identified (Delye *et al.*, 1997; Delye *et al.*, 1998; Hamamoto *et al.*, 2000 and Schnabel and Jones, 2001). One of the mechanisms of resistance includes mis-sense point mutations within the target *CYP51A1* gene (Delye *et al.*, 1998; Albertini *et al.*, 2003). In *Uncinula necator*, causing powdery mildew of grapes, a single mutation at codon position 136 (Y136F) of the CYP51 gene, leading to the substitution of phenylalanine for tyrosine at codon position 136 (Y136F) was found to be responsible for resistance to triadimenol. This point mutation changes a single amino acid in 14-α-demethylase from phenylalanine to tyrosine and reduces the binding affinity of DMI fungicides (Delye *et al.*, 1997). However, most of the researchers suggested that this single change is not the only mechanism for resistance to DMI fungicides (Gisi *et al.*, 2000; Wyand and Brown, 2005).

Another important mechanism of DMI resistance involves the over expression of energy-dependant ABC and MFS transporters encoding drug efflux pumps (Hayashi *et al.*, 2002; Zwiers *et al.*, 2002). ATP-binding cassettes are membrane transporters hypothesized to pump toxic substances outside the fungal cell (Del Sorbo *et al.*, 1997; Gisi *et al.*, 2000). This mechanism has been observed to confer resistance to DMI's in different pathogens such as *Aspergillus nidulans* (De Ward *et al.*, 1981; De Waard *et al.*, 1987), *Penicillium italicum* (De Waard and Van Nistelrooy**,** 1984) and *Botrytis cinerea* (Stehmann and De Waard, 1995). ABC transporters (P-glycoproteins) are known to be responsible for multidrug resistance in prokaryotic and eukaryotic organisms (Schinkel and Borst, 1991; Gottesman and Pastan, 1993). Cloning of *atrA* and *atrB* genes encoding ABC transporters from a filamentous fungus *A. nidulans* demonstrated that an *atrB* transgene rendered *Saccharomyces cerevisiae* resistant to azole fungicide (Del Sorbo *et al.*, 1997).

Changes in the expression level of CYP51 might also contribute to the gradual development of DMI resistance. This mechanism of DMI resistance has been reported for several phytopathogenic fungi like *Candida glabrata*, *Penicillium digitatum* and *V. inaequalis* (Marichal *et al.*, 1997; Hamamoto *et al*/. 2000; Schnabel and Jones, 2001). There are several different mechanisms known to increase CYP51 expression in fungi. One of the mechanisms is an increase in the copy number of the CYP51 gene as in case of *C. glabrata* (Marichal *et al.*, 1997). The presence of 126-bp repeats in the promoter region of CYP51 gene enhanced its expression, which resulted in DMI resistance in *P. digitatum* (Hamamoto *et al.*, 2000). Another important mechanism has been identified in *V. inaequalis* in which over expression of CYP51 in strains resistant to myclobutanil was due to the presence of a 553-bp insertion located in the promoter region (Schnabel and Jones, 2001). However, the high expression of CYP51 was responsible for DMI resistance in some, but not all, field DMI-resistant isolates of this fungus. Villani *et al.* (2016) reported that the relative expression of CYP51A1

was higher for isolates resistant to both difenoconazole and myclobutanil or with resistance to difenoconazole only than the isolates with resistance to myclobutanil only. Furthermore, in difenoconazole resistant isolates, a repeated element, "EL 3,1,2", with the properties of transcriptional enhancer was present two to four times upstream of CYP51A1 gene, conferring difenoconazole resistance. Hence, it was suggested that different resistance mechanisms may operate in different DMI fungicides. Since no definitive study has proven a single genetic origin of resistance, most agree that the populations resistant to DMI fungicides have multiple mechanisms and develop resistance quantitatively over an extended period of time (Delye *et al.*, 1997; Joseph-Horne and Holloman, 1997; Koller *et al.*, 1997; Gisi *et al.*, 2000; Schnabel and Jones, 2001; Wyand and Brown, 2005; Jobin and Carisse, 2007).

Chapter - 3

MATERIALS AND METHODS

The present study was carried out in the Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar during 2014-2017.

3.1 Collection of diseased samples and isolation of *Venturia inaequalis* isolates

Regular surveys were conducted in different apple growing regions of Shopian and Baramulla districts of the Kashmir valley for collection of scab infected plant samples of apple from orchards sprayed with fungicides. Information about the history of fungicides used (name of fungicide, number of years of continuous use, number of years for which satisfactory disease control obtained, current situation of disease control, quantity of respective fungicides used and time of application) was obtained from the orchardists (Table 1). The diseased samples collected from the two districts *viz*., Baramulla and Shopian comprised of 10 samples per orchard and 10 orchards per district, making a total of 200 samples. These samples were brought to the laboratory for isolations and further studies using standard pathological techniques. In addition to this, various diseased samples were also obtained from unsprayed (without fungicidal sprays) orchard from district Baramulla for baseline sensitivity studies.

The isolation of the pathogen isolates was carried out as per the method described by Padder *et al.* (2013). Pathogen populations consisting of 100 isolates from each district (200 isolates from 2 districts) were maintained on PDA medium for further studies. The details of orchard populations are depicted in Table 2.

3.2 Baseline sensitivity assay

The baseline population of the pathogen was obtained from district Baramulla from a backyard orchard with no history of fungicide sprays. In all, thirty isolates were used in baseline sensitivity studies (Table 3). Base line

Table 1: History of fungicide usage for apple scab management in different orchards of district Baramulla and Shopian

Orchards	Location	Fungicides Used	Total No. of sprays	Years of continuous use	Years of satisfactory disease control	Recommended spray schedule		Current level of disease control		
						Yes	No	Poor	Medium	Good
District Baramulla										
B_1	Tangmarg	Captaf, Mancozeb, Index, Indofil Z-78, Superstar, Contaf, Carbendazim, Antracol, Baycor	5-6	7	7	✓				✓
B_2	Pattan	Dithane, Superstar, Grapel, Antracol, Rubigan, Carbendazim, Contaf, Score, Governor	10	10	8		✓		✓	
B_3	Kunzar	Dithane, Superstar, Index, Antracol, Contaf, Score, Governor	5	9	9	✓				✓
B_4	Nowpora	Indofil M-45, Superstar, Grapel, Contaf, Score, Governor, Antracol,	6-7	8	8		✓			✓
B_5	Wagoora	Indofil M-45, Superstar, contaf, Grapel,Score, Antracol, Rubigan	6	9	9	✓				✓
B_6	Ladoora	Dithane, Superstar, Contaf, Score, Baycor. Governor, Taakat, Insist, Index, Wave	10	15	11		✓	✓		
B_7	Parrapora Rafiabad	Dithane, Superstar, Grapel, Anvil, Score, Governor, Antracol, Rubigan, Avtaar, Taakat	9-10	15	10		✓	✓		
B_8	Seelu	Captan, Superstar, Grapel, Score, Contaf, Antracol, Governor, Avtaar	7	8	8		✓		✓	
B_9	Sheeri	Dithane, Superstar, Grapel, Antracol, Carbendazim, Rubigan, Anvil, Governor, Score	7-8	8	8		✓			✓
B_{10}	Boniyar	Dithane, Superstar,Indofil Z-78, Index, Score, Anvil	5-6	5	5		✓			✓

Contd…

Table 1: Contdd

District Shopian										
S_1	Pahnoo	Dithane, Indofil Z-78, Superstar, Graple, Index, Contaf, Score,Governor	8	10	10		✓		✓	
S_2	Wathu	Dithane, Superstar, Boon, Relay, Anvil, Score, Governor	8	9	9		✓		✓	
S_3	Pinjoora	Dithane, Superstar, Grapel, Score, Contaf, Governor Rubigan, Taaqat, Boon, Covert, Noor	10	14	10		✓	✓		
S_4	Imam Sahib	Dithane, Superstar, Grapel, Score, Rubigan, Governor, Boon	7-8	10	10		✓			✓
S_5	Sadew	Dithane, Superstar, Baycor, Contaf, Grapel, Kohinoor, Score, Governor	10	10	10		✓		✓	
S_6	Wachi	Indofil Z-78, Superstar, Antracol, Score, Grapel, Governor, Anvil, Nativo, Cabriotop	8-9	10	10		✓	✓		
S_7	Shirmaal	Dithane, Superstar, Antracol, Index, Anvil, Score	5-6	7	7	✓				✓
S_8	Batpora	Dithane, Superstar, Indofil Z-78, Grapel, Rubigan, Antracol, Score, Avtaar	8-10	5	5		✓			✓
S_9	Mulu	Dithane, Superstar, Boon, Score, Grapel, Anvil, Rubigan, Antracol	6-7	7	7	✓				✓
S_{10}	Maldeer	Indofil Z-78, Superstar, Grapel, Rubigan, Antracol, Score, Contaf, Governor	7-8	8	8		✓			✓

Table 2: Summary information of different *Venturia inaequalis* isolates collected from different orchards of Baramulla and Shopian districts

Orchard/ Location	Location code	Isolate Code	No. of isolates
Baseline population			
Baramulla	Bp	Bp1-Bp30	30
Exposed population			
District Baramulla			
Tangmarg	B_1	B_{1-1} to B_{1-10}	10
Pattan	B_2	B_{2-1} to B_{2-10}	10
Kunzar	B_3	B_{3-1} to B_{3-10}	10
Nowpora	B_4	B_{4-1} to B_{4-10}	10
Wagoora	B_5	B_{5-1} to B_{5-10}	10
Ladoora	B_6	B_{6-1} to B_{6-10}	10
Parrapora Rafiabad	B_7	B_{7-1} to B_{7-10}	10
Seelu	B_8	B_{8-1} to B_{8-10}	10
Sheeri	B_9	B_{9-1} to B_{9-10}	10
Boniyar	B_{10}	B_{10-1} to B_{10-10}	10
District Shopian			
Pahnoo	S_1	S_{1-1} to S_{1-10}	10
Wathu	S_2	S_{2-1} to S_{2-10}	10
Pinjoora	S_3	S_{3-1} to S_{3-10}	10
Imam Sahib	S_4	S_{4-1} to S_{4-10}	10
Sadew	S_5	S_{5-1} to S_{5-10}	10
Wachi	S_6	S_{6-1} to S_{6-10}	10
Shirmaal	S_7	S_{7-1} to S_{7-10}	10
Batpora	S_8	S_{8-1} to S_{8-10}	10
Mulu	S_9	S_{9-1} to S_{9-10}	10
Maldeer	S_{10}	S_{10-1} to S_{10-10}	10
Total			**230**

B-Baramulla, S-Shopian, Bp-baseline population

sensitivity levels of the pathogen (*Venturia inaequalis*) to different fungicides (Table 3) was determined with conventional mycelial growth assays using concentrations *viz.,* 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, and 10.0 µg ml^{-1} of different fungicides namely dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole (on active ingredient basis). Fungicides were separately dissolved in water and dispensed into lukewarm sterilized PDA. Five- mm mycelial discs of each pathogen isolate was placed at the centre of Petri plates containing PDA medium amended with respective fungicide concentrations. Each treatment was replicated thrice and control plates (without fungicide) for each isolate were also maintained for comparison. Inoculated Petri-plates were incubated at 19±1ºC and the radial growth measured after 30 days.

Table 3: Fungicides used in sensitivity studies of *Venturia inaequalis*

Chemical	**Trade Name**
Dodine	Superstar 65WP
Hexaconazole	Contaf 5 EC
Myclobutanil	Index 10WP
Difenoconazole	Score 25 EC
Flusilazole	Governor 40 EC

Percent Relative Growth (RG) for each isolate was calculated as follows (Chapman *et al.,* 2011).

$$RG\ (\%) = \frac{\text{Mean radial growth on the fungicide amended medium}}{\text{Mean radial growth on the unamended medium}} \times 100$$

The same procedure was followed for all the test fungicides.

3.3 Data analysis

ED_{50} and ED_{90} values for each pathogen-fungicide combination were calculated by regression of the relative growth against logarithm of fungicide concentrations. Mean ED_{50} and ED_{90} values of the whole baseline population for each fungicide was calculated by taking the mean of all the individual ED_{50} and ED_{90} values of various isolates.

Minimum inhibitory concentration (MIC) values for each isolate were also calculated using regression equations.

Resistance factor (RF) for each fungicide was calculated by dividing the highest ED_{50} with the mean ED_{50} value in a baseline population.

A discriminatory dose for each fungicide was determined by selecting a dose very close (slightly higher) to the respective mean baseline ED_{50} value.

3.4 Determination of sensitivity levels of pathogen populations collected from fungicide treated orchards

The pathogen population collected from orchards sprayed regularly with fungicides were tested separately for their sensitivity levels to the recommended and commonly used fungicides such as dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole (Table 3).

Fungicide sensitivity tests were carried out by fungal growth inhibition using poisoned food technique (Nene and Thapliyal, 2002). The PDA medium used in sensitivity studies was amended with discriminatory doses of the respective fungicides selected on the basis of base-line sensitivity data. The discriminatory doses for dodine, hexaconazole, myclobutanil, difenoconazole and

flusilazole were 0.20, 0.04, 0.20, 0.03 and 0.03 µg ml^{-1}, respectively. Fungicides (discriminatory doses) were dissolved in water and then dispensed into sterilized PDA medium. Five mm mycelial discs of the pathogen isolate was placed at the centre of the Petri plates containing fungicide amended PDA medium, and the plates were incubated at 19±1°C. Each treatment was replicated thrice and control (PDA without fungicide) was also maintained for comparison. The radial growth was measured after 30 days of incubation. Per cent relative growth (RG) for each isolate was calculated as described previously.

For each fungicide, frequency distributions of RG values of fungicide-exposed pathogen populations were compared to the frequency distributions of the reference baseline populations.

All the isolates collected from the two districts were classified as sensitive (S), shifted (SH) and resistant (R) for each of the fungicide on the basis of RG values when compared to previously described thresholds of RG (Table 4) values (Koller *et al.*, 1997; Chapman *et al.*, 2011).

Table 4: Criteria for classification of isolates as sensitive (S), shifted (SH) and resistant (R) based on relative growth as per Chapman *et al.*, 2011)

Fungicide	Classification based on relative growth (RG)		
	Sensitive	**Shifted**	**Resistant**
Dodine	≤50%	51–90%	>90%
Hexaconazole Myclobutanil Difenoconazole Flusilazole	≤30%	31–69%	≥70%

Moreover, assessment of cross resistance was made by calculating the percentage of isolates resistant to two or more fungicides.

3.5 Molecular characterization of sensitive and resistant isolates

Ten *V. inaequalis* isolates sensitive to all the test fungicides and ten isolates resistant to two or more of the test fungicides were selected for molecular analysis. The sensitive isolates selected were Bp3, S_{3-4}, Bp5, Bp8, Bp12, Bp20, Bp21, Bp22, Bp24 and Bp28whereas the ten resistant isolates comprised of S_{1-8}, S_{2-8}, S_{3-3}, S_{6-3}, S_{6-7}, S_{4-4}, B_{2-2}, B_{6-5}, B_{7-10} and B_{8-7}.

Total genomic fungal DNA from all the isolates was isolated using modified CTAB (Cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980). All the selected sensitive and resistant isolates were grown in 100ml flasks containing 40 ml potato dextrose broth. These flasks were inoculated with 5mm mycelial discs of each isolate and incubated at 19±1ºC for 30 days. After 30 days, the mycelium was harvested and dried under two layers of sterilized blotting filter paper and stored at -80 ºC for further use. The dried mycelium from each isolate was ground into fine powder by constant crushing using autoclaved and pre chilled mortar and pestle in liquid nitrogen, so as to make the mycelium brittle and reduce DNase activity. About 40-50 mg of fine powdered tissue was transferred into 1.5 ml polypropylene centrifuge tube containing about 700 µl pre-heated (65°C) 2X CTAB extraction buffer [100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 1% PVP and 0.5% β-mercaptoethanol (added just before use)]. The powder was suspended in the buffer by inverting and rotating the tubes properly. The tubes were incubated at 65°C for 60 minutes in a water bath. The samples were mixed occasionally while maintaining at 65°C. After incubation, 700 µl of chloroform: isoamyl alcohol (24:1) was added and the tubes were swirled, till it made an emulsion. The tubes were centrifugated at 10,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a clean sterile 1.5 ml polypropylene centrifuge tube using large bore tip and then again 700 µl mixture of chloroform: isoamylalcohol

in the same ratio was added and that step was repeated. After transferring the supernatant to a new tube, equal volume of pre-chilled isopropyl alcohol (approximately 600-700 µl) was added and the tubes were inverted gently several times and kept at -20°C overnight. The microfuge tubes were then centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatant decanted. The DNA pellet was rinsed twice with 70 per cent ethanol for five minutes so as to remove any residual salts followed by re-centrifugation. Pellet was collected and leftover ethanol was dried up completely by turning down microfuge tubes on a blotting paper and allowed to air dry (at room temperature). The pellet was dissolved in 200 µl of 1X TE (Tris EDTA buffer-10mM Tris HCl, 1mM EDTA, pH 8.0), and left for few hours at room temperature to dissolve the DNA. Heat-treated RNase (Fermentas) was then added to a final concentration of 10µg/ml and subsequently incubated at 37ºC for 30 minutes. DNA of all the isolates was obtained in a similar way and stored at -80°C for further use.

3.5.1 Assessment of quality and quantity of DNA

The quantity of DNA was assessed by Agarose gel electrophoresis. Agarose (0.8 g) was dissolved in 100 ml of 0.5X Tris acetate EDTA (TAE) electrophoresis buffer. The mixture was heated till the agarose was dissolved completely and formed a transparent and clear solution. It was cooled down to 60°C with constant stirring, ethidium bromide added to the final concentration of 0.5 µg/ml of buffer. Then the agarose solution was poured into an already prepared gel mould with combs and was left for 20-30 min for solidification. DNA samples for loading were prepared by adding 2 µl loading dye (6X) (0.25% w/v bromophenol blue, 50% glycerol in sterile water) to 8 µl DNA such that the final concentration of loading dye was 1X. The DNA samples were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known concentration was also loaded. The gel was run for about 1-2 hours and visualized under UV transilluminator using photo gel documentation system (Alfa Imager EC, Protein Simple, USA), and the DNA samples photographed. The

intensity of fluorescence of each sample was compared with that of a standard marker and then DNA concentration of each sample ascertained. The quality of DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/poor quality band). The DNA of all the samples was diluted to 25 ng/µl by adding double distilled sterile water and used for PCR.

The DNA from the selected sensitive isolates was mixed to form a sensitive bulk and that of selected resistant isolates was mixed to form a resistant bulk. A set of 46 RAPD primers was used against resistant and sensitive bulk (Table 5) and four ISSR primers were used to screen all the selected resistant and sensitive isolates (Table 5).

3.5.2 Polymerase chain reaction amplification

Amplification of DNA samples of the isolates was carried out with polymerase chain reaction (PCR) (Saiki *et al.,* 1988). PCR reaction was carried out in 0.2 ml PCR tubes with 25 µl reaction volume containing 1X buffer (10 mM Tris-HCl pH 8.0; 50 mM KCl), 1.5 mM MgCl2, 0.2 mM dNTP mix, 1 U of Taq DNA polymerase (Fermentas, Thermo Fisher Scientific,Waltham, MA, USA), 40-50 ng of DNA template, 0.4 µM of primer and 15.8 µl of sterilized distilled water. The reaction mixture of 25 µl in PCR tubes was given short spin or vortexed in microfuge (Thermo Scientific, Thermo Electron Corporation) and placed in 96-well thermal cycler. PCR amplifications for all the RAPD markers were performed in thermal cycler programmed for initial denaturation at 94ºC followed by 35 cycles at 94ºC for 1 minute, annnealing temperature of 37ºC for 1 minute, extension at 72ºC for 2 minutes and a final extension of 10 minutes. For ISSR markers, amplifications were performed using thermal cycler (Eppendorf, Hamburg, Germany) programmed for initial denaturation at 94ºC for 5 minutes followed by 35 cycles with denaturation at 94ºC for 1 minute, annealing for 1 minute at 47ºC for (AG)5 and (CA)5; 54ºC for (CCA)5 and (CGA)5; extension at 72ºC for 2 minutes and a final extension of 10 minutes at 72ºC.

Table 5: Primer sequences of RAPD and ISSR markers used for molecular analysis of sensitive and resistant isolates

Primer	Sequence	Primer	Sequence
RAPD primers			
Primer-1	5'CAGGCCCTTC	Primer-24	5'TGGACCGGTG
Primer-2	5'TGCCGAGCTG	Primer-25	5'CTCACCGTCC
Primer-3	5'ATGCAGCCAC	Primer-26	5'AAAGCTGCGG
Primer-4	5'AATCGGGCTG	Primer-27	5'TGTCATCCCC
Primer-5	5'AGGGGTCTTG	Primer-28	5'AAGCCTCGTC
Primer-6	5'GGTCCCTGAC	Primer-29	5'TGCGTGCTTG
Primer-7	5'GAAACGGGTG	Primer-30	5'GACGGATCAG
Primer-8	5'GTGACGTAGG	Primer-31	5'ACTTCGCCAC
Primer-9	5'GGGTAACGCC	Primer-32	5'ACCGCGAAGG
Primer-10	5'GTGATCGCGT	Primer-33	5'GGACCCAACC
Primer-11	5'CAATCGCCGT	Primer-34	5'GTCGCCGTCA
Primer-12	5'TCGGCGATAG	Primer-35	5'TCTGGTGAGG
Primer-13	5'CAGCACCCAC	Primer-36	5'TGAGCGGACA
Primer-14	5'TCTGTGCTGG	Primer-37	5'ACCTGAACGG
Primer-15	5'TTCCGAACCC	Primer-38	5'TTGGCACGGG
Primer-16	5'AGCCAGCGAA	Primer-39	5'GTGTGCCCCA
Primer-17	5'GACCGCTTGT	Primer-40	5'CTCTGGAGAC
Primer-18	5'TTCGAGCCAG	Primer-41	5'GGTCTACACC
Primer-19	5'GGGGGTCTTT	Primer-42	5'AGCGCCATTG
Primer-20	5'CCGCATCTAC	Primer-43	5'CACCGTATCC
Primer-21	5'GATGACCGCC	Primer-44	5'GGGGTGACGA
Primer-22	5'GAACGGACTC	Primer-45	5'CTTCCCCAAG
Primer-23	5'GTCCCGACGA	Primer-46	5'CATCCGTGCT
ISSR primers			
AG5	5' AGAGAGAGAG 3'	$(CGA)_5$	5' DHBCGACGACGACGACGA 3'
$(CA)_5$	5' DBDCACACACACA 3'	$(CCA)_5$	5' DDBCCACCACCACCACCA 3'

3.5.3 Visualization of PCR products

To 25 µl of the amplified product, 4 µl of 6X loading dye was added to make the final concentration of loading buffer in the reaction samples to 1X. The PCR products were resolved on 1.2 per cent agarose gel. The gel was prepared in 0.5X TAE buffer. Ethidium bromide was added at concentration of 0.5 µg/µl. The gel was run at 5 V/cm, visualized under UV light and photographed using Alfa Imager Gel Documentation System (Alfa Imager EC Protein Simple, USA).

Chapter - 4

EXPERIMENTAL FINDINGS

The present study was aimed at monitoring fungicide resistance in *Venturia inaequalis* populations comprising of 200 isolates exposed to commonly used fungicides namely dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole for apple scab management. To establish the baseline sensitivity, 30 isolates of *V. inaequalis* were collected from an unsprayed apple orchard. For monitoring resistance development in *V. inaequalis*, two hundred isolates were collected from fungicide-exposed orchards of major apple growing districts *viz.,* Baramulla and Shopian of Kashmir valley. The discriminatory doses of respective fungicides, selected from the baseline data were tested against the 200 isolates of the pathogen, and the data obtained was compared with that of baseline population. The selected resistant and sensitive isolates were subsequently screened with RAPD and ISSR markers.

The isolates of the pathogen were isolated from various diseased samples (200 isolates from fungicide-exposed leaves and 30 isolates from fungicide-un-exposed leaves) collected from Baramulla and Shopian districts and identified as *Venturia inaequalis* on the basis of morphological characteristics (Sivanesan and Waller, 1974). In addition, history of the fungicide usage in these orchards was obtained from the respective orchardists (Table 3). Fungicides namely bitertanol, captan, mancozeb, zineb, carbendazim, dodine, hexaconazole, myclobutanil, difenoconazole, flusilazole, fenarimol and propioneb were commonly used in both the districts. The total number of fungicidal sprays in a single growing season varied from 5 to10 in both the districts. Three of the orchardists from district Baramulla (Orchardists of B_1, B_2 and B_5) and two from district Shopian (Orchardists of S_7 and S_9) followed recommended scheme and dosage of fungicides. In case of district Baramulla, the number of years of continuous use of fungicides varied from 5 to 15 with minimum years of use in orchard B_{10} and maximum in B_6 and B_7 orchards. In district Baramulla, orchardists experienced

diminished level of disease control in B2, B6 and B7 orchards where the number of years of continuous fungicide usage was 10, 15 and 15, respectively. In district Shopian, the number of years of continuous use of fungicides varied from 5 to 14 years with minimum years of use in orchard S_8 and maximum in orchard S_3. The diminished level of disease control was recorded in orchard S_2 where fungicide use has been continued for 14 years.

4.1 Sensitivity of baseline population of *Venturia inaequalis* to different fungicides

Baseline sensitivities of *V. inaequalis* to different fungicides *viz.*, dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole were determined with a set of 30 monoconidial isolates from wild type orchard that had never been sprayed with the test fungicides. Each baseline isolate was tested with seven concentrations of each fungicide and the response was recorded in terms of relative growth. ED_{50}, ED_{90} and minimum inhibitory concentration (MIC) values were calculated by regression of the relative growth (RG) against logarithm of fungicide concentration.

4.1.1 Baseline sensitivity to dodine

An appreciable amount of variability was observed in relative growth of different baseline isolates of the pathogen exposed to dodine at different concentrations (Table 6, Plate 1). An increase in relative growth of the isolates with decrease in fungicide concentrations was observed, thereby indicating maximum inhibition of mycelial growth at higher fungicide concentration. The mycelial growth of the isolates *viz.,* Bp17, Bp18, Bp20, Bp21, Bp24, Bp27 and Bp28, was completely inhibited with 10 µg ml^{-1} concentration of dodine. However, isolate Bp16 showed comparatively less inhibition (RG=27.27%) among all the isolates at 10 µg ml^{-1} concentration.

ED_{50} values of dodine for *V. inaequalis* baseline isolates ranged from 0.020 to 0.457 µg ml^{-1} (Table 7). The lowest ED_{50} value of 0.020 µg ml^{-1} was observed for Bp12 isolate followed by that (0.062 µg ml^{-1}) for isolate Bp17.

Table 6: Response of different baseline isolates of *Venturia inaequalis* to different concentrations of dodine

Conc. (μgml^{-1}) / Isolate	Relative growth (%)						
	10	**1**	**0.3**	**0.1**	**0.03**	**0.01**	**0.001**
Bp1	8.51	27.66	46.81	48.94	72.34	76.60	93.62
Bp2	12.50	25.00	43.75	46.88	75.00	84.38	100.00
Bp3	9.76	19.51	43.90	51.22	73.17	78.05	97.56
Bp4	13.79	27.59	48.28	55.17	72.41	77.59	96.55
Bp5	14.93	29.85	38.81	47.76	62.69	65.67	95.52
Bp6	17.24	36.21	48.28	50.00	68.97	70.69	86.21
Bp7	11.54	38.46	57.69	61.54	80.77	82.69	96.15
Bp8	16.90	42.25	50.70	53.52	70.42	73.24	92.96
Bp9	19.44	38.89	48.61	50.00	75.00	80.56	97.22
Bp10	11.94	23.88	44.78	50.75	68.66	74.63	92.54
Bp11	15.38	19.23	34.62	36.54	57.69	65.38	88.46
Bp12	13.56	23.73	30.51	32.20	44.07	47.46	84.75
Bp13	14.29	42.86	59.52	64.29	83.33	86.90	97.62
Bp14	8.96	17.91	47.76	53.73	74.63	79.10	92.54
Bp15	11.27	30.99	50.70	52.11	73.24	78.87	90.14
Bp16	27.27	45.45	68.18	72.73	90.91	95.45	100.00
Bp17	0.00	23.73	40.68	44.07	61.02	69.49	84.75
Bp18	0.00	15.38	42.31	50.00	73.08	76.92	92.31
Bp19	10.96	24.66	41.10	49.32	65.75	73.97	90.41
Bp20	0.00	21.33	40.00	45.33	77.33	81.33	96.00
Bp21	0.00	25.93	37.04	40.74	66.67	70.37	96.30
Bp22	5.56	16.67	44.44	48.61	72.22	77.78	98.61
Bp23	14.10	23.08	42.31	43.59	61.54	64.10	82.05
Bp24	0.00	28.07	42.11	47.37	68.42	71.93	94.74
Bp25	14.29	45.24	59.52	61.90	71.43	76.19	95.24
Bp26	11.27	25.35	45.07	49.30	70.42	73.24	90.14
Bp27	0.00	19.75	41.98	48.15	69.14	72.84	88.89
Bp28	0.00	28.57	46.75	48.05	64.94	71.43	90.91
Bp29	7.23	28.92	45.78	51.81	62.65	67.47	79.52
Bp30	12.12	33.33	45.45	48.48	75.76	81.82	96.97

Table 7: Sensitivity of baseline isolates of *Venturia inaequalis* to dodine

Isolate	Regression equation	ED_{50} (µg ml^{-1})	ED_{90} (µg ml^{-1})	MIC value (µg ml^{-1})
Bp1	Y=-22.1X+31.23 (R^2=0.97)	0.144	7.9	25.1
Bp2	Y=-23.8X+31.37 (R^2=0.96)	0.164	6.3	19.9
Bp3	Y=-23.7X+29.44 (R^2=0.97)	0.135	6.3	15.8
Bp4	Y=-21.6X+34.07 (R^2=0.97)	0.183	12.5	31.6
Bp5	Y=-19.8X+30.71 (R^2=0.97)	0.106	10	31.6
Bp6	Y=-18.7X+33.59 (R^2=0.97)	0.158	17.7	61.65
Bp7	Y=-21.4X+39.66 (R^2=0.95)	0.328	24.2	79.4
Bp8	Y=-21.2X+31.49 (R^2=0.98)	0.158	10.2	29.5
Bp9	Y=-20.0X+38.34 (R^2=0.97)	0.300	25.1	79.4
Bp10	Y=-21.3X+30.97 (R^2=0.97)	0.158	7.9	25.11
Bp11	Y=-19.4X+25.78 (R^2=0.94)	0.026	6.3	19.9
Bp12	Y=-16.4X+22.90 (R^2=0.89)	0.020	3.0	7.0
Bp13	Y=-21.2X+42.74 (R^2=0.95)	0.457	31.6	100.0
Bp14	Y=-23.0X+30.30 (R^2=0.94)	0.141	6.3	19.9
Bp15	Y=-20.6X+34.51 (R^2=0.96)	0.181	12.5	39.8
Bp16	Y=-26.0X+34.21 (R^2=0.92)	0.24	8.5	19.95
Bp17	Y=-21.4X+24.61 (R^2=0.98)	0.062	3.1	7.9
Bp18	Y=-24.9X+24.89 (R^2=0.95)	0.094	3.1	7.9
Bp19	Y=-21.0X+29.72 (R^2=0.98)	0.102	7.9	25.1
Bp20	Y=-25.8X+25.64 (R^2=0.96)	0.112	3.9	7.9
Bp21	Y=-24.0X+23.99 (R^2=0.98)	0.081	3.1	7.9
Bp22	Y=-24.8X+26.92 (R^2=0.97)	0.11	3.9	12
Bp23	Y=-17.7X+29.35 (R^2=0.97)	0.068	12.3	39.8
Bp24	Y=-23.4X+26.72 (R^2=0.98)	0.1	5.0	12.5
Bp25	Y=-21.1X+34.02 (R^2=0.95)	0.177	12.5	39.81
Bp26	Y=-20.8X+31.16 (R^2=0.97)	0.125	10.0	25.1
Bp27	Y=-23.3X+25.21 (R^2=0.97)	0.081	3.9.8	12
Bp28	Y=-22.2X+27.66 (R^2=0.97)	0.096	5.01	15.8
Bp29	Y=-18.2X+30.66 (R^2=0.97)	0.084	12.5	39.8
Bp30	Y=-22.2X+33.89 (R^2=0.97)	0.180	11.7	31.6

ED- Effective dose, MIC-Minimum inhibitory concentration,Y=Relative growth, X=log of conc.

However, the highest ED_{50} value of 0.457 µg ml^{-1} was determined for isolate Bp13 followed by that (0.328 µg ml^{-1}) for isolate Bp7. Similarly, ED_{90} values of dodine for *V. inaequalis* baseline isolates ranged from 3.1 to 31.6 µg ml^{-1} (Table 7). The lowest ED_{90} values of 3.0 µg ml^{-1} was observed for Bp12 isolate followed by 3.1 µg ml^{-1} in isolates Bp17, Bp18 and Bp21. The highest ED_{90} value of 31.6 µg ml^{-1} was assessed for Bp13 isolate followed by 25.1 and 24.2µg ml^{-1} for the isolates Bp9 and Bp7, respectively. The minimum inhibitory concentration (MIC) values of dodine for *V. inaequalis* baseline isolates ranged from 7.0 to 79.4 µg ml^{-1} (Table 7). The lowest MIC value of 7.0 µg ml^{-1} was observed for isolate Bp12 followed by 7.9 µg ml^{-1} for isolates Bp17, Bp18 and Bp21. The highest MIC value of 100µg ml^{-1} was discerned for the isolate Bp13 followed by that (79.4µg ml^{-1}) for Bp7 and Bp9 isolates.

4.1.2 Baseline sensitivity to hexaconazole

A varied response was assessed in baseline isolates of *V. inaequalis* to hexaconazole at different concentrations (Table 8, Plate 2). An increase in relative growth with decrease in hexaconazole concentration was observed in all the isolates of the pathogen. All the pathogen isolates except the isolates Bp1, Bp10, Bp25, Bp27, Bp28 and Bp29 were completely inhibited at 1 µg ml^{-1} concentration of hexaconazole.

The ED_{50} values of hexaconazole for *V. inaequalis* baseline isolates ranged from 0.001 to 0.169 µg ml^{-1} (Table 9). The lowest ED_{50} value of 0.001 µg ml^{-1} was determined for Bp2 and Bp3 isolates followed by 0.003 µg ml^{-1} for the isolate Bp4 and Bp19. The highest ED_{50} value of 0.169 µg ml^{-1} was assessed for Bp10 isolate followed by 0.110 µg ml^{-1} in Bp29 isolate. Similarly, the ED_{90} values of hexaconazole for *V. inaequalis* baseline isolates varied from 0.1 to 5.0 µg ml^{-1} (Table 9). The lowest ED_{90} value of 0.1 µg ml^{-1} was observed for Bp3 isolate followed by 0.2 µg ml^{-1} for the isolate Bp2. However, the highest ED_{90} value of 5.0 µg ml^{-1} was discerned for Bp10 and Bp29 isolates followed by 4.6 µg ml^{-1} for the isolate Bp25. The MIC values of hexaconazole for *V. inaequalis* baseline

Table 8: Response of different baseline isolates of *Venturia inaequalis* to different concentrations of hexaconazole

Conc. (µg ml^{-1}) / Isolate	Relative growth (%)						
	10	**1**	**0.3**	**0.1**	**0.03**	**0.01**	**0.001**
Bp1	0.0	8.5	21.3	42.6	63.8	78.7	93.6
Bp2	0.0	0.0	0.0	0.0	18.8	31.3	43.8
Bp3	0.0	0.0	0.0	0.0	9.8	19.5	39.0
Bp4	0.0	0.0	15.5	24.1	32.8	37.9	55.2
Bp5	0.0	0.0	9.0	11.9	16.4	19.4	32.8
Bp6	0.0	0.0	12.1	36.2	44.8	55.2	69.0
Bp7	0.0	0.0	0.0	23.1	40.4	57.7	82.7
Bp8	0.0	0.0	14.1	36.6	53.5	62.0	78.9
Bp9	0.0	0.0	37.5	55.6	79.2	90.3	95.8
Bp10	0.0	17.1	51.4	60.0	82.9	88.6	94.3
Bp11	0.0	0.0	23.1	34.6	57.7	69.2	100.0
Bp12	0.0	0.0	23.7	30.5	49.2	54.2	78.0
Bp13	0.0	0.0	33.8	38.8	55.0	63.8	86.3
Bp14	0.0	0.0	37.3	44.8	65.7	74.6	89.6
Bp15	0.0	0.0	36.6	42.3	56.3	73.2	93.0
Bp16	0.0	0.0	0.0	0.0	47.8	56.5	95.7
Bp17	0.0	0.0	10.2	16.9	30.5	42.4	84.7
Bp18	0.0	0.0	12.0	32.0	44.0	52.0	74.0
Bp19	0.0	0.0	0.0	8.3	13.9	20.8	72.2
Bp20	0.0	0.0	14.1	22.5	43.7	47.9	70.4
Bp21	0.0	0.0	0.0	7.1	46.4	53.6	82.1
Bp22	0.0	0.0	11.1	16.7	27.8	40.3	55.6
Bp23	0.0	0.0	33.3	38.5	56.4	69.2	79.5
Bp24	0.0	0.0	24.6	31.6	49.1	56.1	77.2
Bp25	0.0	23.8	38.1	48.8	69.0	73.8	88.1
Bp26	0.0	0.0	28.2	50.7	73.2	84.5	93.0
Bp27	0.0	4.9	44.4	54.3	76.5	81.5	92.6
Bp28	0.0	9.4	42.4	47.1	51.8	58.8	72.9
Bp29	0.0	24.1	45.8	50.6	69.9	77.1	92.8
Bp30	0.0	0.0	15.2	30.3	54.5	66.7	81.8

Table 9: Sensitivity of baseline isolates of *Venturia inaequalis* to hexaconazole

Isolate	Regression equation	ED_{50} (µg ml^{-1})	ED_{90} (µg ml^{-1})	MIC value (µg ml^{-1})
Bp1	Y=-26.5X+17.36 (R^2=0.95)	0.054	1.8	3.9
Bp2	Y=-12.1X+1.12 (R^2=0.80)	0.001	0.2	1.2
Bp3	Y=-9.7X-0.04 (R^2=0.75)	0.001	0.1	1.0
Bp4	Y=-14.9X+8.60 (R^2=0.95)	0.003	0.8	3.7
Bp5	Y=-8.4X+4.28 (R^2=0.93)	0.010	0.2	3.1
Bp6	Y=-19.9X+10.96 (R^2=0.92)	0.011	1.0	3.2
Bp7	Y=-23.1X+5.84 (R^2=0.87)	0.012	0.6	1.7
Bp8	Y=-22.7X+12.06 (R^2=0.92)	0.020	1.2	3.1
Bp9	Y=-28.8X+22.13 (R^2=0.89)	0.102	2.5	5.0
Bp10	Y=-26.3X+29.85 (R^2=0.91)	0.169	5.0	12.5
Bp11	Y=-27.2X+13.19 (R^2=0.94)	0.042	1.2	3.0
Bp12	Y=-21.2X+12.28 (R^2=0.94)	0.016	1.2	3.7
Bp13	Y=-23.5X+15.95 (R^2=0.94)	0.033	1.7	3.9
Bp14	Y=-25.5X+18.85 (R^2=0.92)	0.054	1.9	5.0
Bp15	Y=-25.6X+17.25 (R^2=0.94)	0.048	1.9	4.6
Bp16	Y=-25.8X+2.54 (R^2=0.80)	0.015	0.5	1.2
Bp17	Y=-21.1X+5.13 (R^2=0.86)	0.009	0.6	1.7
Bp18	Y=-20.5X+9.87 (R^2=0.93)	0.012	0.9	2.9
Bp19	Y=-16.3X+0.001 (R^2=0.70)	0.003	0.3	1.0
Bp20	Y=-19.3X+8.85 (R^2=0.93)	0.009	0.8	2.5
Bp21	Y=-22.9X+3.95 (R^2=0.82)	0.010	0.5	1.5
Bp22	Y=-15.2X+6.32 (R^2=0.93)	0.003	0.6	2.5
Bp23	Y=-22.8X+16.55 (R^2=0.92)	0.031	1.9	5.0
Bp24	Y=-21.2X+12.71 (R^2=0.94)	0.017	1.3	3.8
Bp25	Y=-23.0X+17.36 (R^2=0.95)	0.083	4.6	12.5
Bp26	Y=-27.9X+18.98 (R^2=0.90)	0.070	1.9	4.6
Bp27	Y=-26.4X+23.94 (R^2=0.91)	0.097	3.2	7.9
Bp28	Y=-19.0X+21.13 (R^2=0.91)	0.029	3.8	12.5
Bp29	Y=-23.8X+27.41 (R^2=0.97)	0.110	5.0	14.1
Bp30	Y=-23.8X+11.53 (R^2=0.92)	0.022	1.1	3.01

ED- Effective dose, MIC-Minimum inhibitory concentration,Y=Relative growth, X=log of conc.

isolates varied from 1.0 to 14.0 µg ml^{-1}. The lowest MIC value of 0.1 µg ml^{-1} was determined for Bp3 isolate followed by that (0.2 µg ml^{-1}) for isolate Bp2. However, the highest MIC value of 14.0 µg ml^{-1} was discerned for Bp29 isolate followed by that (12.5 µg ml^{-1}) for isolates Bp10 and Bp28.

4.1.3 Baseline sensitivity to myclobutanil

A varied response in *V. inaequalis* baseline isolates to myclobutanil at different concentrations was observed (Table 10, Plate 3). An increase in relative growth with decrease in concentration of myclobutanil was recorded. Fifty per-cent of the isolates (Bp2, Bp4, Bp5, Bp7, B p8, Bp9, Bp15, Bp16, Bp17, Bp21, Bp22, Bp26, Bp27, Bp28 and Bp30) were completely inhibited at 10 µg ml^{-1} concentration of myclobutanil

The ED_{50} values of myclobutanil for *V. inaequalis* baseline isolates ranged from 0.016 to 0.501 µg ml^{-1}(Table 11). The lowest ED_{50} value of 0.016 µg ml^{-1} was observed for Bp2 and Bp22 isolates followed by 0.03 µg ml^{-1} for the isolate Bp27. The highest ED_{50} value of 0.501 µg ml^{-1} was determined for the isolate Bp10 followed by that (0.361 µg ml^{-1}) for the isolate Bp7. ED_{90} values of myclobutanil for *V. inaequalis* baseline isolates varied from 1.5 to 39.8 µg ml^{-1} with the lowest ED_{90} value (1.5 µg ml^{-1}) for the isolate Bp22 followed by that (1.8 µg ml^{-1}) for the isolate Bp2, whereas the highest ED_{90} value of 39.8 µg ml^{-1} was observed for the isolate Bp10 followed by that (22.3 µg ml^{-1}) for the isolate Bp23. Similarly, MIC values of myclobutanil for *V. inaequalis* baseline isolates ranged from 4.7 to 100.0 µg ml^{-1}. The lowest MIC value of 4.7 µg $ml^{-1}$was determined for the isolate Bp22 followed by that (5.0 µg ml^{-1}) for the isolate Bp2. The highest MIC value of 100.0 µg $ml^{-1}$was recorded for the isolate Bp10 followed by that (63.1 µg ml^{-1}) for the isolates Bp23, Bp25 and Bp29.

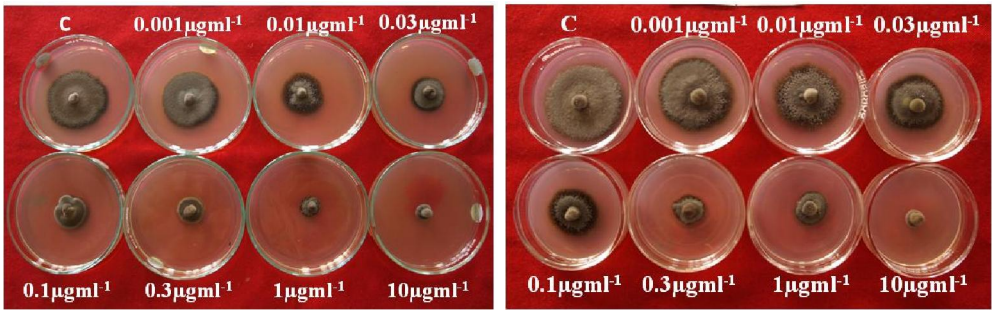
Table 10: Response of different baseline isolates of *Venturia inaequalis* to different concentrations of myclobutanil

Conc. (µg ml^{-1}) / Isolate	Relative growth (%)						
	10	**1**	**0.3**	**0.1**	**0.03**	**0.01**	**0.001**
Bp1	8.51	17.02	51.06	55.32	76.60	82.98	93.62
Bp2	0.00	12.50	25.00	28.13	50.00	53.13	75.00
Bp3	4.88	9.76	39.02	43.90	73.17	75.61	97.56
Bp4	0.00	34.48	48.28	55.17	65.52	72.41	89.66
Bp5	0.00	20.90	49.25	53.73	59.70	67.16	80.60
Bp6	6.90	27.59	39.66	51.72	72.41	79.31	89.66
Bp7	0.00	40.38	61.54	73.08	84.62	88.46	100.00
Bp8	0.00	28.17	50.70	61.97	73.24	78.87	92.96
Bp9	0.00	36.11	61.11	73.61	86.11	88.89	98.61
Bp10	16.42	41.79	62.69	65.67	80.60	86.57	95.52
Bp11	7.69	23.08	42.31	51.92	73.08	75.00	96.15
Bp12	6.78	23.73	44.07	45.76	61.02	64.41	91.53
Bp13	9.52	28.57	46.43	48.81	64.29	70.24	90.48
Bp14	10.45	29.85	50.75	59.70	80.60	83.58	95.52
Bp15	0.00	16.90	36.62	42.25	61.97	64.79	87.32
Bp16	0.00	27.27	45.45	50.00	77.27	81.82	100.00
Bp17	0.00	13.56	47.46	54.24	74.58	81.36	98.31
Bp18	7.69	23.08	50.00	57.69	76.92	84.62	96.15
Bp19	5.48	30.14	43.84	50.68	68.49	71.23	90.41
Bp20	5.33	24.00	40.00	41.33	61.33	66.67	93.33
Bp21	0.00	22.22	40.74	40.74	59.26	66.67	96.30
Bp22	0.00	8.33	19.44	27.78	52.78	58.33	72.22
Bp23	15.38	30.77	56.41	66.67	79.49	82.05	97.44
Bp24	12.28	28.07	47.37	52.63	82.81	89.47	94.74
Bp25	9.52	33.33	45.24	50.00	61.90	66.67	78.57
Bp26	0.00	30.99	36.62	42.25	56.34	64.79	73.24
Bp27	0.00	18.52	39.51	44.44	51.85	61.73	71.60
Bp28	0.00	28.57	46.75	49.35	76.62	83.12	93.51
Bp29	9.64	45.78	53.01	57.83	74.70	81.93	93.98
Bp30	0.00	24.24	42.42	48.48	72.73	78.79	93.94

Table 11: Sensitivitiy of baseline isolates of *Venturia inaequalis* to myclobutanil

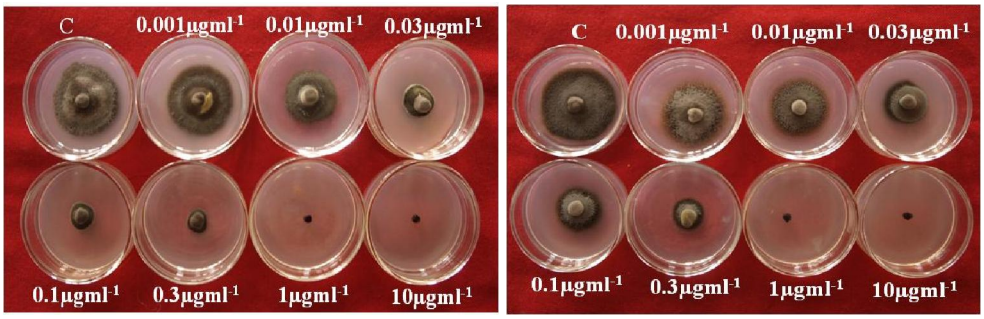
Isolate	Regression equation	ED_{50} (µg ml^{-1})	ED_{90} (µg ml^{-1})	MIC value (µg ml^{-1})
Bp1	Y=-23.7X+31.11 (R^2= 0.93)	0.163	6.3	19.9
Bp2	Y=-19.3X+15.33 (R^2= 0.97)	0.016	1.8	5.0
Bp3	Y=-25.5X+23.38 (R^2= 0.94)	0.090	3.1	7.9
Bp4	Y=-21.5X+30.54 (R^2=0.96)	0.121	7.9	25.1
Bp5	Y=-20.2X+26.91 (R^2=0.93)	0.074	6.3	19.9
Bp6	Y=-22.2X+30.05 (R^2=0.97)	0.127	7.9	19.9
Bp7	Y=-24.7X+39.08 (R^2=0.91)	0.361	12.5	31.6
Bp8	Y=-23.6X+31.34 (R^2=0.95)	0.159	7.9	19.9
Bp9	Y=-25.0X+38.28 (R^2=0.90)	0.341	12.5	31.6
Bp10	Y=-20.2X+43.83 (R^2=0.94)	0.501	39.8	100.0
Bp11	Y=-23.2X+29.31 (R^2=0.97)	0.129	6.6	17.7
Bp12	Y=-20.8X+27.20 (R^2=0.98)	0.079	6.6	19.9
Bp13	Y=-20.2X+30.80 (R^2=0.98)	0.113	10.4	31.6
Bp14	Y=-22.7X+35.71 (R^2=0.95)	0.236	12.5	31.6
Bp15	Y=-22.4X+21.69 (R^2=0.98)	0.051	3.2	9.1
Bp16	Y=-25.7X+28.59 (R^2=0.97)	0.150	5.0	12.5
Bp17	Y=-26.5X+26.10 (R^2=0.95)	0.123	3.9	7.9
Bp18	Y=-24.0X+32.41 (R^2=0.95)	0.192	7.9	19.9
Bp19	Y=-21.2X+30.04 (R^2=0.98)	0.114	7.9	25.1
Bp20	Y=-21.8X+25.43 (R^2=0.98)	0.074	5.0	12.5
Bp21	Y=-23.4X+22.93 (R^2=0.98)	0.064	3.5	9.3
Bp22	Y=-20.1X+13.88 (R^2=0.95)	0.016	1.5	4.7
Bp23	Y=-21.6X+39.39 (R^2=0.95)	0.319	22.3	63.1
Bp24	Y=-23.2X+34.77 (R^2=0.93)	0.220	11.5	30.1
Bp25	Y=-17.1X+32.07 (R^2=0.97)	0.087	19.4	63.1
Bp26	Y=-18.1X+25.22(R^2=0.95)	0.042	6.30	24.5
Bp27	Y=-18.3X+22.61 (R^2=0.96)	0.030	4.8	15.8
Bp28	Y=-24.6X+29.36 (R^2=0.95)	0.141	6.0	15.4
Bp29	Y=-20.5X+38.86 (R^2=0.95)	0.294	25.1	63.1
Bp30	Y=-24.5X+26.79 (R^2=0.97)	0.111	04.8	12.3

ED- Effective dose, MIC-Minimum inhibitory concentration,Y=Relative growth, X=log of conc.



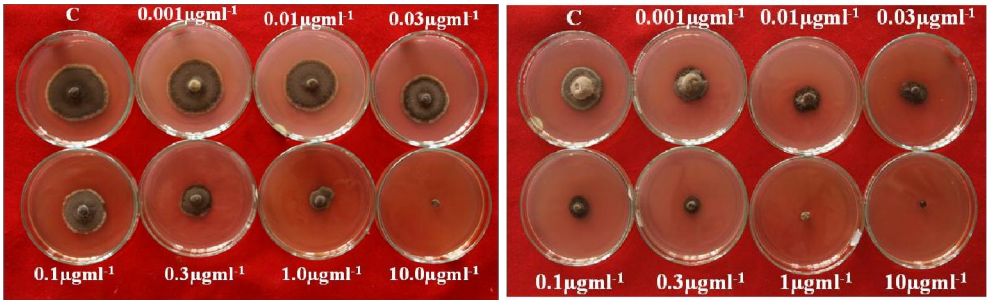
Isolate Bp5 **Isolate Bp8**

Plate 1: ***In vitro*** **response of different** ***Venturia inaequalis*** **baseline isolates to different concentrations of dodine**



Isolate Bp17 **Isolate Bp8**

Plate 2: Response of different ***Venturia inaequalis*** **baseline isolates to different concentrations of hexaconazole**



Isolate Bp4 **Isolate Bp3**

Plate 3: Response of different ***Venturia inaequalis*** **baseline isolates to different concentrations of myclobutanil**

4.1.2 Baseline sensitivity to difenoconazole

A varied response of *V. inaequalis* baseline isolates to difenoconazole at different concentrations was observed (Table 12, Plate 4). The relative growth (RG) of the pathogen isolates increased with decrease in difenoconazole concentration, eight isolates (Bp1, Bp2, Bp3, Bp16, Bp21, Bp26, Bp27 and Bp30) showed complete inhibition of growth at 1 µg ml^{-1} of difenoconazole. The mycelial growth of rest of the isolates was completely inhibited at 10 µg ml^{-1} concentration.

The ED_{50} values of difenoconazole for *V. inaequalis* baseline isolates ranged from 0.002 to 0.137 µg ml^{-1} (Table 13). The lowest ED_{50} value of 0.002 µg ml^{-1} was observed for the isolate Bp27 followed by that (0.003 µg ml^{-1}) for the isolates Bp1, Bp22 and Bp25. The highest ED_{50} value of 0.137 µg ml^{-1} was recorded for the isolate Bp16 followed by that (0.091 µg ml^{-1}) for the isolate Bp19. ED_{90} values of difenoconazole for *V. inaequalis* baseline isolates varied from 0.4 to 4.7 µg ml^{-1}(Table 13). The lowest ED_{90} value of 0.4 µg ml^{-1} was observed for the isolate Bp27 followed by isolate Bp1 (0.6 µg ml^{-1}) and isolate Bp2 (0.7 µg ml^{-1}). The highest ED_{90} value of 4.7 µg ml^{-1} was observed for the isolate Bp16 followed by isolate Bp17 (4.6 µg ml^{-1}) and isolate Bp18 (4.5µg ml^{-1}). The MIC values of difenoconazole for *V. inaequalis* baseline isolates ranged from 1.9 to 11.2 µg ml^{-1} (Table 13). The lowest MIC value of 1.9 µg ml^{-1} was observed for the isolate Bp27 followed by isolate Bp2 (2.2 µg ml^{-1}) and isolate Bp1 (2.6 µg ml^{-1}). The highest MIC value of 11.2 µg ml^{-1} was recorded for the isolate Bp16 followed by that (10.0 µg ml^{-1}) in isolate Bp17, Bp18 and Bp23.

4.1.3 Baseline sensitivity to flusilazole

The baseline isolates of *V. inaequalis* also showed varied response to different concentrations of flusilazole (Table 14, Plate 5). The ED_{50} values of flusilazole for *V. inaequalis* baseline isolates ranged from 0.002 to 0.079 µg ml^{-1} (Table 15). The lowest ED50 value of 0.002 µg ml^{-1} was determined for the

Table 12: Response of different baseline isolates of *Venturia inaequalis* to different concentrations of difenoconazole

Conc. (µg ml^{-1}) / Isolate	Relative growth (%)						
	10	**1**	**0.3**	**0.1**	**0.03**	**0.01**	**0.001**
Bp1	0.00	0.00	8.51	17.02	34.04	40.43	55.32
Bp2	0.00	0.00	12.50	12.50	43.75	46.88	75.00
Bp3	0.00	0.00	14.63	24.39	34.15	39.02	63.41
Bp4	0.00	17.24	27.59	34.48	62.07	75.86	96.55
Bp5	0.00	5.97	17.91	23.88	29.85	47.76	74.63
Bp6	0.00	15.52	27.59	34.48	55.17	68.97	89.66
Bp7	0.00	7.69	23.08	38.46	53.85	61.54	80.77
Bp8	0.00	14.08	22.54	26.76	39.44	54.93	90.14
Bp9	0.00	18.06	30.56	43.06	52.78	58.33	87.50
Bp10	0.00	5.97	29.85	38.81	50.75	59.70	89.55
Bp11	0.00	7.69	23.08	30.77	46.15	51.92	84.62
Bp12	0.00	13.56	27.12	30.51	37.29	50.85	67.80
Bp13	0.00	9.52	21.43	28.57	38.10	42.86	59.52
Bp14	0.00	17.91	20.90	23.88	50.75	62.69	80.60
Bp15	0.00	14.08	22.54	33.80	59.15	70.42	87.32
Bp16	0.00	0.00	54.55	72.73	81.82	81.82	90.91
Bp17	0.00	32.20	37.29	44.07	57.63	62.71	79.66
Bp18	0.00	23.08	38.46	46.15	55.77	65.38	84.62
Bp19	0.00	19.18	38.36	52.05	65.75	76.71	94.52
Bp20	0.00	5.33	21.33	29.33	33.33	42.67	56.00
Bp21	0.00	0.00	29.63	33.33	44.44	48.15	62.96
Bp22	0.00	8.33	13.89	19.44	30.56	33.33	58.33
Bp23	0.00	10.26	46.15	50.00	56.41	58.97	88.46
Bp24	0.00	7.02	17.54	21.05	42.11	43.86	66.67
Bp25	0.00	7.14	19.05	26.19	32.14	35.71	59.52
Bp26	0.00	0.00	16.90	25.35	42.25	47.89	61.97
Bp27	0.00	0.00	4.94	9.88	29.63	33.33	51.85
Bp28	0.00	5.19	20.78	25.97	38.96	45.45	55.84
Bp29	0.00	4.82	26.51	33.73	45.78	48.19	78.31
Bp30	0.00	0.00	18.18	24.24	42.42	46.97	75.76

Table 13: Sensitivity of baseline isolates of *Venturia inaequalis* to difenoconazole

Isolate	Regression equation	ED_{50} (µg ml^{-1})	ED_{90} (µg ml^{-1})	MIC value (µg ml^{-1})
Bp1	Y=-15.5X+6.48 (R^2=0.92)	0.003	0.6	2.6
Bp2	Y=-20.2X+6.85 (R^2=0.88)	0.009	0.7	2.2
Bp3	Y=-16.7X+8.25 (R^2=0.94)	0.005	0.8	3.2
Bp4	Y=-25.6X+19.04 (R^2=0.96)	0.060	2.2	5.5
Bp5	Y=-18.7X+9.70 (R^2=0.93)	0.008	0.9	3.3
Bp6	Y=-23.4X+19.99 (R^2=0.98)	0.041	2.7	7.1
Bp7	Y=-21.9X+15.79 (R^2=0.92)	0.026	1.8	5.2
Bp8	Y=-21.8X+13.42 (R^2=0.93)	0.021	1.4	4.1
Bp9	Y=-21.5X+19.76 (R^2=0.99)	0.038	2.8	8.2
Bp10	Y=-23.1X+15.90 (R^2=0.96)	0.032	1.8	4.9
Bp11	Y=-21.4X+13.32 (R^2=0.92)	0.019	1.4	4.2
Bp12	Y=-16.9X+15.38 (R^2=0.98)	0.010	2.1	8.1
Bp13	Y=-15.3X+13.16 (R^2=0.99)	0.005	1.6	7.2
Bp14	Y=-21.0X+15.50 (R^2=0.94)	0.022	1.8	5.4
Bp15	Y=-23.7X+17.15 (R^2=0.96)	0.038	1.9	5.2
Bp16	Y=-26.4X+27.90 (R^2=0.80)	0.137	4.7	11.2
Bp17	Y=-19.0X+25.61 (R^2=0.97)	0.070	4.6	10.0
Bp18	Y=-20.9X+23.66 (R^2=0.99)	0.052	4.5	10.0
Bp19	Y=-24.7X+24.55 (R^2=0.98)	0.091	3.8	9.7
Bp20	Y=-14.7X+11.96 (R^2=0.97)	0.004	1.3	6.4
Bp21	Y=-17.3X+13.79 (R^2=0.91)	0.009	1.7	6.2
Bp22	Y=-14.2X+9.03 (R^2=0.95)	0.003	0.9	4.3
Bp23	Y=-22.0X+22.17 (R^2=0.92)	0.051	3.5	10.0
Bp24	Y=-17.3X+10.82 (R^2=0.95)	0.007	1.1	3.9
Bp25	Y=-14.6X+10.90 (R^2=0.96)	0.003	1.1	5.4
Bp26	Y=-17.5X+10.07 (R^2=0.94)	0.007	1.0	3.7
Bp27	Y=-14.2X+4.20 (R^2=0.88)	0.002	0.4	1.9
Bp28	Y=-15.3X+12.01 (R^2=0.96)	0.005	1.3	6.0
Bp29	Y=-19.9X+13.80 (R^2=0.95)	0.015	1.5	4.8
Bp30	Y=-20.0X+9.46 (R^2=0.93)	0.010	0.9	2.9

ED- Effective dose, MIC-Minimum inhibitory concentration,Y=Relative growth, X=log of conc.

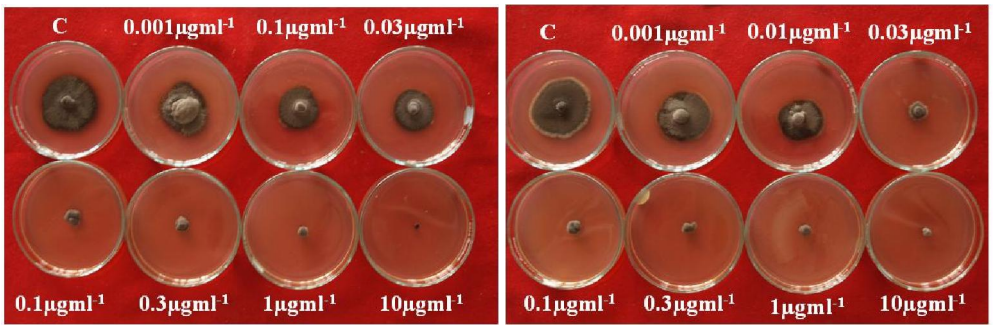
Table 14: Response of different baseline isolates of *Venturia inaequalis* to different concentrations of flusilazole

Conc. (µg ml^{-1}) / Isolate	Relative growth (%)						
	10	**1**	**0.3**	**0.1**	**0.03**	**0.01**	**0.001**
Bp1	0.00	0.00	25.53	38.30	55.32	72.34	97.87
Bp2	0.00	0.00	12.50	37.50	56.25	68.75	93.75
Bp3	0.00	0.00	19.51	29.27	43.90	48.78	87.80
Bp4	0.00	6.90	17.24	36.21	44.83	46.55	58.62
Bp5	0.00	14.93	29.85	41.79	53.73	62.69	74.63
Bp6	0.00	20.69	34.48	37.93	51.72	65.52	82.76
Bp7	0.00	0.00	25.00	38.46	46.15	48.08	73.08
Bp8	0.00	14.08	30.99	36.62	47.89	56.34	73.24
Bp9	0.00	5.56	16.67	27.78	38.89	44.44	63.89
Bp10	0.00	5.97	29.85	38.81	47.76	49.25	65.67
Bp11	0.00	0.00	11.54	26.92	34.62	40.38	50.00
Bp12	0.00	0.00	6.78	13.56	37.29	47.46	64.41
Bp13	0.00	0.00	39.29	45.24	55.95	61.90	85.71
Bp14	0.00	8.96	17.91	20.90	35.82	41.79	68.66
Bp15	0.00	14.08	33.80	42.25	59.15	63.38	73.24
Bp16	0.00	0.00	18.18	36.36	72.73	77.27	90.91
Bp17	0.00	0.00	6.78	13.56	37.29	42.37	67.80
Bp18	0.00	0.00	11.54	15.38	36.54	42.31	69.23
Bp19	0.00	0.00	10.96	12.33	27.40	34.25	57.53
Bp20	0.00	0.00	26.67	32.00	45.33	50.67	66.67
Bp21	0.00	0.00	14.81	22.22	44.44	48.15	77.78
Bp22	0.00	0.00	22.22	30.56	47.22	52.78	72.22
Bp23	0.00	10.26	30.77	37.18	48.72	50.00	61.54
Bp24	0.00	0.00	10.53	21.05	35.09	42.11	63.16
Bp25	0.00	11.90	35.71	42.86	59.52	60.71	71.43
Bp26	0.00	19.72	39.44	45.07	59.15	63.38	87.32
Bp27	0.00	17.28	29.63	37.04	46.91	49.38	64.20
Bp28	0.00	15.58	28.57	32.47	41.56	46.75	77.92
Bp29	0.00	24.10	40.96	45.78	60.24	69.88	95.18
Bp30	0.00	0.00	27.27	42.42	66.67	75.76	96.97

Table 15: Sensitivity of baseline isolates of *Venturia inaequalis* to flusilazole

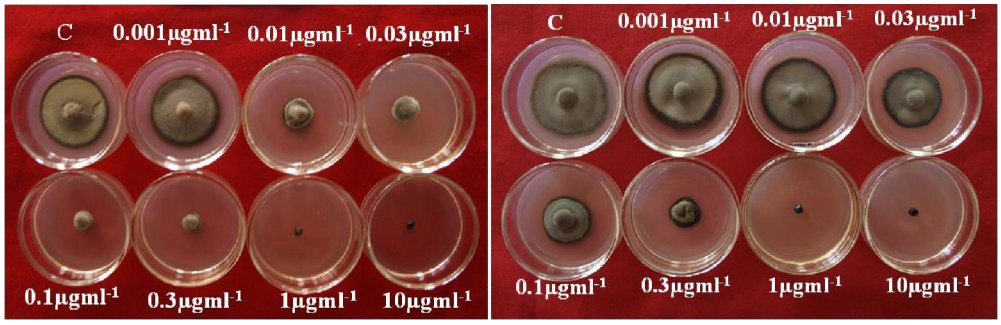
Isolate	Regression equation	ED_{50} (µg ml^{-1})	ED_{90} (µg ml^{-1})	MIC value (µg ml^{-1})
Bp1	Y=-26.9X+14.21 (R^2=0.94)	0.050	1.4	3.3
Bp2	Y=-26.4X+11.75 (R^2=0.92)	0.032	1.2	2.8
Bp3	Y=-22.5X+10.08 (R^2=0.92)	0.017	1.0	2.8
Bp4	Y=-16.2X+13.68 (R^2=0.93)	0.007	1.5	6.3
Bp5	Y=-19.9X+19.62 (R^2=0.98)	0.020	3.0	7.9
Bp6	Y=-20.8X+20.87 (R^2=0.99)	0.038	3.2	10.0
Bp7	Y=-19.5X+13.32 (R^2=0.93)	0.013	1.4	3.9
Bp8	Y=-18.7X+18.11 (R^2=0.99)	0.019	2.5	7.9
Bp9	Y=-16.9X+11.13 (R^2=0.97)	0.005	1.1	3.9
Bp10	Y=-17.5X+16.28 (R^2=0.94)	0.013	1.9	7.9
Bp11	Y=-14.4X+8.70 (R^2=0.98)	0.002	0.7	3.9
Bp12	Y=-18.2X+5.86 (R^2=0.98)	0.004	0.6	2.1
Bp13	Y=-23.0X+17.96 (R^2=0.98)	0.040	1.9	5.0
Bp14	Y=-17.0X+10.55 (R^2=0.95)	0.005	1.1	3.9
Bp15	Y=-19.8X+20.84 (R^2=0.96)	0.039	3.2	10.0
Bp16	Y=-27.2X+14.74 (R^2=0.89)	0.051	1.5	3.16
Bp17	Y=-18.3X+5.45 (R^2=0.89)	0.004	0.5	1.94
Bp18	Y=-18.4X+6.47 (R^2=0.90)	0.004	0.6	1.9
Bp19	Y=-14.9X+5.25 (R^2=0.90)	0.002	0.5	1.9
Bp20	Y=-18.4X+13.06 (R^2=0.93)	0.011	1.4	5.0
Bp21	Y=-20.8X+8.68 (R^2=0.92)	0.011	0.8	2.5
Bp22	Y=-19.9X+12.02 (R^2=0.94)	0.013	1.3	3.9
Bp23	Y=-16.3X+17.57 (R^2=0.94)	0.011	2.5	10
Bp24	Y=-17.2X+7.24 (R^2=0.93)	0.003	0.7	2.5
Bp25	Y=-19.4X+20.76 (R^2=0.93)	0.037	3.1	10.0
Bp26	Y=-21.7X+22.97 (R^2=0.98)	0.054	3.2	10
Bp27	Y=-19.9X+19.62 (R^2=0.98)	0.012	3.1	10.0
Bp28	Y=-18.4X+16.14 (R^2=0.97)	0.015	1.9	6.3
Bp29	Y=-23.4X+24.45 (R^2=0.99)	0.079	3.9	10.0
Bp30	Y=-27.5X+16.40 (R^2=0.94)	0.060	2.0	3.16

ED- Effective dose, MIC-Minimum inhibitory concentration,Y=Relative growth, X=log of conc.



Isolate Bp6 **Isolate Bp4**

Plate 4: Response of different *Venturia inaequalis* baseline isolates to different concentrations of difenoconazole



Isolate Bp16 **Isolate Bp30**

Plate 5: Response of different *Venturia inaequalis* baseline isolates to different concentrations of flusilazole

isolates Bp 11 and Bp19 followed by that (0.003 µg ml^{-1}) for the isolate Bp24. The isolate Bp29 had the highest the ED_{50} value (0.079 µg ml^{-1}) followed by that (0.060 µg ml^{-1}) for the isolate Bp30. The ED_{90} values of *V. inaequalis* baseline isolates to flusilazole ranged from 0.5 to 3.9 µg ml^{-1} with the lowest ED_{90} value (0.5 µg ml^{-1}) recorded for the isolates Bp17 and Bp19 followed by that (0.6 µg ml^{-1}) for the isolates Bp12 and Bp18. The highest ED_{90} value of 3.9 µg $ml^{-1}$was determined for the isolate Bp29 followed by that (3.2 µg ml^{-1}) for the isolates Bp6 and Bp15. Similarly, the MIC values of flusilazole for *V. inaequalis* baseline isolates ranged from 1.9 to 10.0 µg ml^{-1} with the lowest MIC value of 1.9 µg ml^{-1} recorded for the isolate Bp17, Bp18 and Bp19 followed by isolate Bp12 (2.1 µg ml^{-1}). The highest MIC value of 10.0 µg ml^{-1} was observed for the isolates Bp6, Bp15, Bp25 and Bp29 followed by that (7.9 µg ml^{-1}) for the isolates Bp5, Bp8 and Bp10.

4.1.6 Sensitivity characteristics of baseline population

The histograms constructed by plotting frequency of isolates against ED_{50} values for each fungicide revealed that the frequencies of ED_{50} values of all the tested fungicides were lognormally distributed (Fig. 1-5). The mean ED_{50} values of dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole were 0.14, 0.036, 0.17, 0.02 and 0.022 µg ml^{-1}, respectively (Table 16).

The resistance factors of 3.3, 4.7, 3.3, 6.9 and 3.5 were calculated for dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole, respectively (Table 16). These values indicated that the fungicides such as dodine, myclobutanil and flusilazole have narrow range of sensitivity distributions, whereas the fungicides such as difenoconazole and hexaconazole have wider range of sensitivities.

Based on the mean ED_{50} values for each fungicide, discriminatory doses of the respective fungicides were selected based on a dose close to their respective mean ED_{50} values. The discriminatory doses of 0.20, 0.04, 0.20, 0.03 and 0.03 µg ml^{-1} were selected for dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole, respectively.

Table 16: Sensitivity characteristics of baseline population of *Venturia inaequalis* for different fungicides

Fungicide	Range ED_{50} values (µg ml^{-1})	Mean ED_{50} values (µg ml^{-1})	Resistance Factor	Discriminatory dose (µg ml^{-1})
Dodine	0.008 to 0.457	0.14	3.3	0.20
Hexaconazole	0.001 to 0.169	0.03	4.7	0.04
Myclobutanil	0.016 to 0.501	0.15	3.3	0.20
Difenoconazole	0.002 to 0.137	0.02	6.9	0.03
Flusilazole	0.002 to 0.070	0.02	3.5	0.03

4.2 Sensitivity of fungicide-exposed *Venturia inaequalis* populations to fungicides

V. inaequalis populations from apple orchard of district Baramulla and Shopian exposed to different fungicides were assessed for their sensitivity at respective discriminatory doses of different fungicides and the frequency distributions of their RG values were compared to those of baseline isolates at a selected discriminatory dose (Plate 6).

4.2.1 Sensitivity to dodine

The *V. inaequalis* isolates collected from fungicide-exposed orchards of Baramulla and Shopian districts were evaluated for their sensitivity to dodine at a discriminatory dose of 0.2 µg ml^{-1}, and the RG values calculated. Similarly, the RG values of baseline isolates of the pathogen were also calculated at 0.2 µg ml^{-1} discriminatory dose of dodine. The results revealed that the RG values of baseline isolates ranged from 39.17 to 57.38 per cent with a mean RG value of 45.29 per

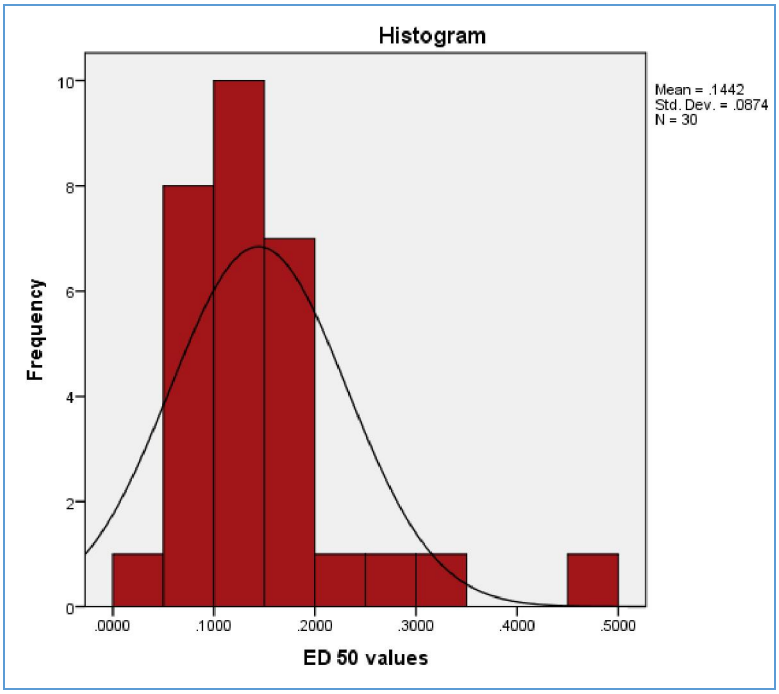


Fig. 1: Frequency distribution of ED_{50} values (dodine) for baseline isolates of *Venturia inaequalis*

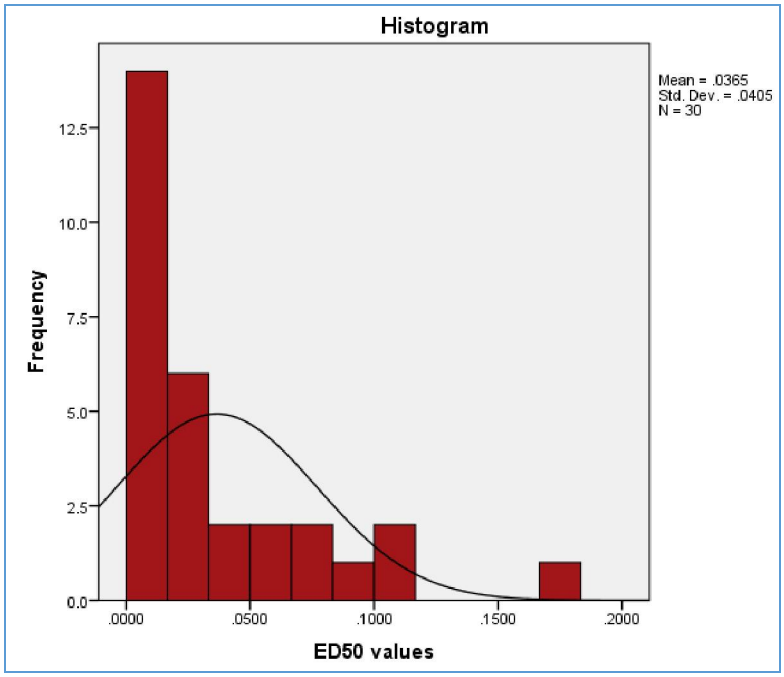


Fig. 2: Frequency distribution of ED_{50} values (hexaconazole) for baseline isolates of *Venturia inaequalis*

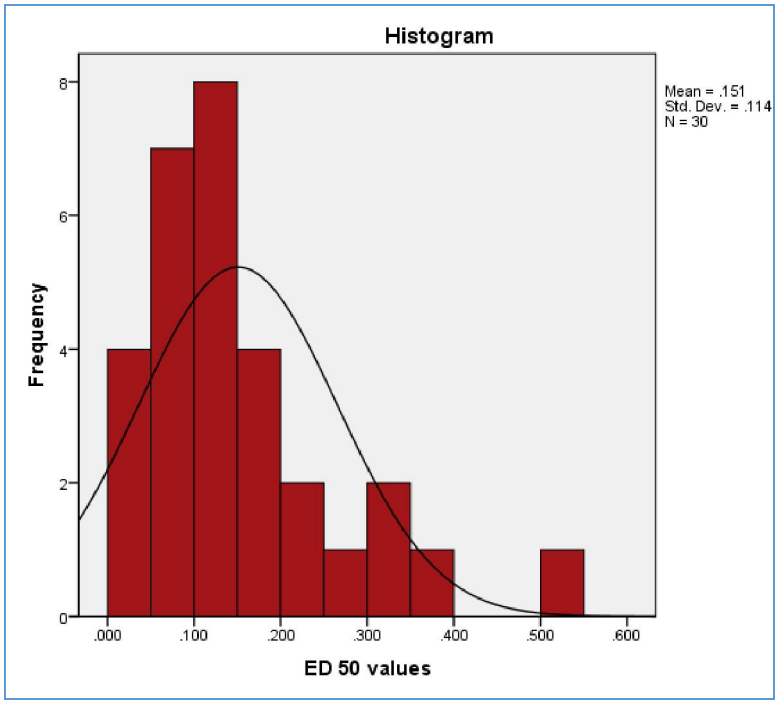


Fig. 3: Frequency distribution of ED_{50} values (myclobutanil) for baseline isolates of *Venturia inaequalis*

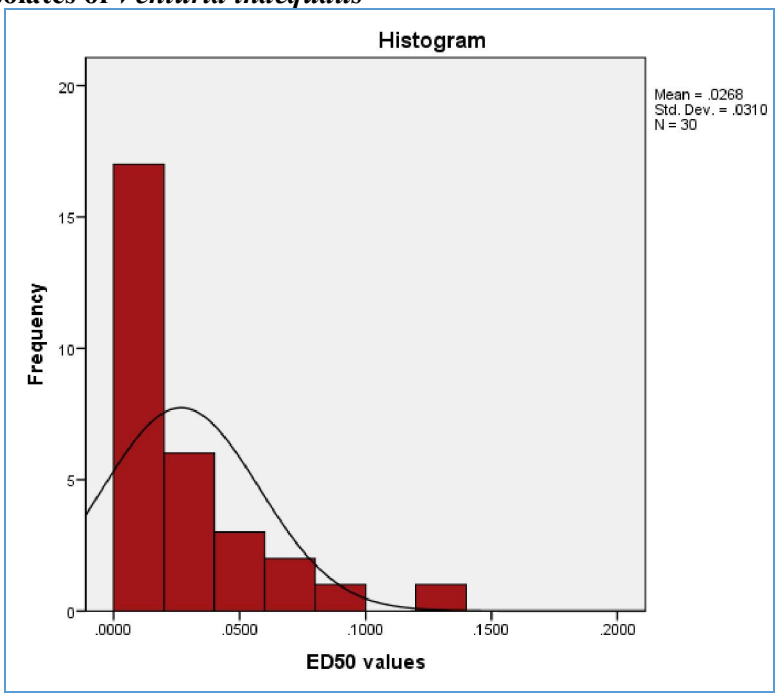


Fig. 4: Frequency distribution of ED_{50} values (difenoconazole) for baseline isolates of *V. inaequalis*

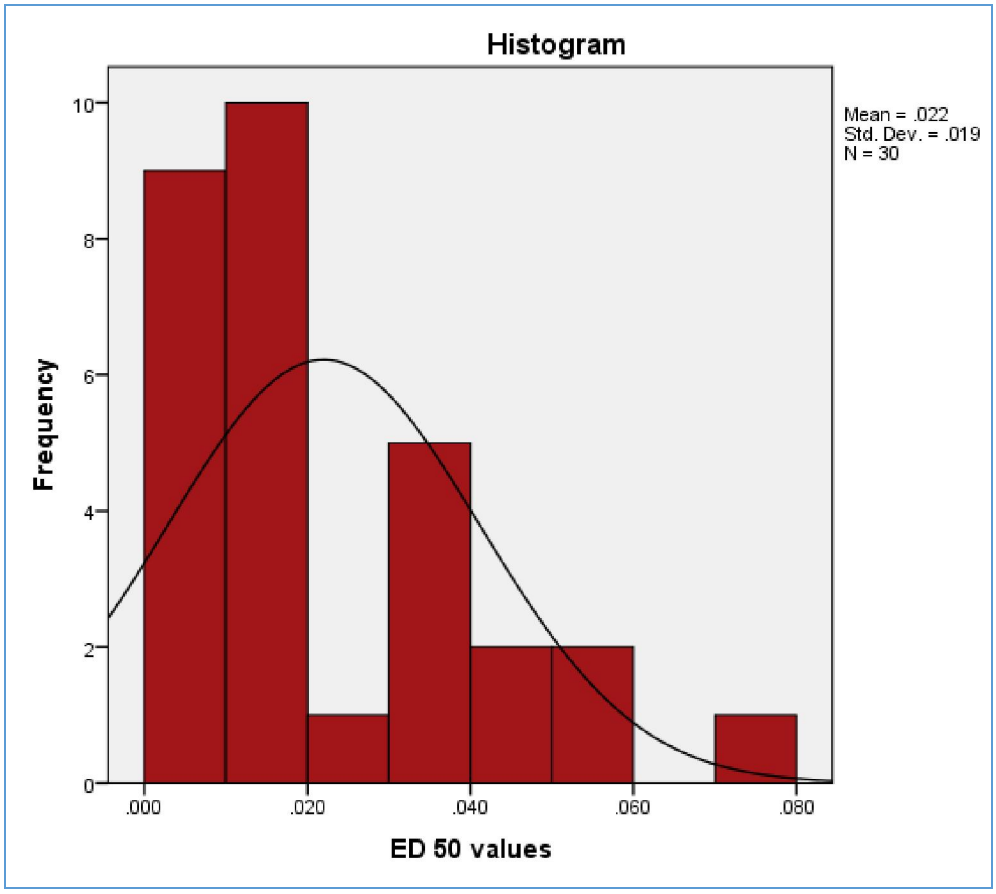
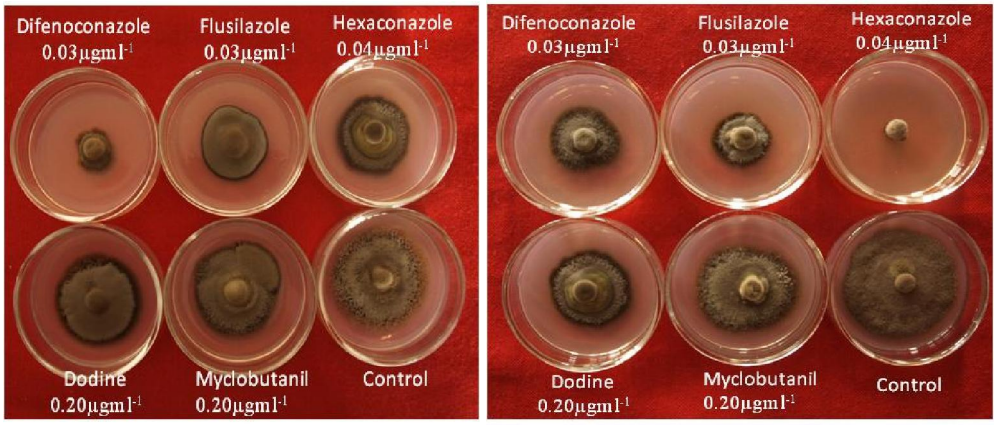


Fig. 5: Frequency distribution of ED_{50} values (flusilazole) for baseline isolates of *V. inaequalis*



Isolate S_{6-3} **Isolate S_{6-7}**

Isolate B_{6-3}

Plate 6: Response of isolates to the respective discriminatory doses of different test fungicides

cent (Table 17). The lowest RG value (39.17%) was observed for isolate Bp11, whereas, the isolate Bp13 showed the highest RG value (57.38%). The RG values of fungicide-exposed population from Baramulla district ranged from 34.37 to 80.76 per cent with a mean value of 53.75 per cent (Table 18); the lowest RG value was observed for the isolate $B_{7\text{-}9}$ (34.37%), whereas, the highest RG value was observed for the isolate $B_{7\text{-}6}$ (80.76%). The RG values of Shopian population varied from 29.5 to 92.10 per cent with a mean value of 51.42 per cent (Table 19); the isolate $S_{3\text{-}4}$ showed the lowest RG value, whereas the isolate $S_{2\text{-}8}$ showed the highest RG value.

The frequency distribution of RG values of the pathogen populations from the fungicide-exposed orchards showed almost similar pattern with a slight variation (Fig. 6a-b). The frequency distribution of dodine sensitivity of the pathogen populations collected from orchards of district Baramulla showed less variation from that of baseline population (Fig. 6a). The RG values of all the baseline isolates ranged from 30 to 60 per cent, whereas majority of the orchard isolates (90%) exhibited RG values between 30 to 70 per cent. A few isolates (8%) exhibited RG values between 70 to 80 per cent, and only 2 per cent of the isolates showed RG values above 80 per cent. These results indicated an evident shift in dodine sensitivity in orchard populations of *V. inaequalis* in district Baramulla as compared to the baseline population.

Similarly, the frequency distribution of dodine sensitivity of pathogen population collected from orchards of district Shopian also showed slight variation from that of baseline population (Fig. 6b). Majority of the orchard isolates (90%) exhibited RG values of 30 to 70 per cent; however, six per cent of the isolates showed RG values between 70 to 80 per cent and only per cent of isolates showed RG values greater than 80 per cent. These results also indicated a slight variation in dodine sensitivity in orchard populations of *V. inaequalis* in district Shopian as compared to the baseline population.

Table 17: Response (relative growth) of baseline isolates of *Venturia inaequalis* to dodine at discriminatory dose of 0.20 µg ml^{-1}

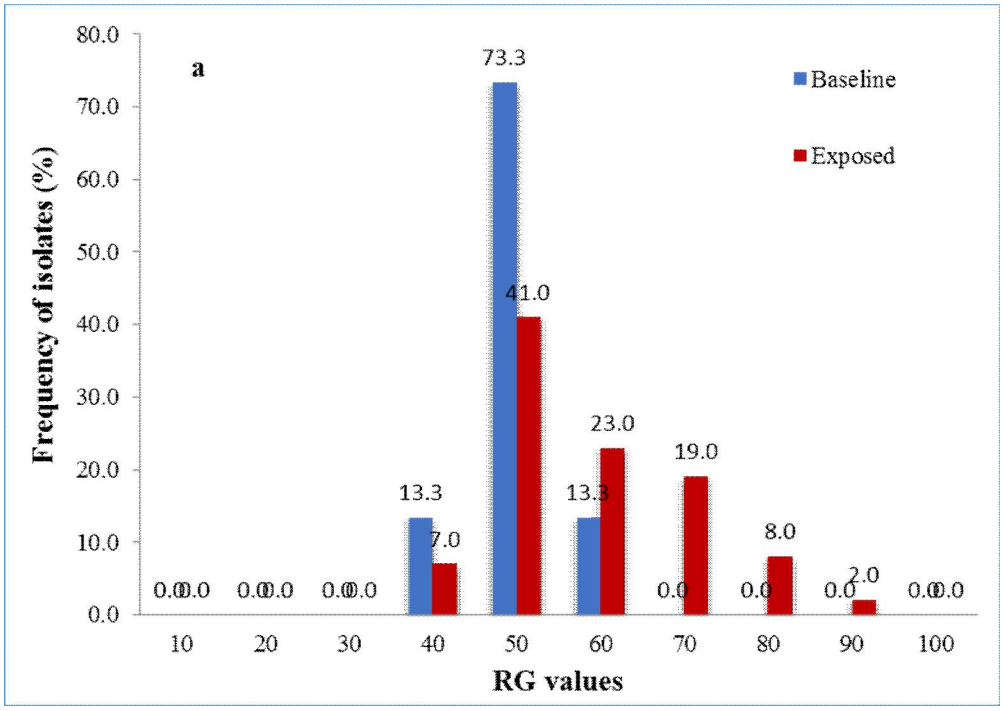
Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
Bp1	46.48	Bp7	39.66	Bp13	57.38	Bp19	44.21	Bp25	48.58
Bp2	47.80	Bp8	46.17	Bp14	46.21	Bp20	43.44	Bp26	45.51
Bp3	45.79	Bp9	52.17	Bp15	56.64	Bp21	40.55	Bp27	41.29
Bp4	49.03	Bp10	45.68	Bp16	52.15	Bp22	44.03	Bp28	43.03
Bp5	44.43	Bp11	39.17	Bp17	39.43	Bp23	41.61	Bp29	43.25
Bp6	46.53	Bp12	34.25	Bp18	42.09	Bp24	42.92	Bp30	49.23

Table 18: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Baramulla to dodine at discriminatory dose of 0.20µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
B_{1-1}	43.47	B_{3-1}	52.38	B_{5-1}	37.03	B_{7-1}	61.53	B_{9-1}	45.45
B_{1-2}	47.82	B_{3-2}	67.50	B_{5-2}	44.44	B_{7-2}	73.80	B_{9-2}	48.88
B_{1-3}	56.25	B_{3-3}	52.50	B_{5-3}	42.30	B_{7-3}	70.96	B_{9-3}	61.29
B_{1-4}	50.00	B_{3-4}	51.42	B_{5-4}	42.10	B_{7-4}	60.50	B_{9-4}	60.24
B_{1-5}	44.00	B_{3-5}	55.88	B_{5-5}	59.52	B_{7-5}	48.48	B_{9-5}	46.80
B_{1-6}	40.74	B_{3-6}	56.25	B_{5-6}	45.07	B_{7-6}	80.76	B_{9-6}	42.30
B_{1-7}	50.00	B_{3-7}	53.33	B_{5-7}	41.97	B_{7-7}	42.37	B_{9-7}	65.71
B_{1-8}	55.00	B_{3-8}	45.00	B_{5-8}	46.75	B_{7-8}	45.16	B_{9-8}	56.41
B_{1-9}	43.33	B_{3-9}	50.00	B_{5-9}	45.78	B_{7-9}	34.37	B_{9-9}	42.85
B_{1-10}	41.93	B_{3-10}	45.83	B_{5-10}	45.45	B_{7-10}	71.92	B_{9-10}	47.36
B_{2-1}	77.90	B_{4-1}	64.70	B_{6-1}	53.33	B_{8-1}	48.83	B_{10-1}	34.61
B_{2-2}	75.51	B_{4-2}	66.00	B_{6-2}	42.50	B_{8-2}	67.56	B_{10-2}	30.50
B_{2-3}	54.28	B_{4-3}	62.50	B_{6-3}	70.00	B_{8-3}	60.52	B_{10-3}	59.52
B_{2-4}	43.75	B_{4-4}	58.13	B_{6-4}	40.54	B_{8-4}	53.52	B_{10-4}	47.76
B_{2-5}	55.00	B_{4-5}	47.05	B_{6-5}	82.50	B_{8-5}	57.35	B_{10-5}	50.70
B_{2-6}	78.57	B_{4-6}	65.51	B_{6-6}	38.88	B_{8-6}	53.73	B_{10-6}	68.18
B_{2-7}	66.66	B_{4-7}	50.00	B_{6-7}	51.02	B_{8-7}	80.00	B_{10-7}	40.67
B_{2-8}	66.66	B_{4-8}	46.77	B_{6-8}	58.97	B_{8-8}	51.40	B_{10-8}	42.30
B_{2-9}	68.75	B_{4-9}	37.31	B_{6-9}	48.71	B_{8-9}	53.06	B_{10-9}	41.09
B_{2-10}	69.23	B_{4-10}	68.33	B_{6-10}	79.16	B_{8-10}	45.83	B_{10-10}	40.00

Table 19: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Shopian to dodine at discriminatory dose of 0.20µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
S_{1-1}	58.06	S_{3-1}	51.85	S_{5-1}	43.90	S_{7-1}	53.96	S_{9-1}	48.14
S_{1-2}	45.80	S_{3-2}	50.94	S_{5-2}	87.09	S_{7-2}	39.47	S_{9-2}	46.42
S_{1-3}	50.00	S_{3-3}	50.00	S_{5-3}	74.32	S_{7-3}	45.07	S_{9-3}	46.66
S_{1-4}	51.38	S_{3-4}	29.50	S_{5-4}	77.27	S_{7-4}	50.00	S_{9-4}	36.20
S_{1-5}	68.60	S_{3-5}	49.05	S_{5-5}	64.28	S_{7-5}	58.82	S_{9-5}	44.44
S_{1-6}	48.30	S_{3-6}	43.63	S_{5-6}	55.26	S_{7-6}	58.33	S_{9-6}	46.29
S_{1-7}	49.09	S_{3-7}	37.70	S_{5-7}	36.00	S_{7-7}	43.85	S_{9-7}	36.66
S_{1-8}	43.47	S_{3-8}	42.59	S_{5-8}	51.78	S_{7-8}	44.28	S_{9-8}	57.69
S_{1-9}	31.25	S_{3-9}	50.00	S_{5-9}	46.15	S_{7-9}	33.33	S_{9-9}	52.38
S_{1-10}	42.30	S_{3-10}	60.00	S_{5-10}	80.59	S_{7-10}	43.63	S_{9-10}	52.00
S_{2-1}	56.00	S_{4-1}	60.86	S_{6-1}	55.55	S_{8-1}	50.00	S_{10-1}	50.00
S_{2-2}	75.75	S_{4-2}	55.50	S_{6-2}	42.50	S_{8-2}	59.42	S_{10-2}	72.13
S_{2-3}	53.33	S_{4-3}	60.86	S_{6-3}	71.05	S_{8-3}	56.25	S_{10-3}	65.00
S_{2-4}	33.33	S_{4-4}	55.73	S_{6-4}	47.82	S_{8-4}	30.88	S_{10-4}	47.05
S_{2-5}	33.82	S_{4-5}	45.45	S_{6-5}	36.66	S_{8-5}	37.14	S_{10-5}	58.10
S_{2-6}	30.98	S_{4-6}	55.55	S_{6-6}	43.85	S_{8-6}	31.50	S_{10-6}	52.38
S_{2-7}	38.00	S_{4-7}	40.74	S_{6-7}	38.00	S_{8-7}	43.05	S_{10-7}	40.38
S_{2-8}	92.10	S_{4-8}	62.50	S_{6-8}	61.77	S_{8-8}	66.23	S_{10-8}	50.00
S_{2-9}	58.18	S_{4-9}	61.11	S_{6-9}	56.33	S_{8-9}	56.14	S_{10-9}	44.44
S_{2-10}	60.34	S_{4-10}	52.63	S_{6-10}	59.01	S_{8-10}	55.00	S_{10-10}	74.28



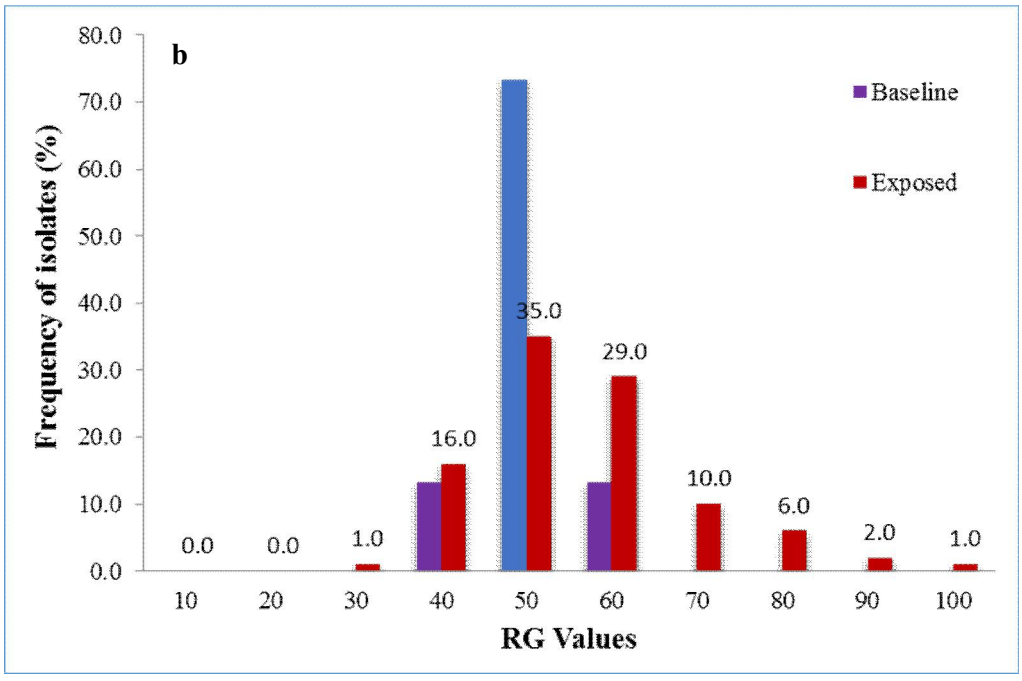


Fig. 6: Comparison between frequency distributions *V. inaequalis* baseline and exposed populations to dodine at 0.20 µg ml^{-1} discriminatory dose. a) Baramulla population, b) Shopian population

4.2.2 Sensitivity to hexaconazole

The *V. inaequalis* isolates collected from the fungicide-exposed orchards of district Baramulla and Shopian were assessed for their sensitivity to hexaconazole at a discriminatory dose of 0.04 µg ml^{-1}, and the RG values calculated. The RG values of baseline isolates at the same discriminatory dose of hexaconazole were also calculated. The results revealed that the RG values of baseline isolates ranged from 13.40 to 66.40 per cent with a mean value of 42.71 per cent (Table 20). The isolate Bp4 showed the lowest RG value, whereas the isolate Bp10 showed the highest RG value. The RG values of the fungicide-exposed population from district Baramulla ranged from 26.66 to 92.30 per cent with a mean RG value of 51.19 per cent (Table 21); the isolate B_{9-2} showed the lowest RG value, whereas the isolate B_{6-9} exhibited the highest RG value. The RG values of the pathogen population from district Shopian ranged from 21.12 to 89.65 per cent with a mean RG value of 51.45 per cent (Table 22); the isolate S_{2-6} showed the lowest RG value, whereas the isolate S_{3-3} showed the highest RG value.

The frequency distribution of RG values of *V. inaequalis* populations collected from fungicide exposed orchards of Baramulla and Shopian districts showed almost similar pattern with a slight variation (Fig. 7a-b). The frequency distribution of hexaconazole sensitivity of the pathogen isolates from Baramulla was more or less similar to that of baseline population (Fig. 7a). The RG values of majority of the baseline isolates ranged from 30 to 70 per cent. Majority of the orchard isolates (84%) exhibited RG values between 30 to 70 per cent, whereas 12 per cent of the isolates exhibited RG values greater than 70 per cent. These results indicate that there is no pronounced shift in hexaconazole sensitivity in orchard populations of district Baramulla from that of baseline population.

The frequency distribution of hexaconazole sensitivity of the pathogen isolates from Shopian was also more or less similar to that of baseline population (Fig. 7b). Majority of the orchard isolates (84%) also exhibited RG values of 30 to

Table 20: Response (relative growth) of baseline isolates of *Venturia inaequalis* to hexaconazole at discriminatory dose of 0.04 µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
Bp1	54.23	Bp7	37.97	Bp13	48.65	Bp19	22.72	Bp25	57.60
Bp2	18.05	Bp8	43.61	Bp14	54.35	Bp20	35.80	Bp26	57.77
Bp3	29.37	Bp9	62.24	Bp15	52.87	Bp21	35.83	Bp27	60.76
Bp4	13.40	Bp10	66.40	Bp16	38.47	Bp22	27.44	Bp28	47.65
Bp5	16.02	Bp11	51.10	Bp17	34.49	Bp23	48.31	Bp29	60.62
Bp6	38.66	Bp12	41.78	Bp18	38.44	Bp24	42.21	Bp30	44.61

Table 21: Response (relative growth) of exposed orchard of *Venturia inaequalis* isolates collected from district Baramulla to hexaconazole at discriminatory dose of 0.04µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
B_{1-1}	39.13	B_{3-1}	69.04	B_{5-1}	40.74	B_{7-1}	44.61	B_{9-1}	40.90
B_{1-2}	34.78	B_{3-2}	57.50	B_{5-2}	33.33	B_{7-2}	73.80	B_{9-2}	26.66
B_{1-3}	53.12	B_{3-3}	52.50	B_{5-3}	46.15	B_{7-3}	80.64	B_{9-3}	51.61
B_{1-4}	62.50	B_{3-4}	37.14	B_{5-4}	38.59	B_{7-4}	68.42	B_{9-4}	87.34
B_{1-5}	66.00	B_{3-5}	38.23	B_{5-5}	38.09	B_{7-5}	45.45	B_{9-5}	85.10
B_{1-6}	51.85	B_{3-6}	43.75	B_{5-6}	47.88	B_{7-6}	86.53	B_{9-6}	57.69
B_{1-7}	40.47	B_{3-7}	50.00	B_{5-7}	34.56	B_{7-7}	38.98	B_{9-7}	40.00
B_{1-8}	40.00	B_{3-8}	37.50	B_{5-8}	41.55	B_{7-8}	45.16	B_{9-8}	33.33
B_{1-9}	36.66	B_{3-9}	60.00	B_{5-9}	48.19	B_{7-9}	67.18	B_{9-9}	35.71
B_{1-10}	45.16	B_{3-10}	58.33	B_{5-10}	45.45	B_{7-10}	78.94	B_{9-10}	47.36
B_{2-1}	44.18	B_{4-1}	48.52	B_{6-1}	31.10	B_{8-1}	72.09	B_{10-1}	38.46
B_{2-2}	69.38	B_{4-2}	60.00	B_{6-2}	48.14	B_{8-2}	62.16	B_{10-2}	37.28
B_{2-3}	68.57	B_{4-3}	62.50	B_{6-3}	53.09	B_{8-3}	55.26	B_{10-3}	40.47
B_{2-4}	70.83	B_{4-4}	60.46	B_{6-4}	24.32	B_{8-4}	33.80	B_{10-4}	53.73
B_{2-5}	55.00	B_{4-5}	44.11	B_{6-5}	55.00	B_{8-5}	32.35	B_{10-5}	53.52
B_{2-6}	83.33	B_{4-6}	70.68	B_{6-6}	33.33	B_{8-6}	40.29	B_{10-6}	45.45
B_{2-7}	51.85	B_{4-7}	40.00	B_{6-7}	24.48	B_{8-7}	72.50	B_{10-7}	47.45
B_{2-8}	68.33	B_{4-8}	41.93	B_{6-8}	48.71	B_{8-8}	42.85	B_{10-8}	50.00
B_{2-9}	28.75	B_{4-9}	67.16	B_{6-9}	92.30	B_{8-9}	63.26	B_{10-9}	49.31
B_{2-10}	37.17	B_{4-10}	70.00	B_{6-10}	50.00	B_{8-10}	62.50	B_{10-10}	40.00

Table 22: Response (relative growth) of exposed orchard of *Venturia inaequalis* to hexaconazole isolates collected from district Shopian at discriminatory dose of 0.04 µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
S_{1-1}	66.12	S_{3-1}	62.96	S_{5-1}	45.36	S_{7-1}	63.49	S_{9-1}	59.25
S_{1-2}	50.00	S_{3-2}	66.03	S_{5-2}	69.35	S_{7-2}	43.23	S_{9-2}	60.71
S_{1-3}	42.85	S_{3-3}	89.65	S_{5-3}	89.18	S_{7-3}	42.25	S_{9-3}	63.33
S_{1-4}	44.44	S_{3-4}	34.42	S_{5-4}	59.09	S_{7-4}	47.14	S_{9-4}	39.65
S_{1-5}	65.60	S_{3-5}	67.92	S_{5-5}	74.28	S_{7-5}	57.35	S_{9-5}	62.96
S_{1-6}	72.58	S_{3-6}	63.63	S_{5-6}	60.52	S_{7-6}	65.00	S_{9-6}	62.96
S_{1-7}	69.09	S_{3-7}	49.18	S_{5-7}	28.00	S_{7-7}	61.40	S_{9-7}	53.33
S_{1-8}	71.01	S_{3-8}	33.33	S_{5-8}	37.50	S_{7-8}	62.85	S_{9-8}	38.46
S_{1-9}	32.50	S_{3-9}	54.76	S_{5-9}	32.69	S_{7-9}	32.05	S_{9-9}	54.76
S_{1-10}	63.46	S_{3-10}	40.00	S_{5-10}	68.65	S_{7-10}	58.18	S_{9-10}	44.00
S_{2-1}	64.00	S_{4-1}	65.21	S_{6-1}	42.59	S_{8-1}	57.40	S_{10-1}	50.00
S_{2-2}	43.93	S_{4-2}	48.88	S_{6-2}	32.50	S_{8-2}	42.02	S_{10-2}	62.29
S_{2-3}	53.33	S_{4-3}	43.47	S_{6-3}	78.94	S_{8-3}	56.25	S_{10-3}	70.00
S_{2-4}	25.75	S_{4-4}	36.06	S_{6-4}	58.69	S_{8-4}	27.94	S_{10-4}	52.94
S_{2-5}	23.52	S_{4-5}	68.18	S_{6-5}	36.66	S_{8-5}	24.28	S_{10-5}	64.86
S_{2-6}	21.12	S_{4-6}	61.11	S_{6-6}	50.87	S_{8-6}	24.65	S_{10-6}	52.38
S_{2-7}	23.77	S_{4-7}	40.74	S_{6-7}	80.00	S_{8-7}	26.38	S_{10-7}	30.76
S_{2-8}	65.78	S_{4-8}	50.00	S_{6-8}	50.30	S_{8-8}	57.14	S_{10-8}	36.66
S_{2-9}	52.72	S_{4-9}	40.74	S_{6-9}	45.45	S_{8-9}	47.36	S_{10-9}	38.88
S_{2-10}	51.72	S_{4-10}	39.47	S_{6-10}	45.90	S_{8-10}	48.33	S_{10-10}	57.14

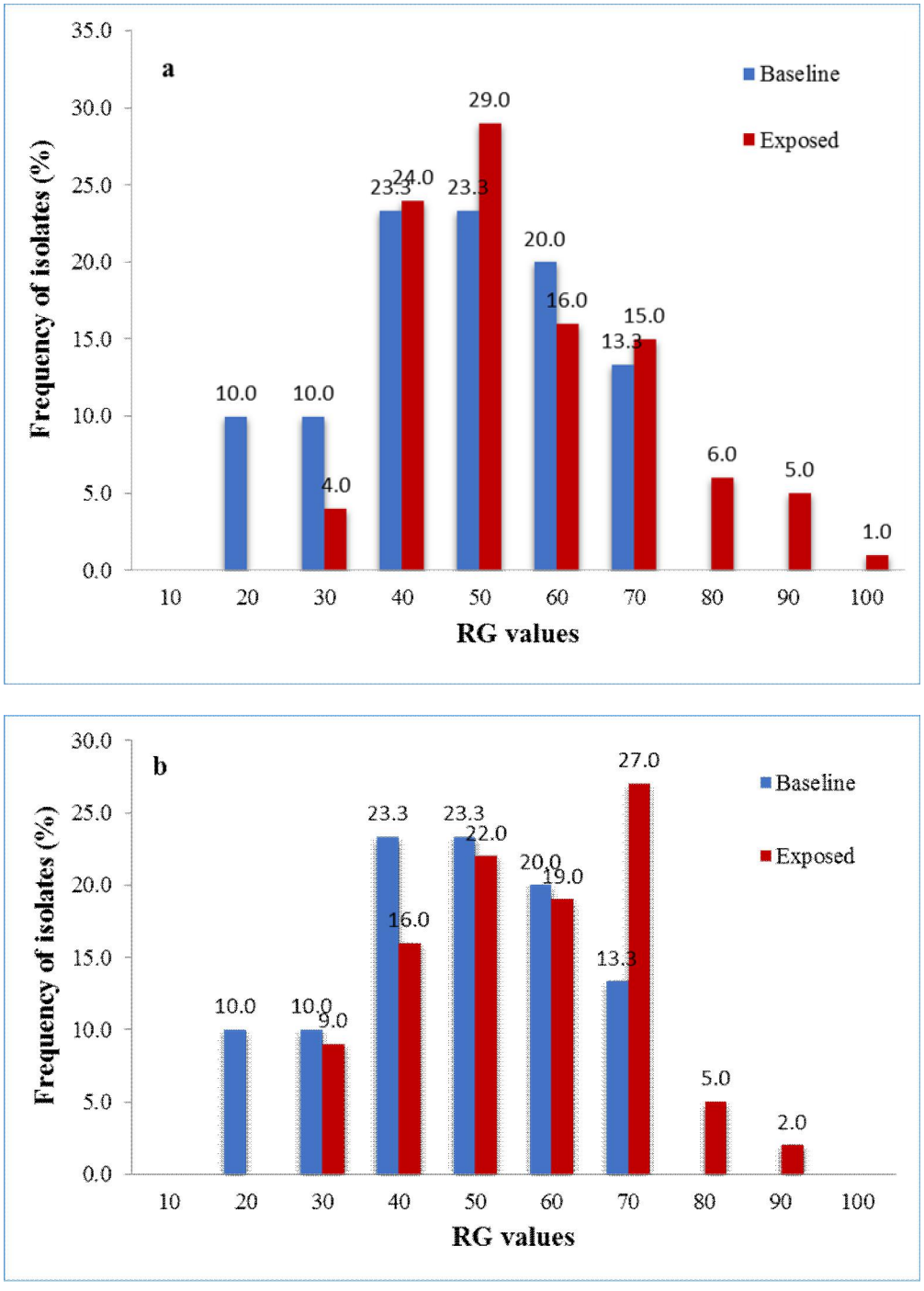


Fig 7: Comparison between frequency distributions of baseline and exposed *V. inaequalis* populations to hexaconazole at 0.04 µg ml^{-1} discriminatory dose. a) Baramulla population, b) Shopian population

70 per cent, and only 7 per cent of isolates showed RG values greater than 70 per cent. These results also indicated no evident shift in hexaconazole sensitivity in orchard populations of district Shopian as compared to baseline population.

4.2.3 Sensitivity to myclobutanil

V. inaequalis isolates collected from district Baramulla and Shopian were tested for their sensitivity to myclobutanil at a discriminatory dose of 0.2 µg ml^{-1}, and the RG values calculated. The RG values of the baseline isolates at the same discriminatory dose were also calculated. The RG values of the baseline population ranged from 27.75 to 57.77 per cent with a mean RG value of 44.68 per cent (Table 23); the isolate Bp22 showed the lowest RG value, whereas the isolate Bp10 showed the highest RG value. The RG values of the fungicide-exposed pathogen population from Baramulla ranged from 19.44 to 100 with a mean RG value of 62.37 per cent (Table 24); the isolate B_{5-2} showed the lowest RG value, whereas the isolate B_{7-6} showed the highest RG value. The RG values of *V. inaequalis* population from district Shopian ranged from 27.94 to 91.80 per cent with a mean RG value of 64.04 per cent (Table 25); the isolate S_{2-5} exhibited the lowest RG value, whereas the isolate S_{4-4} exhibited the highest RG value.

The frequency distribution of RG values of fungicide-exposed orchard populations of *V. inaequalis* collected from Baramulla and Shopian populations differed from that of baseline population (Fig. 8a-b). The frequency distribution of myclobutanil sensitivity of the isolates of the pathogen collected from Baramulla differed from that of baseline population (Fig 8a); the RG values of majority of the baseline isolates ranged from 30 to 60 per cent, whereas majority of orchard isolates (87%) of the pathogen exhibited RG values between 40 to 90 per cent, six per cent of the isolates exhibited RG values greater than 90 per cent. These results indicated that there is a major shift in myclobutanil sensitivity in orchard populations of *V. inaequalis* collected from Baramulla district in comparison to the baseline population.

Table 23: Response (relative growth) of baseline isolates of *Venturia inaequalis* to myclobutanil at discriminatory dose of 0.20µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
Bp1	47.49	Bp7	56.16	Bp13	44.77	Bp19	44.70	Bp25	43.88
Bp2	28.68	Bp8	47.61	Bp14	51.42	Bp20	40.50	Bp26	37.72
Bp3	41.03	Bp9	55.55	Bp15	37.15	Bp21	39.12	Bp27	35.27
Bp4	45.39	Bp10	57.77	Bp16	46.37	Bp22	27.75	Bp28	46.36
Bp5	40.90	Bp11	45.37	Bp17	44.39	Bp23	54.32	Bp29	53.03
Bp6	45.40	Bp12	41.57	Bp18	48.98	Bp24	48.12	Bp30	43.72

Table 24: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Baramulla to myclobutanil at discriminatory dose of 0.20µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
B_{1-1}	39.13	B_{3-1}	57.14	B_{5-1}	40.74	B_{7-1}	61.53	B_{9-1}	43.18
B_{1-2}	60.86	B_{3-2}	70.00	B_{5-2}	19.44	B_{7-2}	85.71	B_{9-2}	66.66
B_{1-3}	75.00	B_{3-3}	65.00	B_{5-3}	56.41	B_{7-3}	83.87	B_{9-3}	90.32
B_{1-4}	55.00	B_{3-4}	54.28	B_{5-4}	47.36	B_{7-4}	71.05	B_{9-4}	66.26
B_{1-5}	68.00	B_{3-5}	58.82	B_{5-5}	45.23	B_{7-5}	58.18	B_{9-5}	80.85
B_{1-6}	55.55	B_{3-6}	59.37	B_{5-6}	36.61	B_{7-6}	100.00	B_{9-6}	53.84
B_{1-7}	66.66	B_{3-7}	62.00	B_{5-7}	39.50	B_{7-7}	49.15	B_{9-7}	82.85
B_{1-8}	52.50	B_{3-8}	52.50	B_{5-8}	46.75	B_{7-8}	51.61	B_{9-8}	51.28
B_{1-9}	66.66	B_{3-9}	70.00	B_{5-9}	53.01	B_{7-9}	71.87	B_{9-9}	76.78
B_{1-10}	64.51	B_{3-10}	68.75	B_{5-10}	42.42	B_{7-10}	61.40	B_{9-10}	70.17
B_{2-1}	43.02	B_{4-1}	64.70	B_{6-1}	51.11	B_{8-1}	55.80	B_{10-1}	42.30
B_{2-2}	93.87	B_{4-2}	70.00	B_{6-2}	74.07	B_{8-2}	71.62	B_{10-2}	44.06
B_{2-3}	94.28	B_{4-3}	75.00	B_{6-3}	84.72	B_{8-3}	65.78	B_{10-3}	46.42
B_{2-4}	62.50	B_{4-4}	65.11	B_{6-4}	68.91	B_{8-4}	54.92	B_{10-4}	62.68
B_{2-5}	80.00	B_{4-5}	58.82	B_{6-5}	85.00	B_{8-5}	63.97	B_{10-5}	36.61
B_{2-6}	78.57	B_{4-6}	72.41	B_{6-6}	69.44	B_{8-6}	58.20	B_{10-6}	36.36
B_{2-7}	74.04	B_{4-7}	53.33	B_{6-7}	44.89	B_{8-7}	93.00	B_{10-7}	47.45
B_{2-8}	80.00	B_{4-8}	48.38	B_{6-8}	92.30	B_{8-8}	54.85	B_{10-8}	50.00
B_{2-9}	83.75	B_{4-9}	65.67	B_{6-9}	58.97	B_{8-9}	67.34	B_{10-9}	43.83
B_{2-10}	79.48	B_{4-10}	60.00	B_{6-10}	72.91	B_{8-10}	70.83	B_{10-10}	40.00

Table 25: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Shopian to myclobutanil at discriminatory dose of 0.20µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
S_{1-1}	73.38	S_{3-1}	68.51	S_{5-1}	56.09	S_{7-1}	69.84	S_{9-1}	64.81
S_{1-2}	66.60	S_{3-2}	75.47	S_{5-2}	83.87	S_{7-2}	56.39	S_{9-2}	67.85
S_{1-3}	41.42	S_{3-3}	96.55	S_{5-3}	91.89	S_{7-3}	38.02	S_{9-3}	70.00
S_{1-4}	44.44	S_{3-4}	39.34	S_{5-4}	78.78	S_{7-4}	42.85	S_{9-4}	44.82
S_{1-5}	80.50	S_{3-5}	92.45	S_{5-5}	87.14	S_{7-5}	63.23	S_{9-5}	70.37
S_{1-6}	88.70	S_{3-6}	89.09	S_{5-6}	76.31	S_{7-6}	71.66	S_{9-6}	81.48
S_{1-7}	61.80	S_{3-7}	96.72	S_{5-7}	42.00	S_{7-7}	50.87	S_{9-7}	73.33
S_{1-8}	84.05	S_{3-8}	68.51	S_{5-8}	94.64	S_{7-8}	71.40	S_{9-8}	65.38
S_{1-9}	40.00	S_{3-9}	71.42	S_{5-9}	65.38	S_{7-9}	39.74	S_{9-9}	66.66
S_{1-10}	67.30	S_{3-10}	73.33	S_{5-10}	77.60	S_{7-10}	58.18	S_{9-10}	64.00
S_{2-1}	80.00	S_{4-1}	80.43	S_{6-1}	55.55	S_{8-1}	64.81	S_{10-1}	50.00
S_{2-2}	77.27	S_{4-2}	55.50	S_{6-2}	47.50	S_{8-2}	57.97	S_{10-2}	68.85
S_{2-3}	83.33	S_{4-3}	50.00	S_{6-3}	97.36	S_{8-3}	71.87	S_{10-3}	73.75
S_{2-4}	28.78	S_{4-4}	91.80	S_{6-4}	60.86	S_{8-4}	32.35	S_{10-4}	52.94
S_{2-5}	27.94	S_{4-5}	45.45	S_{6-5}	33.33	S_{8-5}	28.57	S_{10-5}	67.56
S_{2-6}	28.16	S_{4-6}	58.33	S_{6-6}	52.63	S_{8-6}	30.13	S_{10-6}	71.42
S_{2-7}	48.25	S_{4-7}	61.11	S_{6-7}	40.00	S_{8-7}	45.83	S_{10-7}	40.38
S_{2-8}	97.36	S_{4-8}	81.25	S_{6-8}	72.36	S_{8-8}	68.83	S_{10-8}	70.00
S_{2-9}	67.27	S_{4-9}	66.66	S_{6-9}	69.09	S_{8-9}	61.40	S_{10-9}	61.11
S_{2-10}	68.10	S_{4-10}	55.26	S_{6-10}	70.49	S_{8-10}	58.33	S_{10-10}	74.28

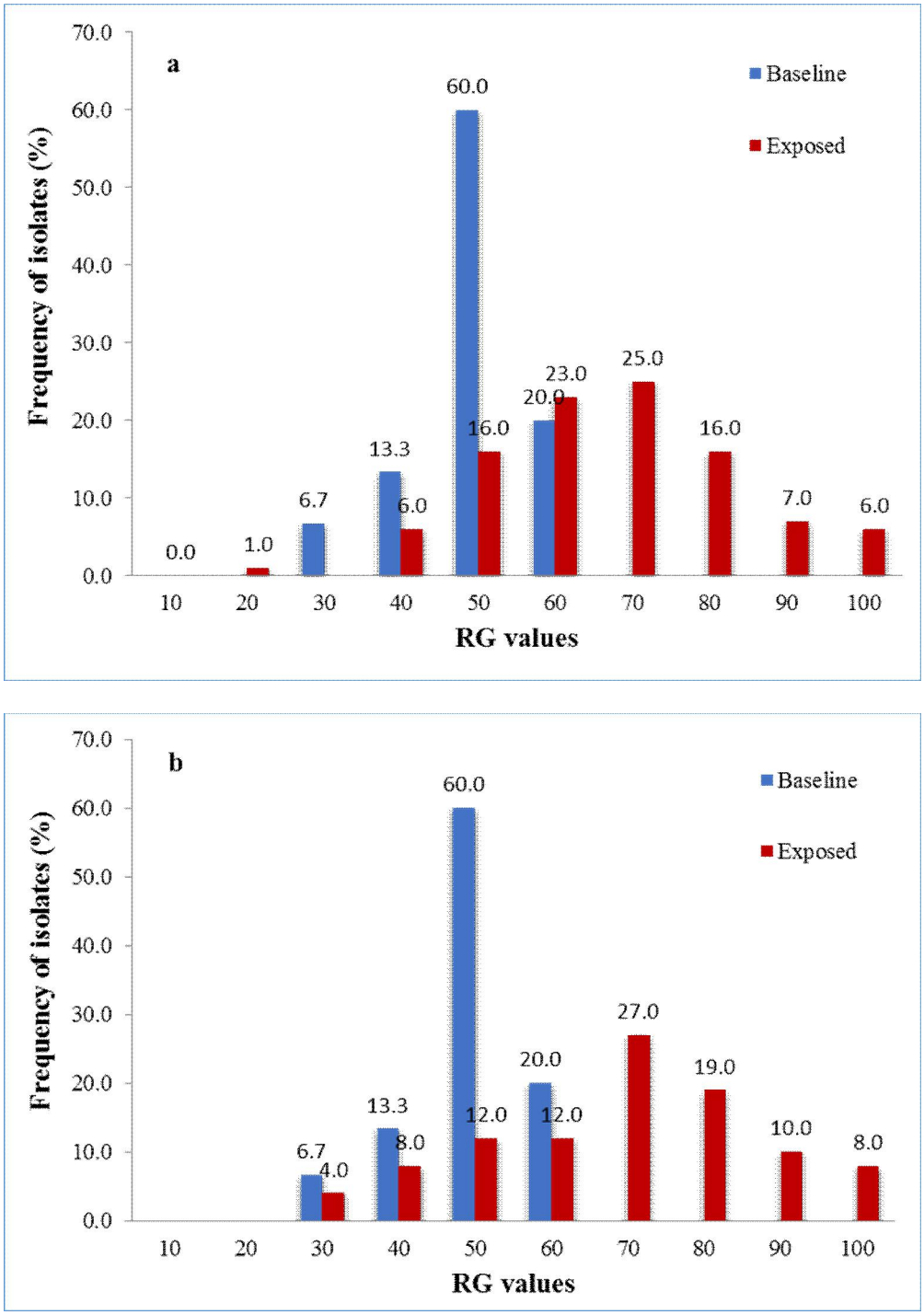


Fig. 8: Comparison between frequency distributions of baseline and exposed *V. inaequalis* populations to myclobutanil at 0.20 µg ml^{-1} discriminatory dose. a) Baramulla population, b) Shopian population

The frequency distribution of myclobutanil sensitivity of orchard isolates of the pathogen from district Shopian also differed from that of baseline population (Fig. 8b). Majority of the pathogen isolates (80%) exhibited RG values of 40 to 90 per cent, and 8 per cent of isolates exhibited RG values greater than 90 per cent. These results indicate that there is a major shift in myclobutanil sensitivity in orchard populations of *V. inaequalis* in district Shopian.

4.2.4 Sensitivity to difenoconazole

The *V. inaequalis* isolates collected from fungicide-exposed orchards of district Baramulla and Shopian were assessed for their sensitivity to difenoconazole at a discriminatory dose of 0.03 µg ml^{-1}, and the RG values calculated. Similarly, the RG values of the baseline isolates at the same discriminatory dose of fungicide were also calculated. The results revealed that the RG values of baseline population ranged from 25.81 to 62.23 per cent with a mean RG value of 44.35 per cent (Table 26). Among all the baseline isolates, isolate Bp27 exhibited the lowest RG value, whereas the isolate Bp19 exhibited the highest RG value. The RG values of the fungicide-exposed population from Baramulla varied from 29.62 to 80.00 per cent with a mean RG value of 54.03 per cent (Table 27); the isolate B_{5-7} exhibited the lowest RG value, whereas the isolates B_{7-1}, B_{8-7} and B_{9-2} exhibited the highest RG value. The RG values of Shopian population varied from 29.50 to 96.55 per cent with a mean value of 53.40 per cent (Table 28), isolate S_{3-4} exhibited the lowest RG value, whereas the isolate S_{3-3} exhibited the highest RG value

The frequency distribution of RG values of the fungicide exposed populations of *V. inaequalis* showed more or less similar pattern as that of the baseline population (Fig. 9a-b). The frequency distribution of difenoconazole sensitivity of pathogen collected from Baramulla orchards exhibited a slight variation from that of the baseline population (Fig. 9a). The RG values of majority of baseline isolates ranged from 30 to 60 per cent, whereas majority of the fungicide-exposed isolates (91%) exhibited RG values of 30 to 70 per cent. Eight

Table 26: Response (relative growth) of baseline isolates of *Venturia inaequalis* to difenoconazole at discriminatory dose of 0.03µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
Bp1	30.17	Bp7	49.18	Bp13	36.43	Bp19	62.23	Bp25	33.21
Bp2	37.61	Bp8	46.61	Bp14	47.46	Bp20	34.44	Bp26	36.77
Bp3	33.66	Bp9	52.53	Bp15	53.23	Bp21	40.09	Bp27	25.81
Bp4	57.96	Bp10	51.12	Bp16	68.11	Bp22	30.74	Bp28	35.33
Bp5	38.18	Bp11	45.87	Bp17	54.56	Bp23	55.62	Bp29	44.15
Bp6	55.66	Bp12	41.12	Bp18	55.53	Bp24	37.23	Bp30	39.93

Table 27: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Baramulla to difenoconazole at discriminatory dose of 0.03 µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
B_{1-1}	47.82	B_{3-1}	47.61	B_{5-1}	44.44	B_{7-1}	80.00	B_{9-1}	52.27
B_{1-2}	69.56	B_{3-2}	65.00	B_{5-2}	30.55	B_{7-2}	69.04	B_{9-2}	80.00
B_{1-3}	59.37	B_{3-3}	55.00	B_{5-3}	56.41	B_{7-3}	64.51	B_{9-3}	64.51
B_{1-4}	57.50	B_{3-4}	40.00	B_{5-4}	42.10	B_{7-4}	63.15	B_{9-4}	60.24
B_{1-5}	60.00	B_{3-5}	44.11	B_{5-5}	32.14	B_{7-5}	39.39	B_{9-5}	61.70
B_{1-6}	40.74	B_{3-6}	39.06	B_{5-6}	42.25	B_{7-6}	78.84	B_{9-6}	40.38
B_{1-7}	52.38	B_{3-7}	60.00	B_{5-7}	29.62	B_{7-7}	62.71	B_{9-7}	62.85
B_{1-8}	62.50	B_{3-8}	52.50	B_{5-8}	38.96	B_{7-8}	66.12	B_{9-8}	66.66
B_{1-9}	48.33	B_{3-9}	64.00	B_{5-9}	45.78	B_{7-9}	45.31	B_{9-9}	46.42
B_{1-10}	45.16	B_{3-10}	62.50	B_{5-10}	42.42	B_{7-10}	64.91	B_{9-10}	47.36
B_{2-1}	58.13	B_{4-1}	75.00	B_{6-1}	37.70	B_{8-1}	41.86	B_{10-1}	46.15
B_{2-2}	75.50	B_{4-2}	58.00	B_{6-2}	35.10	B_{8-2}	67.56	B_{10-2}	37.28
B_{2-3}	44.28	B_{4-3}	57.50	B_{6-3}	70.90	B_{8-3}	61.84	B_{10-3}	38.09
B_{2-4}	47.91	B_{4-4}	60.46	B_{6-4}	40.54	B_{8-4}	36.61	B_{10-4}	50.74
B_{2-5}	55.00	B_{4-5}	35.29	B_{6-5}	77.50	B_{8-5}	41.91	B_{10-5}	59.15
B_{2-6}	66.66	B_{4-6}	63.79	B_{6-6}	36.11	B_{8-6}	40.29	B_{10-6}	54.54
B_{2-7}	55.55	B_{4-7}	58.33	B_{6-7}	48.97	B_{8-7}	80.00	B_{10-7}	57.62
B_{2-8}	61.66	B_{4-8}	62.90	B_{6-8}	58.97	B_{8-8}	57.14	B_{10-8}	55.76
B_{2-9}	56.25	B_{4-9}	47.76	B_{6-9}	46.15	B_{8-9}	65.30	B_{10-9}	65.75
B_{2-10}	39.74	B_{4-10}	60.00	B_{6-10}	62.50	B_{8-10}	64.58	B_{10-10}	33.33

Table 28: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Shopian to difenoconazole at discriminatory dose of 0.03µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
S_{1-1}	46.77	S_{3-1}	61.11	S_{5-1}	43.90	S_{7-1}	42.85	S_{9-1}	55.55
S_{1-2}	75.00	S_{3-2}	56.60	S_{5-2}	62.90	S_{7-2}	60.15	S_{9-2}	51.78
S_{1-3}	45.71	S_{3-3}	96.55	S_{5-3}	86.48	S_{7-3}	43.66	S_{9-3}	66.66
S_{1-4}	47.22	S_{3-4}	29.50	S_{5-4}	59.09	S_{7-4}	45.71	S_{9-4}	34.48
S_{1-5}	62.60	S_{3-5}	58.49	S_{5-5}	68.57	S_{7-5}	54.41	S_{9-5}	55.55
S_{1-6}	33.80	S_{3-6}	47.27	S_{5-6}	50.00	S_{7-6}	41.66	S_{9-6}	50.00
S_{1-7}	47.20	S_{3-7}	34.42	S_{5-7}	52.00	S_{7-7}	47.36	S_{9-7}	38.33
S_{1-8}	69.50	S_{3-8}	31.48	S_{5-8}	60.71	S_{7-8}	60.00	S_{9-8}	42.30
S_{1-9}	53.75	S_{3-9}	64.28	S_{5-9}	50.00	S_{7-9}	57.69	S_{9-9}	61.90
S_{1-10}	42.30	S_{3-10}	62.22	S_{5-10}	58.20	S_{7-10}	41.81	S_{9-10}	60.00
S_{2-1}	54.00	S_{4-1}	54.34	S_{6-1}	44.44	S_{8-1}	46.29	S_{10-1}	52.50
S_{2-2}	43.93	S_{4-2}	42.22	S_{6-2}	45.00	S_{8-2}	40.57	S_{10-2}	57.37
S_{2-3}	53.33	S_{4-3}	45.65	S_{6-3}	50.00	S_{8-3}	53.12	S_{10-3}	72.50
S_{2-4}	46.96	S_{4-4}	52.45	S_{6-4}	89.13	S_{8-4}	44.11	S_{10-4}	50.00
S_{2-5}	50.73	S_{4-5}	40.90	S_{6-5}	56.66	S_{8-5}	47.14	S_{10-5}	64.86
S_{2-6}	47.88	S_{4-6}	66.66	S_{6-6}	56.14	S_{8-6}	43.83	S_{10-6}	42.85
S_{2-7}	43.66	S_{4-7}	38.88	S_{6-7}	96.00	S_{8-7}	47.22	S_{10-7}	50.00
S_{2-8}	71.05	S_{4-8}	64.58	S_{6-8}	57.64	S_{8-8}	51.94	S_{10-8}	55.00
S_{2-9}	50.90	S_{4-9}	48.14	S_{6-9}	61.81	S_{8-9}	50.87	S_{10-9}	57.40
S_{2-10}	53.44	S_{4-10}	47.36	S_{6-10}	59.01	S_{8-10}	53.33	S_{10-10}	57.14

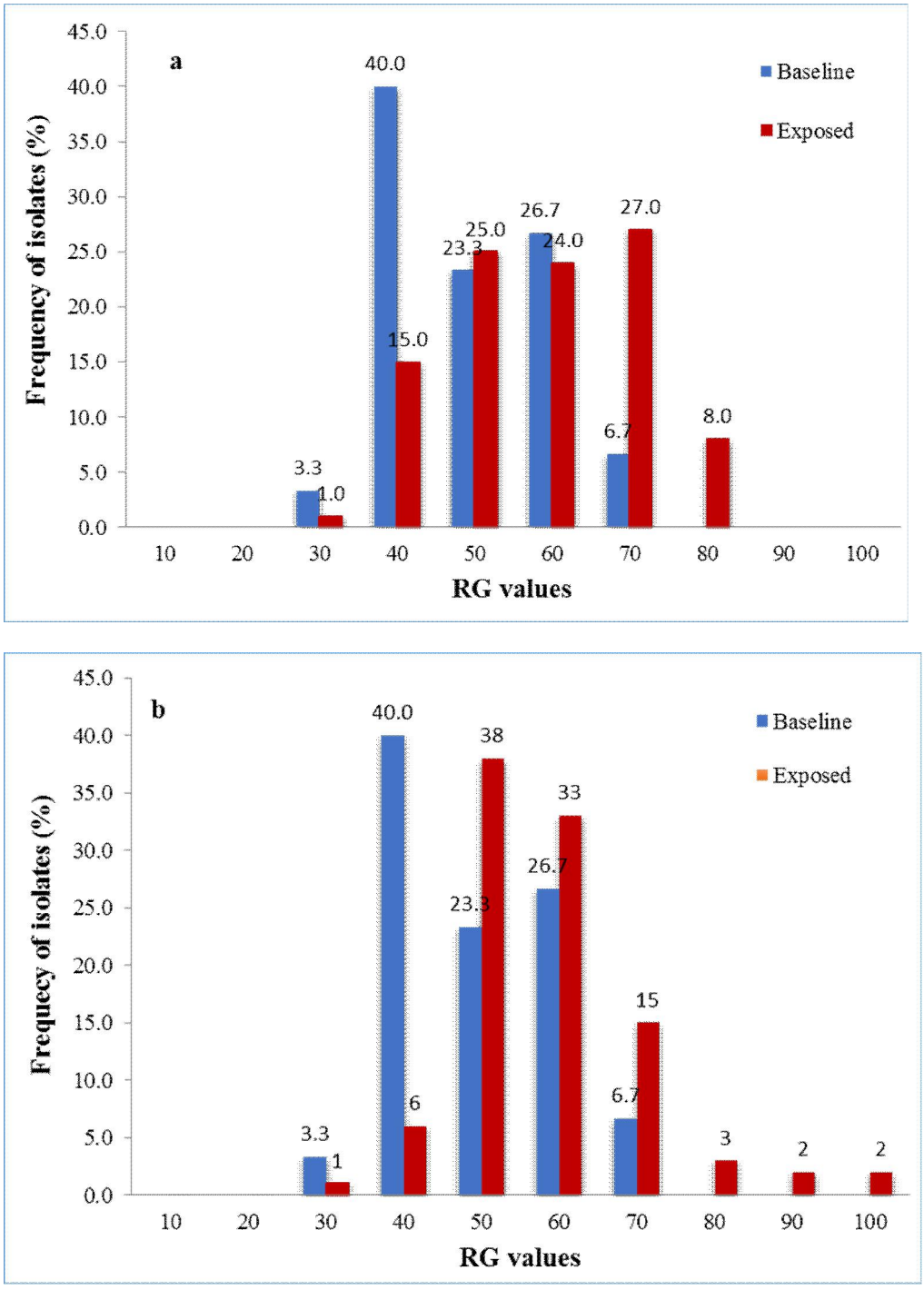


Fig. 9: Comparison between frequency distributions of *V. inaequalis* baseline and exposed populations to difenoconazole at 0.03 µg ml^{-1} discriminatory dose. a) Baramulla population, b) Shopian population

per cent of isolates exhibited RG values greater than 70 per cent and none of the isolates exhibited RG value greater than 80 per cent.

Similarly, the frequency distribution of difenoconazole sensitivity of the fungicide-exposed isolates from district Shopian also varied slightly from that of the baseline population (Fig. 9b). Majority of the orchard isolates (92%) exhibited RG values of 30 to 70 per cent, with a few isolates (7%) exhibiting RG values greater than 70 per cent.

4.2.5 Sensitivity to flusilazole

The *V. inaequalis* isolates collected from fungicide-exposed orchards of district Baramulla and Shopian were evaluated for their sensitivity to flusilazole at a discriminatory dose of 0.03 μg ml^{-1}, and the RG values calculated. Similarly, the RG values of baseline isolates at the same discriminatory dose of fungicide were also calculated. The results revealed that the RG values of baseline population varied between 30.67 to 60.3 per cent with a mean RG value of 44.47 per cent (Table 29); isolate Bp11 exhibited the lowest RG value, whereas the isolate Bp29 exhibited the highest RG value. The RG values of the pathogen isolates collected from fungicide-exposed orchards of Baramulla ranged from 30.55 to 82.85 per cent with a mean RG value of 57.61per cent (Table 30); isolate B_{5-2} exhibited the lowest RG value, whereas the isolate B_{2-3} exhibited the highest RG value. Similarly, RG values of the pathogen isolates from Shopian ranged from 25.00 to 96.00 per cent with a mean value of 58.65 per cent (Table 31); isolate S_{1-4} exhibited the lowest RG value, whereas the isolate S_{6-7} exhibited the highest RG value.

The frequency distribution of RG values of the fungicide-exposed *V. inaequalis* populations showed varied pattern from that of the baseline population (Fig. 10a-b). The frequency distribution of flusilazole sensitivity of pathogen isolates collected from fungicide-exposed orchards of Baramulla varied from that of baseline population (Fig. 10a). The RG values of majority of the baseline

Table 29: Response of baseline isolates (relative growth) of *Venturia inaequalis* to flusilazole at discriminatory dose of 0.03µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
Bp1	55.15	Bp7	42.97	Bp13	52.98	Bp19	30.78	Bp25	51.78
Bp2	51.96	Bp8	46.67	Bp14	36.46	Bp20	36.69	Bp26	56.87
Bp3	44.31	Bp9	36.85	Bp15	52.16	Bp21	40.31	Bp27	43.34
Bp4	40.18	Bp10	43.25	Bp16	56.20	Bp22	42.38	Bp28	44.13
Bp5	49.88	Bp11	30.67	Bp17	33.40	Bp23	42.46	Bp29	60.03
Bp6	52.57	Bp12	33.56	Bp18	34.43	Bp24	33.38	Bp30	58.30

Table 30: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Baramulla to flusilazole at discriminatory dose of 0.03µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
B_{1-1}	45.65	B_{3-1}	47.61	B_{5-1}	44.44	B_{7-1}	80.00	B_{9-1}	52.27
B_{1-2}	54.34	B_{3-2}	65.00	B_{5-2}	30.55	B_{7-2}	69.04	B_{9-2}	80.00
B_{1-3}	68.75	B_{3-3}	55.00	B_{5-3}	56.41	B_{7-3}	64.51	B_{9-3}	64.51
B_{1-4}	55.00	B_{3-4}	40.00	B_{5-4}	42.10	B_{7-4}	63.15	B_{9-4}	60.24
B_{1-5}	56.00	B_{3-5}	44.11	B_{5-5}	32.14	B_{7-5}	39.39	B_{9-5}	61.70
B_{1-6}	48.14	B_{3-6}	39.06	B_{5-6}	42.25	B_{7-6}	78.84	B_{9-6}	40.38
B_{1-7}	47.61	B_{3-7}	60.00	B_{5-7}	29.62	B_{7-7}	62.71	B_{9-7}	62.85
B_{1-8}	50.00	B_{3-8}	52.50	B_{5-8}	38.96	B_{7-8}	66.12	B_{9-8}	66.66
B_{1-9}	56.66	B_{3-9}	64.00	B_{5-9}	45.78	B_{7-9}	45.31	B_{9-9}	46.42
B_{1-10}	54.83	B_{3-10}	62.50	B_{5-10}	42.42	B_{7-10}	64.91	B_{9-10}	47.36
B_{2-1}	69.76	B_{4-1}	75.00	B_{6-1}	37.70	B_{8-1}	41.86	B_{10-1}	46.15
B_{2-2}	77.55	B_{4-2}	58.00	B_{6-2}	35.10	B_{8-2}	67.56	B_{10-2}	37.28
B_{2-3}	82.85	B_{4-3}	57.50	B_{6-3}	70.90	B_{8-3}	61.84	B_{10-3}	38.09
B_{2-4}	43.75	B_{4-4}	60.46	B_{6-4}	40.54	B_{8-4}	36.61	B_{10-4}	50.74
B_{2-5}	57.50	B_{4-5}	35.29	B_{6-5}	77.50	B_{8-5}	41.91	B_{10-5}	59.15
B_{2-6}	73.80	B_{4-6}	63.79	B_{6-6}	36.11	B_{8-6}	40.29	B_{10-6}	54.54
B_{2-7}	59.25	B_{4-7}	58.33	B_{6-7}	48.97	B_{8-7}	80.00	B_{10-7}	57.62
B_{2-8}	81.66	B_{4-8}	62.90	B_{6-8}	58.97	B_{8-8}	57.14	B_{10-8}	55.76
B_{2-9}	51.25	B_{4-9}	47.76	B_{6-9}	46.15	B_{8-9}	65.30	B_{10-9}	65.75
B_{2-10}	55.12	B_{4-10}	60.00	B_{6-10}	62.50	B_{8-10}	64.58	B_{10-10}	33.33

Table 31: Response (relative growth) of exposed orchard isolates of *Venturia inaequalis* collected from district Shopian to flusilazole at discriminatory dose of 0.03µg ml^{-1}

Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value	Isolate	RG value
S_{1-1}	42.74	S_{3-1}	53.70	S_{5-1}	46.34	S_{7-1}	39.68	S_{9-1}	53.70
S_{1-2}	87.50	S_{3-2}	62.26	S_{5-2}	77.40	S_{7-2}	73.30	S_{9-2}	57.14
S_{1-3}	32.85	S_{3-3}	79.31	S_{5-3}	81.08	S_{7-3}	29.57	S_{9-3}	63.33
S_{1-4}	25.00	S_{3-4}	31.14	S_{5-4}	90.90	S_{7-4}	37.14	S_{9-4}	37.93
S_{1-5}	70.10	S_{3-5}	69.81	S_{5-5}	64.28	S_{7-5}	57.35	S_{9-5}	66.66
S_{1-6}	64.50	S_{3-6}	74.54	S_{5-6}	52.63	S_{7-6}	56.66	S_{9-6}	72.22
S_{1-7}	60.00	S_{3-7}	31.14	S_{5-7}	52.00	S_{7-7}	66.66	S_{9-7}	36.66
S_{1-8}	82.60	S_{3-8}	62.96	S_{5-8}	53.57	S_{7-8}	65.71	S_{9-8}	61.53
S_{1-9}	32.50	S_{3-9}	75.55	S_{5-9}	50.00	S_{7-9}	37.17	S_{9-9}	57.14
S_{1-10}	67.30	S_{3-10}	75.55	S_{5-10}	71.64	S_{7-10}	60.00	S_{9-10}	64.00
S_{2-1}	72.00	S_{4-1}	69.50	S_{6-1}	48.14	S_{8-1}	57.40	S_{10-1}	52.50
S_{2-2}	53.03	S_{4-2}	68.88	S_{6-2}	35.00	S_{8-2}	44.92	S_{10-2}	65.57
S_{2-3}	80.00	S_{4-3}	52.17	S_{6-3}	89.47	S_{8-3}	71.87	S_{10-3}	70.00
S_{2-4}	43.93	S_{4-4}	67.21	S_{6-4}	56.52	S_{8-4}	42.64	S_{10-4}	57.35
S_{2-5}	50.00	S_{4-5}	52.27	S_{6-5}	80.00	S_{8-5}	47.14	S_{10-5}	62.16
S_{2-6}	50.70	S_{4-6}	66.66	S_{6-6}	70.17	S_{8-6}	47.94	S_{10-6}	47.61
S_{2-7}	44.75	S_{4-7}	40.74	S_{6-7}	96.00	S_{8-7}	43.05	S_{10-7}	55.76
S_{2-8}	85.52	S_{4-8}	39.58	S_{6-8}	65.30	S_{8-8}	59.74	S_{10-8}	46.66
S_{2-9}	61.81	S_{4-9}	64.81	S_{6-9}	63.63	S_{8-9}	54.38	S_{10-9}	46.29
S_{2-10}	64.65	S_{4-10}	57.89	S_{6-10}	65.57	S_{8-10}	58.33	S_{10-10}	64.28

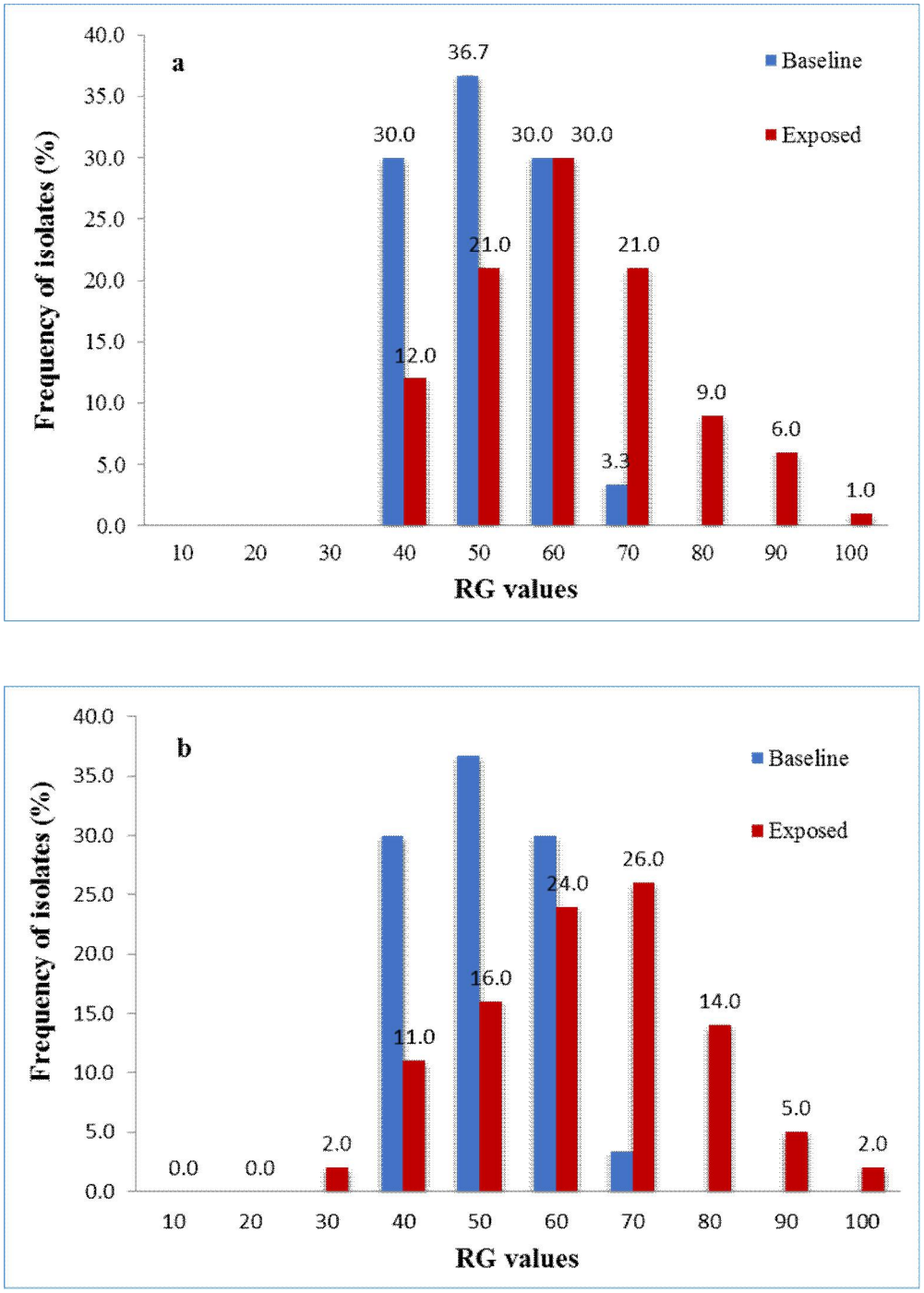


Fig. 10: Comparison between frequency distributions of baseline and exposed *V. inaequalis* populations to flusilazole at 0.03 µg ml^{-1} discriminatory dose. a) Baramulla population, b) Shopian population

isolates ranged from 30 to 60 per cent, whereas majority of the fungicide exposed isolates (93%) exhibited RG values of 30 to 80 per cent, only 7 per cent of isolates exhibited RG values greater than 80 per cent.

The frequency distribution of flusilazole sensitivity of fungicide-exposed pathogen populations from district Shopian also differed from that of the baseline population (Fig 10b). The RG values of majority of the baseline isolates ranged from 30 to 60 per cent, whereas majority of the isolates (91%) collected from fungicide exposed orchards exhibited RG values between 30 to 80 per cent, only 7 per cent of isolates exhibited RG values greater than 80 per cent.

4.3 Categorisation of *Venturia inaequalis* isolates

Shift in the sensitivity of *V. inaequalis* populations collected from Baramulla and Shopian orchards to various fungicides *viz.,* dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole, was observed, and the pathogen isolates categorized as 'sensitive', 'shifted' and 'resistant' (Table 32) on the basis of the criteria described under section 3.3.

4.3.1 Composition of isolate categories

On the basis of responses of the pathogen isolates to different fungicides across the districts, the proportion of the isolates falling in different categories were ascertained (Fig. 11a). Dodine-'resistant' isolates comprised only 0.5 per cent of the total population, whereas 56.5 and 43 per cent exhibited 'shifted' and 'sensitive' response to the fungicide. Resistance to hexaconazole was observed in only 10.5 per cent of the total isolate population, whereas 36 and 53.5 per cent of the isolates depicted 'shifted' and 'sensitive' response to the fungicide. Resistance to myclobutanil was found in 35.5 per cent of the isolates, whereas, 41 per cent of the isolates were 'shifted' response, and only 23.5 per cent of the isolates 'sensitive' response to the fungicide. Majority of the pathogen isolates (49.5%) showed 'shifted' response to difenconazole, whereas only 7.5 per cent of isolates were found resistant and 43 per cent sensitive to this fungicide. Flusilazole-

Table 32: Categorisation of *Venturia inaequalis* isolates

V. inaequalis Isolate	Sensitivity to fungicide				
	Dodine	**Hexaconazole**	**Myclobutanil**	**Difenoconazole**	**Flusilazole**
B_{1-1}	S	S	S	S	S
B_{1-2}	S	S	SH	SH	SH
B_{1-3}	SH	SH	R	SH	SH
B_{1-4}	S	SH	SH	SH	SH
B_{1-5}	S	SH	SH	SH	SH
B_{1-6}	S	SH	SH	S	S
B_{1-7}	S	S	SH	SH	S
B_{1-8}	SH	S	SH	SH	S
B_{1-9}	S	S	SH	S	SH
B_{1-10}	S	S	SH	S	SH
B_{2-1}	SH	S	S	SH	SH
B_{2-2}	SH	SH	R	R	R
B_{2-3}	SH	SH	R	S	R
B_{2-4}	S	R	SH	S	S
B_{2-5}	SH	SH	R	SH	SH
B_{2-6}	SH	R	R	SH	R
B_{2-7}	SH	SH	R	SH	SH
B_{2-8}	SH	SH	R	SH	R
B_{2-9}	SH	S	R	SH	SH
B_{2-10}	SH	S	R	S	SH
B_{3-1}	SH	SH	SH	S	SH
B_{3-2}	SH	SH	R	SH	SH
B_{3-3}	SH	SH	SH	SH	SH
B_{3-4}	SH	S	SH	S	S
B_{3-5}	SH	S	SH	S	SH
B_{3-6}	SH	S	SH	S	SH
B_{3-7}	SH	S	SH	SH	SH
B_{3-8}	SH	S	SH	SH	S
B_{3-9}	S	SH	R	SH	S
B_{3-10}	S	SH	SH	SH	S
B_{4-1}	SH	S	SH	R	R
B_{4-2}	SH	SH	R	SH	SH
B_{4-3}	SH	SH	R	SH	R
B_{4-4}	SH	SH	SH	SH	SH
B_{4-5}	S	S	SH	S	S
B_{4-6}	SH	R	R	SH	SH
B_{4-7}	S	S	SH	SH	SH

Contd…

Table 32: Contd…

B_{4-8}	S	S	S	SH	SH
B_{4-9}	S	SH	SH	S	SH
B_{4-10}	SH	R	SH	SH	R
B_{5-1}	S	S	S	S	S
B_{5-2}	S	S	S	S	S
B_{5-3}	S	S	SH	SH	S
B_{5-4}	S	S	S	S	S
B_{5-5}	SH	S	S	S	SH
B_{5-6}	S	S	S	S	SH
B_{5-7}	S	S	S	S	S
B_{5-8}	S	S	S	S	S
B_{5-9}	S	S	SH	S	SH
B_{5-10}	S	S	S	S	SH
B_{6-1}	SH	S	SH	S	S
B_{6-2}	S	S	R	S	S
B_{6-3}	SH	SH	R	R	SH
B_{6-4}	S	S	SH	S	S
B_{6-5}	SH	SH	R	R	R
B_{6-6}	S	S	SH	S	S
B_{6-7}	SH	S	S	S	SH
B_{6-8}	SH	S	R	SH	R
B_{6-9}	SH	S	SH	S	SH
B_{6-10}	SH	S	R	SH	SH
B_{7-1}	SH	S	SH	R	R
B_{7-2}	SH	R	R	SH	R
B_{7-3}	SH	R	R	SH	R
B_{7-4}	SH	SH	R	SH	R
B_{7-5}	S	S	SH	S	SH
B_{7-6}	SH	R	R	R	R
B_{7-7}	S	S	S	SH	SH
B_{7-8}	S	S	SH	SH	SSHH
B_{7-9}	S	SH	R	S	SH
B_{7-10}	SH	R	SH	SH	R
B_{8-1}	SH	R	SH	S	SH
B_{8-2}	SH	SH	R	SH	SH
B_{8-3}	SH	SH	SH	SH	SH
B_{8-4}	SH	S	SH	S	S
B_{8-5}	SH	S	SH	S	SH
B_{8-6}	SH	S	SH	S	SH
B_{8-7}	SH	R	R	R	R
B_{8-8}	SH	S	SH	SH	S

Contd…

Table 32: Contd…

B_{8-9}	SH	SH	SH	SH	S
B_{8-10}	S	SH	R	SH	S
B_{9-1}	S	S	S	SH	S
B_{9-2}	S	S	SH	R	SH
B_{9-3}	SH	SH	R	SH	R
B_{9-4}	SH	R	SH	SH	SH
B_{9-5}	S	R	R	SH	SH
B_{9-6}	S	SH	SH	S	S
B_{9-7}	SH	S	R	SH	SH
B_{9-8}	SH	S	SH	SH	SH
B_{9-9}	S	S	R	S	SH
B_{9-10}	S	S	R	S	SH
B_{10-1}	S	S	S	S	S
B_{10-2}	S	S	S	S	S
B_{10-3}	SH	S	S	S	SH
B_{10-4}	S	S	SH	SH	S
B_{10-5}	SH	S	S	SH	SH
B_{10-6}	SH	S	S	SH	S
B_{10-7}	S	S	S	SH	S
B_{10-8}	S	S	S	SH	SH
B_{10-9}	S	S	S	SH	S
B_{10-10}	S	S	S	S	S
S_{1-1}	S	SH	R	S	S
S_{1-2}	S	S	SH	R	R
S_{1-3}	S	S	S	S	S
S_{1-4}	SH	S	S	S	S
S_{1-5}	SH	SH	R	SH	R
S_{1-6}	S	R	R	S	SH
S_{1-7}	S	SH	SH	S	SH
S_{1-8}	S	R	R	SH	R
S_{1-9}	S	S	S	SH	S
S_{1-10}	S	SH	SH	S	SH
S_{2-1}	SH	SH	R	SH	R
S_{2-2}	SH	S	R	S	SH
S_{2-3}	SH	SH	R	SH	R
S_{2-4}	S	S	S	S	S
S_{2-5}	S	S	S	SH	S
S_{2-6}	S	S	S	S	SH
S_{2-7}	S	S	S	S	S
S_{2-8}	R	SH	R	R	R
S_{2-9}	SH	SH	SH	SH	SH

Contd…

Table 32: Contd…

S_{2-10}	SH	SH	SH	SH	SH
S_{3-1}	SH	SH	SH	SH	SH
S_{3-2}	SH	SH	R	SH	SH
S_{3-3}	S	R	R	R	R
S_{3-4}	S	S	S	S	S
S_{3-5}	S	SH	R	SH	SH
S_{3-6}	S	SH	R	S	R
S_{3-7}	S	S	R	S	S
S_{3-8}	S	S	SH	S	SH
S_{3-9}	S	SH	R	SH	R
S_{3-10}	SH	S	SR	SH	R
S_{4-1}	SH	SH	R	SH	SH
S_{4-2}	SH	S	SH	S	SH
S_{4-3}	SH	S	S	S	SH
S_{4-4}	SH	S	R	SH	SH
S_{4-5}	SH	SH	S	S	SH
S_{4-6}	SH	SH	SH	SH	SH
S_{4-7}	SH	S	SH	S	S
S_{4-8}	SH	S	R	SH	S
S_{4-9}	SH	S	SH	S	SH
S_{4-10}	SH	S	SH	S	SH
S_{5-1}	S	S	SH	S	S
S_{5-2}	SH	SH	R	SH	R
S_{5-3}	SH	R	R	R	R
S_{5-4}	SH	SH	R	SH	R
S_{5-5}	SH	R	R	SH	SH
S_{5-6}	SH	SH	R	S	SH
S_{5-7}	S	S	S	SH	SH
S_{5-8}	SH	S	R	SH	SH
S_{5-9}	S	S	SH	S	S
S_{5-10}	SH	SH	SH	SH	R
S_{6-1}	SH	S	SH	S	S
S_{6-2}	S	S	S	S	S
S_{6-3}	SH	R	R	S	R
S_{6-4}	S	SH	SH	R	SH
S_{6-5}	S	S	S	SH	R
S_{6-6}	S	SH	SH	SH	R
S_{6-7}	S	R	S	R	R
S_{6-8}	SH	SH	R	SH	SH
S_{6-9}	SH	S	SH	SH	SH
S_{6-10}	SH	S	R	SH	SH

Contd…

Table 32: Contd…

S_{7-1}	SH	SH	SH	S	S
S_{7-2}	S	S	SH	SH	R
S_{7-3}	S	S	S	S	S
S_{7-4}	S	S	S	S	S
S_{7-5}	SH	SH	SH	SH	SH
S_{7-6}	SH	SH	R	S	SH
S_{7-7}	S	SH	SH	S	SH
S_{7-8}	S	SH	R	SH	SH
S_{7-9}	S	S	S	SH	S
S_{7-10}	S	SH	SH	S	SH
S_{8-1}	S	SH	SH	S	SH
S_{8-2}	SH	S	SH	S	S
S_{8-3}	SH	SH	R	SH	R
S_{8-4}	S	S	S	S	S
S_{8-5}	S	S	S	S	S
S_{8-6}	S	S	S	S	S
S_{8-7}	S	S	S	S	S
S_{8-8}	SH	SH	SH	SH	SH
S_{8-9}	SH	S	SH	SH	SH
S_{8-10}	SH	S	SH	SH	SH
S_{9-1}	S	SH	SH	SH	SH
S_{9-2}	S	SH	SH	SH	SH
S_{9-3}	S	SH	R	SH	SH
S_{9-4}	S	S	S	S	S
S_{9-5}	S	SH	R	SH	SH
S_{9-6}	S	SH	R	S	R
S_{9-7}	S	SH	R	S	S
S_{9-8}	SH	S	SH	S	SH
S_{9-9}	SH	SH	SH	SH	SH
S_{9-10}	SH	S	SH	SH	SH
S_{10-1}	S	S	S	SH	SH
S_{10-2}	SH	SH	SH	SH	SH
S_{10-3}	SH	R	R	R	R
S_{10-4}	S	SH	SH	S	SH
S_{10-5}	SH	SH	SH	SH	SH
S_{10-6}	SH	SH	R	S	S
S_{10-7}	S	S	S	S	SH
S_{10-8}	S	S	R	SH	S
S_{10-9}	S	S	SH	SH	S
S_{10-10}	SH	SH	R	SH	SH

S = ‘Sensitive’, SH = ‘Shifted’, R = ‘Resistant’

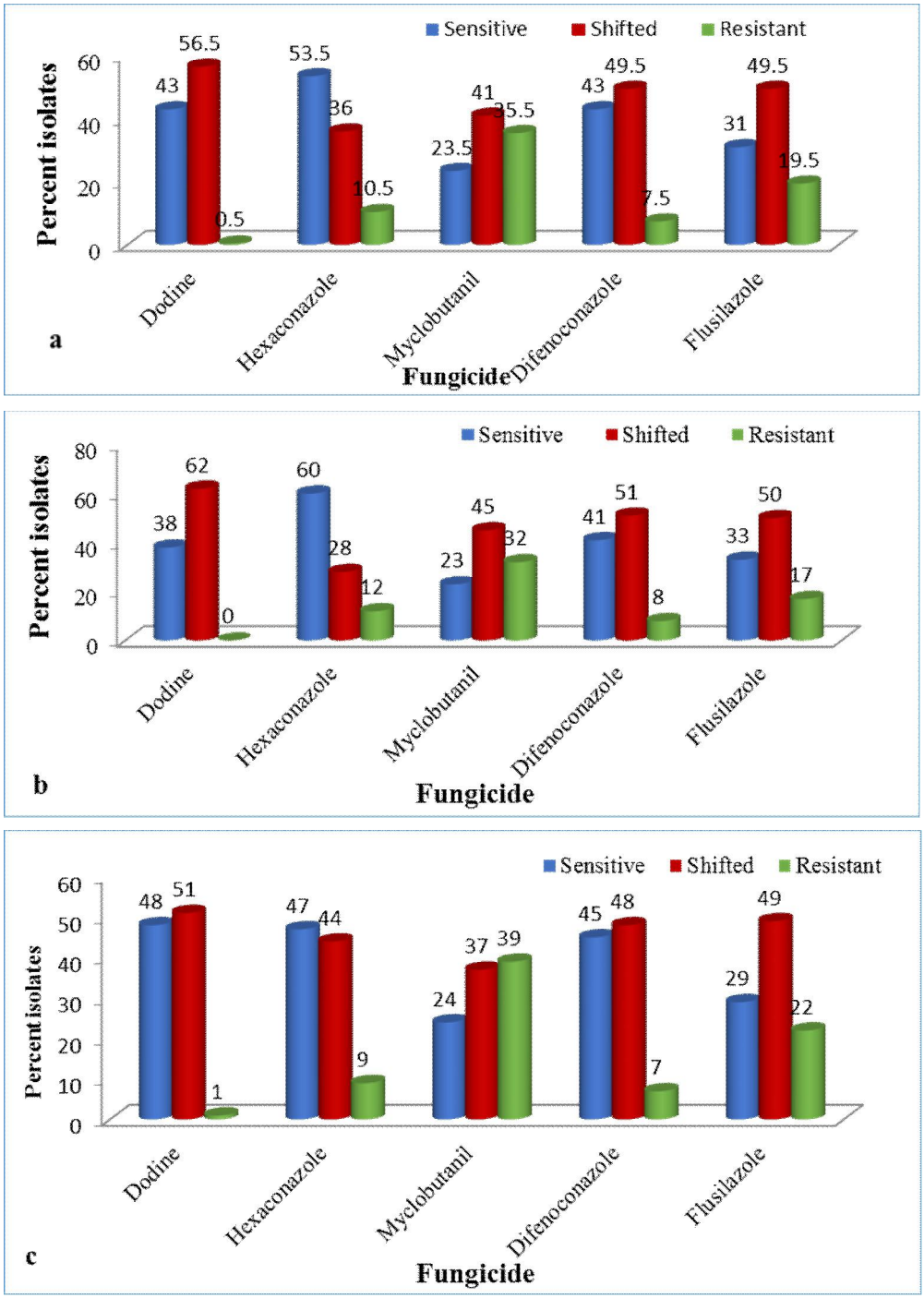


Fig 11: Per cent distribution of *Venturia inaequalis* isolates classified as sensitive, shifted and resistant to different fungicides tested. a) Baramulla population, b) Shopian population and c) combined population

'resistant' isolates comprised 19.5 per cent of the total population, wheras 49.5 per cent isolates were identified as 'shifted' and 31 percent 'sensitive' to this fungicide.

4.3.2 District-wise distribution of isolate categories

Resistance of *V. inaequalis* isolates to different fungicides at varying frequencies was detected in both the districts Baramulla and Shopian (Fig. 11 b-c). Thirty-eight per cent of *V. inaequalis* isolates from Baramulla and 48 per cent isolates from Shopian were found sensitive to dodine (Fig. 11b-c), whereas 62 per cent and 51 per cent of isolates exhibited 'shifted' response in Baramulla and Shopian, respectively. However, none of the isolates from district Baramulla was identified as dodine-'resistant', and only 1 per cent of isolates from Shopian exhibited 'resistant' response to dodine.

Hexaconazole-'sensitive' isolates comprised 60 and 47 per cent of the total population in Baramulla and Shopian, respectively. However, 28 per cent of isolates from district Baramulla and 44 per cent isolates from district Shopian exhibited 'shifted' response. Hexaconazole-'resistant' isolates comprised 12 and 9 per cent of the total population in district Baramulla and Shopian, respectively.

The 'sensitive', 'shifted' and 'resistant' response of the isolates to myclobutanil comprised 23, 45 and 32 per cent of the total population, respectively in district Baramulla, whereas 24, 37 and 39 per cent isolates showed such responses, respectively, to myclobutanil in district Shopian.

Difenoconazole-'sensitive' isolates comprised 41 and 45 per cent of the population, respectively, in district Baramulla and Shopian, respectively. However, 51 and 48 per cent isolates exhibited 'shifted' response to the fungicide, respectively, in district Baramulla and Shopian. The difenoconazole- 'resistant' isolates comprised 8 and 7 per cent of the total population in the two districts, respectively.

Thirty-three per cent of isolates from district Baramulla and 29 per cent

from district Shopian were found sensitive to flusilazole. However, 50 per cent of isolates in Baramulla and 49 per cent of isolates in Shopian exhibited 'shifted' response to flusilazole resistance, whereas the flusilazole 'resistant' response was displayed by 17 and 22 per cent of the total isolate population, respectively, in Baramulla and Shopian.

4.3.3 Orchard-wise distribution of isolate categories

The pathogen isolates from various orchards varied in their responses to different fungicides (Table 33 to 37). In district Baramulla, none of the isolates was found resistant to dodine (Table 33). However, 90 per cent of the isolates ($B_{2\text{-}1}$, $B_{2\text{-}3}$, $B_{2\text{-}3,}$ $B_{2\text{-}5}$, $B_{2\text{-}6}$, $B_{2\text{-}7}$, $B_{2\text{-}8}$, $B_{2\text{-}9}$ and $B_{2\text{-}10}$) in orchard B_2 exhibited 'shifted' response to dodine, whereas 10 per cent of isolates in orchard B_5 ($B_{5\text{-}5}$) exhibited such a response. In district shopian, dodine resistance was observed in a single orchard i.e., orchard S_2 with only 10 per cent of dodine resistant isolates ($S_{2\text{-}8}$). The highest proportion of isolates (100%) exhibited 'shifted' response to dodine in orchard S_4 and the lowest proportion (30%) behaved so in orchards S_1 ($S_{1\text{-}1}$, $S_{1\text{-}4}$, $S_{1\text{-}5}$), S_3 ($S_{3\text{-}1}$, $S_{3\text{-}2}$, $S_{3\text{-}10}$), S_7 ($S_{7\text{-}1,}$ $S_{7\text{-}5,}$ $S_{7\text{-}6}$) and S_9 ($S_{9\text{-}8,}$ $S_{9\text{-}9,}$ $S_{9\text{-}10}$).

The distribution of hexaconazole 'resistant' isolates also varied among orchards in both the districts (Table 34). In case of district Baramulla, highest proportion (40%) of hexaconazole resistant isolates was found in orchard B_7 ($B_{7\text{-}2}$, $B_{7\text{-}3}$, $B_{7\text{-}6}$, $B_{7\text{-}10}$); however, none of the isolates from orchards B_1, B_2, B_3, B_5, B_6 and B_{10} showed 'resistant' response to the fungicide. The highest number of isolates (100%) exhibiting 'shifted' response to hexaconazole was observed in orchard B_{10} and lowest in B_6 and B_9 orchards. In district Shopian, hexaconazole-'resistant' isolates comprised 20 per cent of the total isolates in orchards S_1 ($S_{1\text{-}6}$, $S_{1\text{-}8}$), S_5 ($S_{5\text{-}3,}$ $S_{5\text{-}5}$) and S_6 ($S_{6\text{-}3,}$ $S_{6\text{-}7}$); however, none of the isolates in orchards S_2, S_4, S_7, S_8 and S_9 showed 'resistant' response to hexaconazole. The highest number of isolates exhibiting 'shifted' response to hexaconazole was observed in orchard S_9 (70%; $S_{9\text{-}1,}$ $S_{9\text{-}2,}$ $S_{9\text{-}3,}$ $S_{9\text{-}5,}$ $S_{9\text{-}6,}$ $S_{9\text{-}7}$ and $S_{9\text{-}9}$).

Myclobutanil resistance in *V. inaequalis* isolates was prevalent in both Baramulla and Shopian districts (Table 35). In district Baramulla, the highest percentage (80%) of myclobutanil-'resistant' isolates was observed in orchard B_2 (B_{2-2}, B_{2-3}, B_{2-5} , B_{2-6},B_{2-7}, B_{2-8}, B_{2-9}, B_{2-10}) followed by 50 per cent such isolates each in orchard B_6 (B_{6-2}, B_{6-3}, B_{6-5}, B_{6-8}, B_{6-10}), and B_9 (B_{9-3}, B_{9-5}, B_{9-7}, B_{9-9}, B_{9-10}). The lowest proportion of myclobutanil-'resistant' isolates (10%) was found in orchard B_1 (B_{1-3}). The highest proportion of 'shifted' isolates (80%) was found each in orchards B_1 (B_{1-2}, B_{1-4}, B_{1-5}, B_{1-6}, B_{1-7}, B_{1-8}, B_{1-9}, B_{1-10}) and B_3 (B_{3-1}, B_{3-3}, B_{3-4}, B_{3-5}, B_{3-6}, B_{3-7}, B_{3-8}, B_{3-10}). In district shopian, the highest proportion (70%) of myclobutanil-'resistant' isolates (S_{3-2}, S_{3-3}, S_{3-5}, S_{3-6}, S_{3-7}, S_{3-9}, S_{3-10}) was found in orchard S_3 followed by (60%) in orchard S_5 (S_{5-2}, S_{5-3}, S_{5-4}, S_{5-5}, S_{5-6}, S_{5-8}); the lowest proportion (10%) of such isolates was found in orchard S_8 (S_{8-3}). Myclobutanil-'resistant' isolates comprised 40 per cent of the total isolates in each of the orchards S_1 (S_{1-1}, S_{1-5}, S_{1-6}, S_{1-8}), S_2 (S_{2-1}, S_{2-2}, S_{2-3}, S_{2-8}), S_9 (S_{9-3}, S_{9-5}, S_{9-6}, S_{9-7}) and S_{10} (S_{10-3}, S_{10-6}, S_{10-8}, S_{10-10}). However, the highest number of isolates (50%) were found to exhibit 'shifted' response to myclobutanil in each of the orchard S_7 (S_{7-1}, S_{7-2}, S_{7-5}, S_{7-7} and S_{7-10}), S_8 (S_{8-1}, S_{8-2}, S_{8-8}, S_{8-9} and S_{8-10}) and S_9 (S_{9-1}, S_{9-2}, S_{9-8}, S_{9-9} and S_{9-10}).

The resistance to difenoconazole was less prevalent among orchards in both the districts (Table 36). In district Baramulla, the highest percentage (20%) of difenoconazole-'resistant' isolates was observed in orchards B_6 (B_{6-3}, B_{6-5}) and B_7 (B_{7-1}, B_{7-6}); however, none of the isolates exhibited 'resistant' response in orchards B_1, B_3, B_5 and B_{10}. The highest percentage (70%) of the isolates (B_{4-2}, B_{4-3}, B_{4-4}, B_{4-6}, B_{4-7}, B_{4-8} and B_{4-10}) showed 'shifted' response in orchard B_4. In district shopian, 20 per cent of 'resistant' isolates was observed in orchard S_6 (S_{6-4}, S_{6-7}). Ten per cent of 'resistant' isolates was found in each of the orchards S_1 (S_{1-2}), S_2 (S2-8), S_3 (S_{3-3}), S_5 (S_{5-3}) and S_{10} (S_{10-3}). None of the isolates in rest of the orchards exhibited "resistance" to difenoconazole. However, the greatest proportion (60%) of the isolates exhibiting 'shifted' response" was observed in S_5

Table 33: Orchard-wise distribution of *Venturia inaequalis* isolates as 'sensitive' (S), 'shifted' (SH) and 'resistant' (R) to dodine

No./ per cent isolates classified as				No/ per cent isolates classified as			
Orchards	**S**	**SH**	**R**	**Orchards**	**S**	**SH**	**R**
DistrictBaramulla				**District Shopian**			
B_1	B_{1-1}, B_{1-2}, B_{1-4}, B_{1-5}, B_{1-6}, B_{1-7}, B_{1-9}, B_{1-10} (80%)	B_{1-3}, B_{1-8} (20%)	-	S_1	S_{1-2}, S_{1-3}, S_{1-6}, S_{1-7}, S_{1-8}, S_{1-9}, S_{1-10} (70%)	S_{1-1}, S_{1-4}, S_{1-5} (30%)	-
B_2	B_{2-4} (10%)	B_{2-1} to B_{2-3}, B_{2-5} to B_{2-10} (90%)	-	S_2	S_{2-4}, S_{2-5}, S_{2-6}, S_{2-7} (40%)	S_{2-1}, S2-2, S2-3, S2-9, S2-10 (50%)	S_{2-8} (10%)
B_3	B_{3-9} B_{3-10} (20%)	B_{3-1}, B_{3-2}, B_{3-3}, B_{3-4}, B_{3-5}, B_{3-6}, B_{3-7}, B_{3-8} (80%)	-	S_3	S_{3-3}, S_{3-4}, S_{3-5}, S_{3-6}, S_{3-7}, S_{3-8}, S_{3-9} (70%)	S_{3-1}, S_{3-2}, S_{3-10} (30%)	-
B_4	B_{4-5} B_{4-7} to B_{4-9} (40%)	B_{4-1} to B_{4-4} B_{4-6} B_{4-8} B_{4-10} (60%)	-	S_4	-	S_{4-1}, S_{4-2}, S_{4-3}, S_{4-4}, S_{4-5}, S_{4-6}, S_{4-7}, S_{4-8}, S_{4-9}, S_{4-10} (100%)	-
B_5	B_{5-1} B_{5-2}, B_{5-3}, B_{5-4}, B_{5-6} B_{5-7}, B_{5-8}, B_{5-9}, B_{5-10} (90%)	B_{5-5} (10%)	-	S_5	S_{5-1}, S_{5-7}, S_{5-9} (30%)	S_{5-2}, S_{5-3}, S_{5-4}, S_{5-5}, S_{5-6}, S_{5-8}, S_{5-10} (70%)	-
B_6	B_{6-2} B_{6-4} B_{6-6} B_{6-9} (40%)	B_{6-1} B_{6-3} B_{6-5} B_{6-7} B_{6-8} B_{6-10} (60%)	-	S_6	S_{6-2}, S_{6-5}, S_{6-6}, S_{6-7} (40%)	S_{6-1}, S_{6-3}, S_{6-4}, S_{6-8}, S_{6-9}, S_{6-10} (60%)	-
B_7	B_{7-5} B_{7-7} to B_{7-9} (40%)	B_{7-1} to B_{7-4}, B_{7-6}, B_{7-10} (60%)	-	S_7	S_{7-2}, S_{7-3}, S_{7-4}, S_{7-7}, S_{7-8}, S_{7-9}, S_{7-10} (70%)	S_{7-1}, S_{7-5}, S_{7-6} (30%)	-
B_8	B_{8-1}, B_{8-10} (20%)	B_{8-2} to B_{8-9} (80%)	-	S_8	S_{8-1}, S_{8-4}, S_{8-5}, S_{8-6}, S_{8-7} (50%)	S_{8-2}, S_{8-3}, S_{8-8}, S_{8-9}, S_{8-10} (50%)	-
B_9	B_{9-1} B_{9-2}, B_{9-5}, B_{9-6}, B_{9-9}, B_{9-10} (60%)	B_{9-3}, B_{9-4}, B_{9-7}, B_{9-8} (40%)	-	S_9	S_{9-1}, S_{9-2}, S_{9-3}, S_{9-4}, S_{9-5}, S_{9-6}, S_{9-7} (70%)	S_{9-8}, S_{9-9}, S_{9-10} (30%)	-
B_{10}	B_{10-1}, B_{10-2}, B_{10-4}, B_{10-7} to B_{10-10} (70%)	B_{10-3}, B_{10-5} B_{10-6} (30%)	-	S_{10}	S_{10-1}, S_{10-4}, S_{10-7}, S_{10-8}, S_{10-9} (50%)	S_{10-2}, S_{10-3}, S_{10-5}, S_{10-6}, S_{10-10} (50%)	-

Table 34: Orchard-wise distribution (%) of *Venturia inaequalis* isolates as 'sensitive' (S), 'shifted' (SH) and 'resistant' (R) to hexaconazole

No./ per cent isolates classified as				No./ per cent isolates classified as			
Orchards	**S**	**SH**	**R**	**Orchards**	**S**	**SH**	**R**
District Baramulla				**District Shopian**			
B_1	B_{1-1}, B_{1-2}, B_{1-7}, B_{1-8} B_{1-9}, B_{1-10} (60%)	B_{1-3}, B_{1-4}, B_{1-5}, B_{1-6} (40%)	-	S_1	S_{1-2}, S_{1-3}, S_{1-4}, S_{1-9} (40%)	S_{1-1}, S_{1-5}, S_{1-7}, S_{1-10} (40%)	S_{1-6}, S_{1-8} (20%)
B_2	B_{2-1} B_{2-9} B_{2-10} (30%)	B_{2-2}, B_{2-3}, B_{2-5} B_{2-7} B_{2-8}(50%)	B_{2-4} B_{2-6} (20%)	S_2	S_{2-2}, S_{2-4}, S_{2-5}, S_{2-6}, S_{2-7} (50%)	S_{2-1}, S_{2-3}, S_{2-8} S_{2-9}, S_{2-10} (50%)	-
B_3	B_{3-4}, B_{3-5}, B_{3-6}, B_{3-7}, B_{3-8} (50%)	B_{3-1}, B_{3-2}, B_{3-3}, B_{3-9}, B_{3-10} (50%)	-	S_3	S_{3-4}, S_{3-7}, S_{3-8}, S_{3-10} (40%)	S_{3-1}, S_{3-2} ,S_{3-5}, S_{3-6}, S_{3-9} (50%)	S_{3-3}(10%)
B_4	B_{4-1} B_{4-5} B_{4-7} B_{4-8} (40%)	B_{4-2}, $B_{4-3}$$B_{4-4}$,B_{4-9} (40%)	B_{4-6} , B_{4-10}(20%)	S_4	S_{4-2}, S_{4-3}, S_{4-4}, S_{4-7}, S_{4-8}, S_{4-9}, S_{4-10}(70%)	S_{4-1}, S_{4-5}, S_{4-6}, (30%)	-
B_5	B_{5-1} toB_{5-10} (100%)	-	-	S_5	S_{5-1}, S_{5-7}, S_{5-8}, S_{5-9} (30%)	S_{5-2}, S_{5-4}, S_{5-6}, S_{5-10} (70%)	S_{5-3}, S_{5-5}
B_6	B_{6-1} B_{6-2} B_{6-4} B_{6-6} B_{6-7} B_{6-8} B_{6-9} B_{6-10} (80%)	B_{6-3} B_{6-5} (20%)	-	S_6	S_{6-1}, S_{6-2}, S_{6-5}, S_{6-9}, S_{6-10} (50%)	S_{6-4}, S_{6-6}, S_{6-8}, (30%)	S_{6-3}, S_{6-7} (20%)
B_7	B_{7-1} B_{7-5} B_{7-7} B_{7-8} (40%)	B_{7-4}, B_{7-9} (60%)	B_{7-2}, B_{7-3}, B_{7-6}, B_{7-10}(40%)	S_7	S_{7-2}, S_{7-3}, S_{7-4}, S_{7-9} (40%)	S_{7-1}, S_{7-5}, S_{7-6} S_{7-7}, S_{7-8}, , S_{7-10} (60%)	-
B_8	B_{8-4}, B_{8-5} B_{8-6}, B_{8-8} (40%)	B_{8-2} B_{8-3}, B_{8-9} B_{8-1}0 (40%)	B_{8-1}, B_{8-7} (20%)	S_8	S_{8-2}, S_{8-4}, S_{8-5}, S_{8-6}, S_{8-7},S_{8-9}, S_{8-10} (70%)	S_{8-1}, S_{8-3}, S_{8-8}, (30%)	-
B_9	B_{9-1} B_{9-2}, B_{9-7}, B_{9-8}, B_{9-9}, B_{9-10} (60%)	B_{9-3}, B_{9-6} (20%)	B_{9-4}, B_{9-5} (20%)	S_9	S_{9-4}, S_{9-8}, S_{9-10} (30%)	S_{9-1}, S_{9-2}, S_{9-3}, S_{9-5}, S_{9-6}, S_{9-7}, S_{9-9} (70%)	-
B_{10}	-	B_{10-1}, to B_{10-10} (100%)	-	S_{10}	S_{10-1}, S_{10-7}, S_{10-8}, S_{10-9} (40%)	S_{10-2}, S_{10-4}, S_{10-5}, S_{10-6}, S_{10-10} (50%)	S_{10-3} (10%)

Table 35: Orchard-wise distribution (%) of *Venturia inaequalis* isolates as 'sensitive' (S), 'shifted' (SH) and 'resistant' (R) to myclobutanil

District No. od isolatws classified as				No/ per cent isolates classified as			
District/ Orchards	**S**	**SH**	**R**	**Orchards**	**S**	**SH**	**R**
Baramulla				**District Shopian**			
B_1	B_{1-1} (10%)	B_{1-2}, B_{1-4}, B_{1-5}, B_{1-6}, B_{1-7}, B_{1-8}, B_{1-9}, B_{1-10} (80%)	B_{1-3}(10%)	S_1	S_{1-3}, S_{1-4}, S_{1-9} (30%)	S_{1-2}, S_{1-7}, S_{1-10} (30%)	S_{1-1}, S_{1-5}, S_{1-6}, S_{1-8} (40%)
B_2	B_{2-1} (10%)	B_{2-4} (10%)	B_{2-2}, B_{2-3}, B_{2-5} B_{2-6}, B_{2-7}, B_{2-8} B_{2-9}, B_{2-10} (80%)	S_2	S_{2-4}, S_{2-5}, S_{2-6}, S_{2-7} (40%)	S_{2-9}, S_{2-10} (20%)	S_{2-1}, S_{2-2}, S_{2-3}, S_{2-8} (40%)
B_3	-	B_{3-1}, B_{3-3}, B_{3-4}, B_{3-5}, B_{3-6}, B_{3-7}, B_{3-8}, B_{3-10} (80%)	B_{3-2}, B_{3-9} (20%)	S_3	S_{3-4}, S_{3-8} (20%)	S_{3-1} (10%)	S_{3-2}, S_{3-3}, S_{3-5}, S_{3-6}, S_{3-7}, S_{3-9}, S_{3-10}(70%)
B_4	B_{4-8} (10%)	B_{4-1} B_{4-4} B_{4-5} B_{4-7} B_{4-9} B_{4-10} (60%)	B_{4-2} B_{4-3} B_{4-6}(30%)	S_4	S_{4-3}, S_{4-5}(20%)	S_{4-2}, S_{4-6}, S_{4-7}, S_{4-9}, S_{4-10} (50%)	S_{4-1}, S_{4-4}, S_{4-8}(30%)
B_5	B_{5-1} B_{5-2}, B_{5-4}, B_{5-5}, B_{5-6} B_{5-7}, B_{5-8}, B_{5-10} (80%)	B_{5-3}, B_{5-9} (20%)	-	S_5	S_{5-7}, (10%)	S_{5-1}, S_{5-9},S_{5-10} (30%)	S_{5-2}, S_{5-3}, S_{5-4}, S_{5-5}, S_{5-6}, S_{5-8}(60%)
B_6	B_{6-7} (10%)	B_{6-1} B_{6-4} B_{6-6} B_{6-9} (40%)	B_{6-2} B_{6-3} B_{6-5} B_{6-8} B_{6-10}(50%)	S_6	S_{6-2}, S_{6-5}, S_{6-7} (30%)	S_{6-1}, S_{6-4}, , S_{6-6} S_{6-9} (40%)	S_{6-3}, S_{6-8}, S_{6-10} (30%)
B_7	B_{7-7} (10%)	B_{7-1}, B_{7-5}, B_{7-8}, B_{7-9}, B_{7-10} (50%)	B_{7-2} B_{7-3} B_{7-4} B_{7-6} (40%)	S_7	S_{7-3}, S_{7-4}, S_{7-9}, (30%)	S_{7-1}, S_{7-2}, S_{7-5}, S_{7-7}, S_{7-10} (50%)	S_{7-6}, S_{7-8} (20%)
B_8	-	B_{8-1}, B_{8-3}, B_{8-4}, B_{8-5}, B_{8-6}, B_{8-8}, B_{8-9} (70%)	B_{8-2} B_{8-7} B_{8-10} (30%)	S_8	S_{8-4}, S_{8-5}, S_{8-6}, S_{8-7}(40%)	S_{8-1}, S_{8-2}, S_{8-8}, S_{8-9}, S_{8-10} (50%)	S_{8-3}(10%)
B_9	B_{9-1} (10%)	B_{9-2}, B_{9-4}, B_{9-6}, B_{9-8} (40%)	B_{9-3}, B_{9-5}, B_{9-7}, B_{9-9}, B_{9-10}(50%)	S_9	S_{9-4} (10%)	S_{9-1}, S_{9-2}, S_{9-8}, S_{9-9}, S_{9-10} (50%)	S_{9-3}, S_{9-5}, S_{9-6}, S_{9-7} (40%)
B_{10}	B_{10-1}, B_{10-2}, B_{10-3},B_{10-5} to B_{10-10} (90%)	B_{10-4} (10%)	-	S_{10}	S_{10-1}, S_{10-7} (20%)	S_{10-2}, S_{10-4}, S_{10-5}, S_{10-9} (40%)	S_{10-3}, S_{10-6}, S_{10-8}, S_{10-10}(40%)

Table 36: Orchard-wise distribution of *Venturia inaequalis* isolates as 'sensitive' (S), 'shifted' (SH) and 'resistant' (R) difenoconazole

No./ per cent of isolates classified as				No/ per cent isolates classified as			
Orchards	**S**	**SH**	**R**	**Orchards**	**S**	**SH**	**R**
District Baramulla				**District Shopian**			
B_1	B_{1-1}, B_{1-6}, B_{1-9}, B_{1-10} (40%)	B_{1-2}, B_{1-3}, B_{1-4}, B_{1-5}, B_{1-7}, B_{1-8} (60%)	-	S_1	S_{1-1}, S_{1-3}, S_{1-4}, S_{1-6}, S_{1-7}, S_{1-10} (60%)	S_{1-5}, S_{1-8}, S_{1-9} (30%)	S_{1-2} (10%)
B_2	B_{2-3} B_{2-4} B_{2-10} (30%)	B_{2-1} B_{2-5} B_{2-6} B_{2-7} B_{2-8} B_{2-9} (60%)	B_{2-2} (10%)	S_2	S2-2, S_{2-4}, S_{2-6}, S_{2-7}, S2-9, S2-10 (60%)	S2-1, S2-3, S_{2-5}, (30%)	S_{2-8} (10%)
B_3	B_{3-1}, B_{3-4}, B_{3-5}, B_{3-6} (40%)	B_{3-2}, B_{3-3}, B_{3-7}, B_{3-8} , B_{3-9} B_{3-10} (60%)	-	S_3	S_{3-4}, S_{3-6}, S_{3-7}, S_{3-8} (40%)	S_{3-1}, S_{3-2}, S_{3-5}, S_{3-9}, S_{3-10} (50%)	S_{3-3} (10%)
B_4	B_{4-5} B_{4-9} (20%)	B_{4-2} B_{4-3} B_{4-4} B_{4-6} B_{4-7} B_{4-8} B_{4-10} (70%)	B_{4-1} (10%)	S_4	S_{4-2}, S_{4-3}, S_{4-5}, S_{4-7}, S_{4-9}, S_{4-10}(60%)	S_{4-1}, S_{4-4}, S_{4-6}, S_{4-8}, (40%)	-
B_5	B_{5-1} B_{5-2}, B_{5-4}, B_{5-5}, B_{5-6} B_{5-7}, B_{5-8}, B_{5-9}, B_{5-10} (90%)	B_{5-3} (10%)	-	S_5	S_{5-1}, S_{5-6}, S_{5-9} (30%)	S_{5-2}, S_{5-4}, S_{5-5}, S_{5-7},S_{5-8}, S_{5-10} (60%)	S_{5-3} (10%)
B_6	B_{6-1} B_{6-2} B_{6-4} B_{6-6} B_{6-7} B_{6-9} (60%)	B_{6-8} B_{6-10} (20%)	B_{6-3}, B_{6-5} (20%)	S_6	S_{6-1}, S_{6-2}, S_{6-3}, (40%)	S_{6-5}, S_{6-6} S_{6-8}, S_{6-9}, S_{6-10} (60%)	S_{6-4}, S_{6-7}
B_7	B_{7-2} B_{7-3}, B_{7-4} B_{7-7}, B_{7-8}, B_{7-10} (60%)	B_{7-5} , B_{7-9} (20%)	B_{7-1}, B_{7-6} (20%)	S_7	S_{7-1}, S_{7-3}, S_{7-4}, S_{7-6} S_{7-7}, S_{7-10} (60%)	S_{7-2}, S_{7-5}, S_{7-8}, S_{7-9}, (40%)	-
B_8	B_{8-1}, B_{8-4}, B_{8-5}, B_{8-6} (40%)	B_{8-2} , B_{8-3}, B_{8-8} B_{8-9}, B_{8-10} (50%)	B_{8-7} (10%)	S_8	S_{8-1}, S_{8-2}, S_{8-4}, S_{8-5}, S_{8-6}, S_{8-7} (60%)	S_{8-3}, S_{8-8}, S_{8-9}, S_{8-10} (40%)	-
B_9	B_{9-6}, B_{9-9}, B_{9-10} (30%)	B_{9-1} B_{9-3}, B_{9-4}, B_{9-5}, B_{9-7}, B_{9-8} (60%)	B_{9-2} (10%)	S_9	S_{9-4}, S_{9-6}, S_{9-7} S_{9-8} (40%)	S_{9-1}, S_{9-2}, S_{9-3}, S_{9-5}, S_{9-9}, S_{9-10} (60%)	-
B_{10}	B_{10-1}, B_{10-2}, B_{10-3}, B_{10-10} (40%)	B_{10-4} to B_{10-9} (60%)	-	S_{10}	S_{10-4}, S_{10-6}, S_{10-7} (30%)	S_{10-1}, S_{10-2}, S_{10-5}, S_{10-8}, S_{10-9}, S_{10-10} (600%)	S_{10-3} (10%)

Table 37: Orchard-wise distribution of *Venturia inaequalis* isolates as 'sensitive' (S), 'shifted' (SH) and 'resistant' (R) to flusilazole

No/ per cent isolates classified as				No/ per cent isolates classified as			
Orchards	**S**	**SH**	**R**	**Orchards**	**S**	**SH**	**R**
Baramulla				**District Shopian**			
B_1	$B_{1-1}B_{1-6}$, B_{1-7}, B_{1-8} (40%)	, B_{1-2}, B_{1-3}, B_{1-4}, B_{1-5}, B_{1-9}, B_{1-10} (60%)	-	S_1	S_{1-1}, S_{1-3}, S_{1-4}, S_{1-9} (40%)	S_{1-6}, S_{1-7}, S_{1-10} (30%)	S_{1-2}, S_{1-5}, S_{1-8} (30%)
B_2	B_{2-4} (10%)	B_{2-1} B_{2-5} B_{2-7} B_{2-9} B_{2-10} (50%)	B_{2-2} B_{2-3}, B_{2-6}, B_{2-8} (40%)	S_2	S_{2-4}, S_{2-5}, S_{2-7} (30%)	S2-2, S2-3, S_{2-6}, S2-9, S2-10 (50%)	S2-1, S2-8 (20%)
B_3	B_{3-8}, B_{3-9} B_{3-10} (30%)	B_{3-1}, B_{3-2}, B_{3-3}, B_{3-4}, B_{3-5}, B_{3-6}, B_{3-7} (80%)	-	S_3	S_{3-4}, S_{3-7} (20%)	S_{3-1}, S_{3-2}, S_{3-5}, S_{3-8} (40%)	S_{3-3}, S_{3-6}, S_{3-9}, S_{3-10}(40%)
B_4	B_{4-5} (10%)	B_{4-2} B_{4-4} B_{4-6} B_{4-7} B_{4-8} B_{4-9} (60%)	B_{4-1} B_{4-3} B_{4-10}(30%)	S_4	S_{4-7}, S_{4-8}(20%)	S_{4-1}, S_{4-2}, S_{4-3}, S_{4-4}, S_{4-5}, S_{4-6}, S_{4-9}, S_{4-10} (80%)	-
B_5	B_{5-1} B_{5-2}, B_{5-3}, B_{5-4}, B_{5-7}, B_{5-8} (60%)	B_{5-5} B_{5-6} B_{5-9}, B_{5-10} (40%)	-	S_5	S_{5-1}, S_{5-9} (20%)	S_{5-5}, S_{5-6}, S_{5-7}, S_{5-8}, (40%)	S_{5-2}, S_{5-3}, S_{5-4}, S_{5-10}(40%)
B_6	B_{6-1} B_{6-2} B_{6-4} B_{6-6} (40%)	B_{6-3} B_{6-7} B_{6-9} B_{6-10} (40%)	B_{6-5} B_{6-8}(20%)	S_6	S_{6-1}, S_{6-2}, (20%)	S_{6-4}, S_{6-8}, S_{6-9}, S_{6-10} (40%)	S_{6-3}, S_{6-5}, S_{6-6}, S_{6-7}(40%)
B_7	-	B_{7-5} B_{7-7} to B_{7-9} (40%)	B_{7-1} to B_{7-4}, B_{7-6}, B_{7-10}(60%)	S_7	S_{7-1}, S_{7-3}, S_{7-4}, S_{7-9}, (40%)	S_{7-5}, S_{7-6} S_{7-7}, S_{7-8}, S_{7-10} (50%)	S_{7-2} (10%)
B_8	B_{8-4}, B_{8-8}, B_{8-9}, B_{8-10} (40%)	B_{8-1}, B_{8-2} B_{8-3}, B_{8-5} B_{8-6}, (50%)	B_{8-7}(10%)	S_8	S_{8-2}, S_{8-4}, S_{8-5}, S_{8-6}, S_{8-7} (50%)	S_{8-1}, S_{8-8}, S_{8-9}, S_{8-10} (40%)	S_{8-3}(10%)
B_9	B_{9-1}, B_{9-6}, (20%)	B_{9-2}, B_{9-4}, B_{9-5}, B_{9-7}, B_{9-8} B_{9-9}, B_{9-10} (70%)	B_{9-3}(10%)	S_9	S_{9-4}, S_{9-7} (20%)	S_{9-1}, S_{9-2}, S_{9-3}, S_{9-5}, S_{9-8}, S_{9-9}, S_{9-10} (70%)	S_{9-6} (10%)
B_{10}	B_{10-1}, B_{10-2}, B_{10-4}, B_{10-6}, B_{10-7} B_{10-9} B_{10-10} (70%)	B_{10-3}, B_{10-5} B_{10-8} (30%)	-	S_{10}	S_{10-6}, S_{10-8}, S_{10-9} (30%)	S_{10-1}, S_{10-2}, S_{10-4}, S_{10-5}, S_{10-7}, S_{10-10} (60%)	S_{10-3}(10%)

(S_{5-2}, S_{5-4}, S_{5-5}, S_{5-7}, S_{5-8}, S_{5-10}), S_9 (S_{9-1}, S_{9-2}, S_{9-3}, S_{9-5}, S_{9-9}, S_{9-10}) and S_{10} (S_{10-1}, S_{10-2}, S_{10-5}, S_{10-8}, S_{10-9}, S_{10-10}) orchards.

The distribution of flusilazole-'resistant' isolates also varied among orchards in both the districts (Table 37). In district Baramulla, the highest proportion (60%) of flusilazole-'resistant' isolates was observed in orchard B_7 (B_{7-1}, B_{7-2}, B_{7-3}, B_{7-4}, B_{7-6}, B_{7-10}) followed by that (40%) in orchard B_2 (B_{2-2}, B_{2-3}, B_{2-6}, B_{2-8}), whereas the highest proportion of shifted isolates (70%) was observed in orchard B_9. The lowest proportion (10%) of flusilazole-'resistant' isolates was observed in orchards B_8 (B_{8-7}) and B_9 (B_{9-3}), and none of the isolates in orchard B_{10} was found resistant to flusilazole. In district Shopian, the highest proportion (40%) of flusilazole resistant isolates was found in orchards S_3 (S_{3-3}, S_{3-6}, S_{3-9}, S_{3-10}), S_5 (S_{5-2}, S_{5-3}, S_{5-4}, S_{5-10}) and S_6 (S_{6-3}, S_{6-5}, S_{6-6}, S_{6-7}), whereas the lowest proportion (10%) of such isolates existed in orchards "S_7", "S_8", "S_9" and "S_{10}". None of the isolates in orchard S_4 was found 'resistant' to flusilazole. However, the highest proportion (80%) of the isolates exhibiting 'shifted' response (S_{4-1}, S_{4-2}, S_{4-3}, S_{4-4}, S_{4-5}, S_{4-6}, S_{4-9}, S_{4-10}) was found in orchard S4.

4.4 Assessment of cross resistance

All the isolates from district Baramulla and Shopian were assessed for their resistance to multiple fungicides (cross resistance). Out of 81 resistant isolates, 38 isolates were found to be resistant two or more fungicides. Resistant isolates were divided into different categories (Table 38, Fig. 12). None of the isolates exhibited 'resistant' response to all the five fungicides. However, the isolate S2-8 showed resistant response to four of the test fungicides viz., dodine, myclobutanil, difenoconazole and flusilazole; six per cent of isolates (B_{7-6}, B_{8-7}, S_{3-3}, S_{5-3}, S_{10-3}) showed 'resistant' response to all the DMI fungicides *viz.,* hexaconazole, myclobutanil, difenoconazole and flusilazole. Seven per cent of isolates (B2-6, B_{7-2}, B_{7-3}, S_{1-8}) were resistant to three of the fungicides *viz*., hexaconazole, myclobutanil and flusilazole; two per cent of resistant isolates (B_{2-2}, B_{6-5}) exhibited 'resistant' to myclobutanil, difenoconazole and flusilazole.

Table 38: ***Venturia inaequalis*** **isolates resistant to single or multiple fungicides**

Fungicides	**Resistant isolates**
Isolates resistant to single fungicide	
Difenconazole	S_{6-4}, B_{9-2}
Flusilazole	S_{7-2}, S_{6-5}, S_{6-6}
Myclobutanil	B_{1-3}, B_{2-5}, B_{2-7}, B_{2-9},B_{2-10}, B_{3-2}, B_{3-9}, B_{4-2}, B_{6-2}, B_{6-10}, B_{7-9}, B_{8-2}, B_{8-10}, B_{9-7}, B_{9-9}, B_{9-10},S_{1-1},S_{2-2}, S_{3-2}, S_{3-5}, S_{3-7}, S_{4-1}, S_{4-4}, S_{4-8}, S_{5-6}, S_{5-8},S_{6-8}, S_{6-10}, S_{7-6}, S_{7-8}, S_{9-3}, S_{9-5}, S_{9-7}, S_{10-6}, S_{10-8}, S_{10-10}
Hexaconazole	B_{8-1},B_{9-4}
Isolates resistant to two fungicides	
Difenconazole and Flusilazole	B4-1, B7-1,S1-2
Myclobutanil and Flusilazole	B_{2-3}, B_{2-8}, B_{4-3}, B_{6-8}, B_{7-4}, B_{9-3}, S_{1-5}, S_{2-1}, S_{2-3}, S_{3-6}, S_{3-9}, S_{3-10}, S_{5-2}, S_{5-4}, S_{5-10}, S_{8-3}, S_{9-6}
Flusilazole and Hexaconazole	B_{4-10}, B_{7-10}
Myclobutanil and Hexaconazole	B_{4-6},B_{9-5},S_{1-6}, S_{5-5}
Isolates resistant to three fungicides	
Flusilazole, Myclobutanil and Hexaconazole	B_{2-6}, B_{7-2}, B_{7-3}, S_{1-8},
Difenoconazole, Flusilazole and Myclobutanil	B_{2-2}, B_{6-5}
Isolates resistant to four fungicides	
Difenoconazole, Flusilazole, Myclobutanil and Hexaconazole	B_{7-6}, B_{8-7},S_{3-3},S_{5-3}, S_{10-3}
Difenconazole, Flusilazole, Myclobutanil and Dodine	S_{2-8}

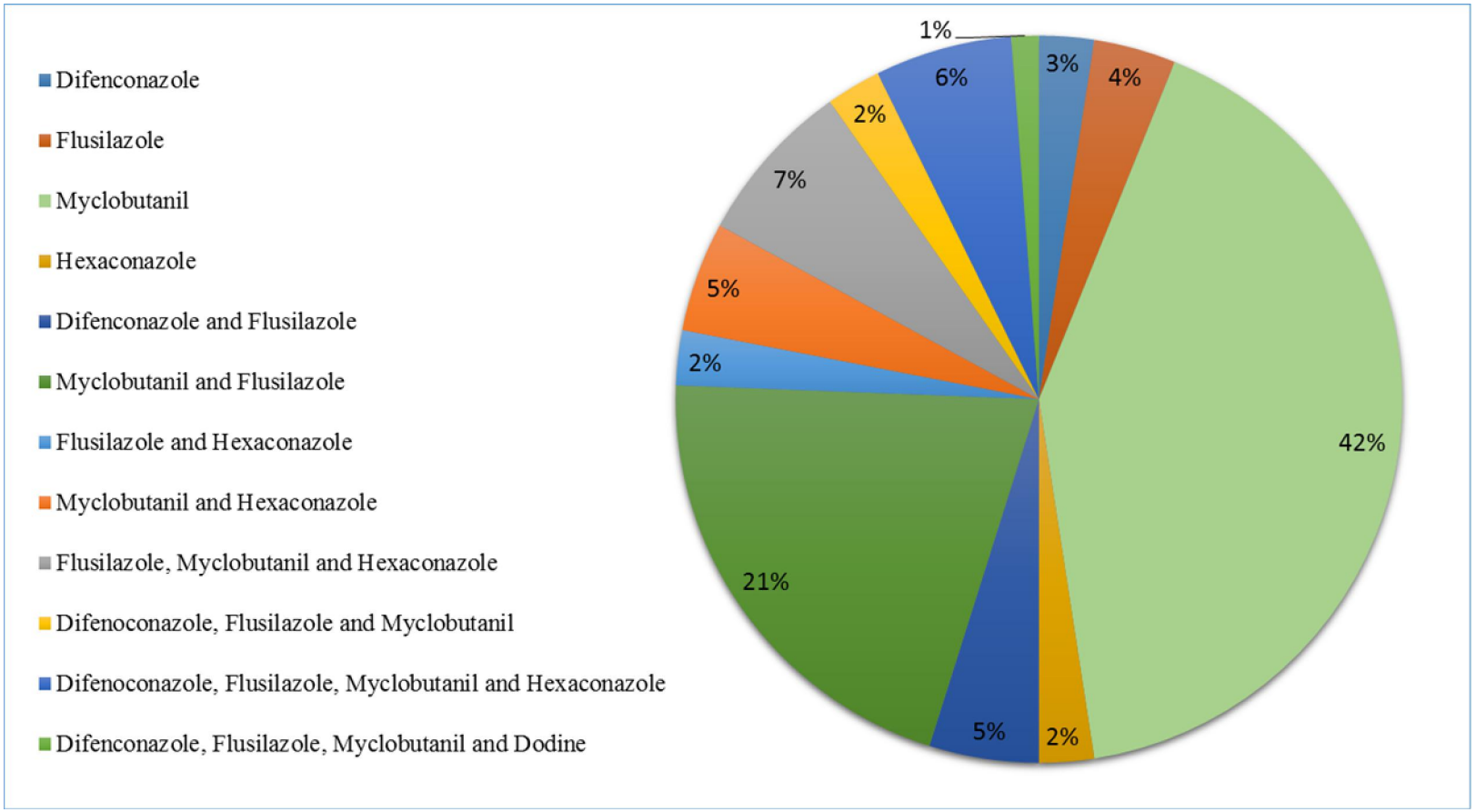


Fig. 12: Per cent distribution of *Venturia inaequalis* isolates based on their resistance to single or multiple fungicides

Most of the cross resistant isolates exhibited 'resistant' response to two of the fungicides. Twenty-one per cent of the isolates (B_{2-3}, B_{2-8}, B_{4-3}, B_{6-8}, B_{7-4}, B_{9-3}, S_{1-5}, S_{2-1}, S_{2-3}, S_{3-6}, S_{3-9}, S_{3-10}, S_{5-2}, S_{5-4}, S_{5-10},S_{8-3} and S_{9-6}) were resistant to myclobutanil as well as flusilazole, whereas 5 per cent of the 'resistant' isolates (B_{4-6}, B_{9-5}, S_{1-6}, S_{5-5}) were found resistant to both myclobutanil as well as hexaconazole; 2 per cent of the isolates (B_{4-10}, B_{7-10}) to flusilazole as well as hexaconazole, and 5 per cent of the isolates (B_{4-1}, B_{7-1}, S_{1-2}) were found 'resistant' to both difenoconazole as well as flusilazole. Apart from the cross-resistant isolates, 42 per cent of resistant isolates (B_{1-3}, B_{2-5}, B_{2-7}, B_{2-9}, B_{2-10}, B_{3-2}, B_{3-9}, B_{4-2}, B_{6-2}, B_{6-10}, B_{7-9}, B_{8-2}, B_{8-10}, B_{9-7}, B_{9-9}, B_{9-10},S_{1-1},S_{2-2}, S_{3-2}, S_{3-5}, S_{3-7}, S_{4-1}, S_{4-4}, S_{4-8}, S_{5-6}, S_{5-8}, S_{6-8}, S_{6-10}, S_{7-6}, S_{7-8}, S_{9-3}, S_{9-5}, S_{9-7}, S_{10-6}, S_{10-8} and S_{10-10}) exhibited 'resistant' response to myclobutanil only.

4.5 Molecular characterisation of selected pathogen isolates

Molecular analysis of selected resistant and sensitive isolates of the pathogen was carried out with random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. For RAPD analysis, DNA of selected sensitive (Bp3, S_{3-4}, Bp5, Bp8, Bp12, Bp20, Bp21, Bp22, Bp24 and Bp28) and resistant (S_{1-8}, S_{2-8}, S_{3-3}, S_{6-3}, S_{6-7}, S_{4-4}, B_{2-2}, B_{6-5}, B_{7-10} and B_{8-7}) isolates was combined to form sensitive and resistant bulks, respectively. A set of 46 RAPD primers were used to screen the sensitive and resistant bulks. The findings revealed that RAPD profile of sensitive and resistant bulk was similar except for primer P16 and P17 (Plate 7) with a single locus difference between resistant and sensitive. In addition, four ISSR markers were selected for genotyping of selected sensitive and resistant isolates. The isolates showed distinct banding pattern (Plate 8). Cluster analysis was conducted on taxonomic distance matrix with the Unweighted Pair Group Method based Arithmetic Average (UPGMA) and dendrogram generated (Fig. 13). Dendrogram based on ISSR data showed a single cluster at 72 per cent of similarity coefficient, whereas at 84 per cent similarity, various isolates were grouped into two clusters (cluster I and cluster II) and one

independent lineage. Cluster I comprised of 17 isolates and cluster II comprised of two isolates (S_{6-7} and B_{7-10}), whereas, the isolate Bp5 formed an independent lineage. At 86 per cent similarity coefficient, cluster I was further divided into four sub-clusters *viz.,* cluster Ia, Ib, Ic and Id. Cluster Ia comprised of three isolates *viz.,* Bp3, S_{3-4} and B_{8-7} in which two sensitive isolates Bp3 and S_{3-4} were grouped together. Cluster Ib comprised of six isolates *viz.,* Bp8, Bp20, Bp12, S_{3-3}, S_{6-3} and S_{4-4} in which sensitive isolates Bp8 and Bp20 were grouped together, Bp12 grouped separately and resistant isolates S_{3-3}, S_{6-3} and S_{4-4} were grouped together. Cluster Ic comprised of six isolates *viz.,* Bp21, Bp22, Bp24, Bp28, S_{1-8}, S_{2-8} in which two sensitive isolates Bp21 and Bp22 were grouped together. Another two sensitive isolates namely Bp 24 and Bp 28 were grouped together and resistant isolates S_{1-8} and S_{2-8} were separately grouped. Cluster Id comprised of only two resistant isolates B_{2-2} and B_{6-5}.These results indicated that the sensitive isolates of the pathogen were more closely related to each other as compared to resistant isolates.

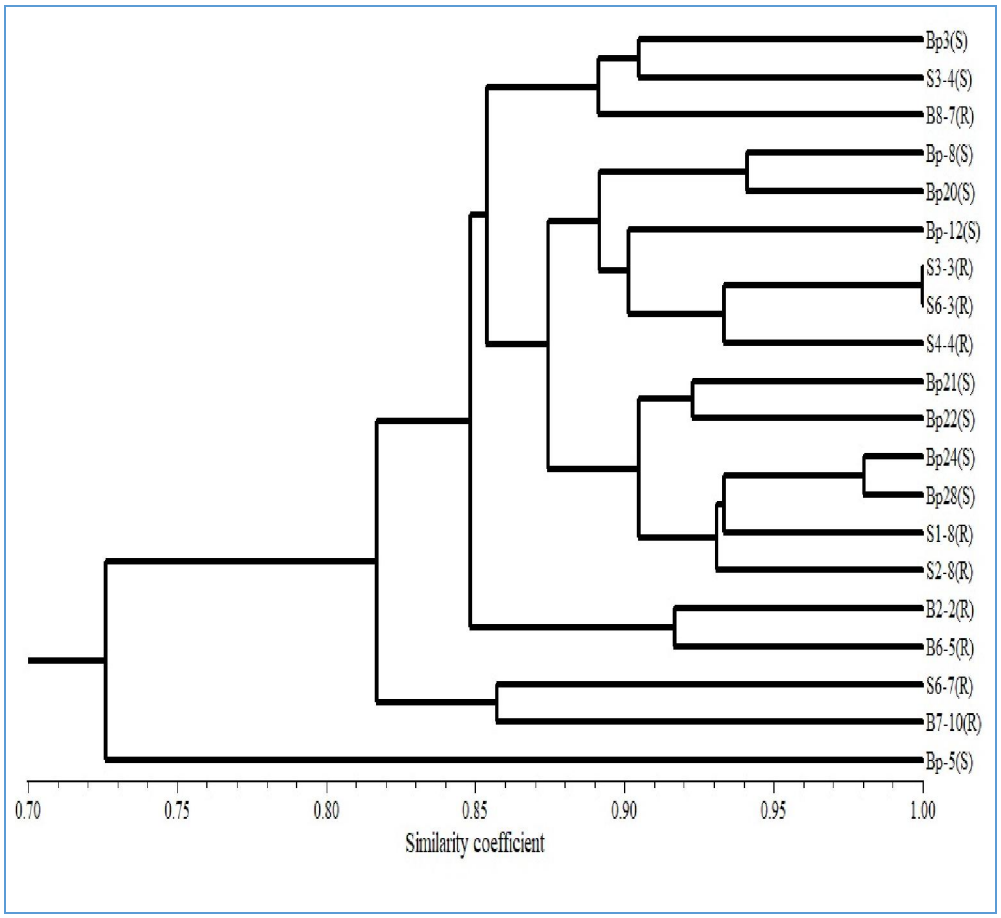


Fig 13: UPGMA based dendrogram showing relationship between fungicide 'sensitive' and 'resistant' strains of *V. inaequalis* using ISSR primers

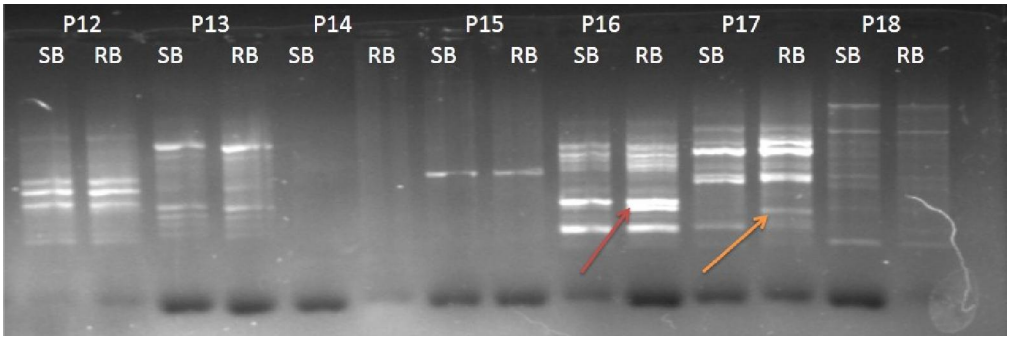


Plate 7: DNA amplification profiles of sensitive and resistant bulk isolates of *Venturia inaequalis* with RAPD primers

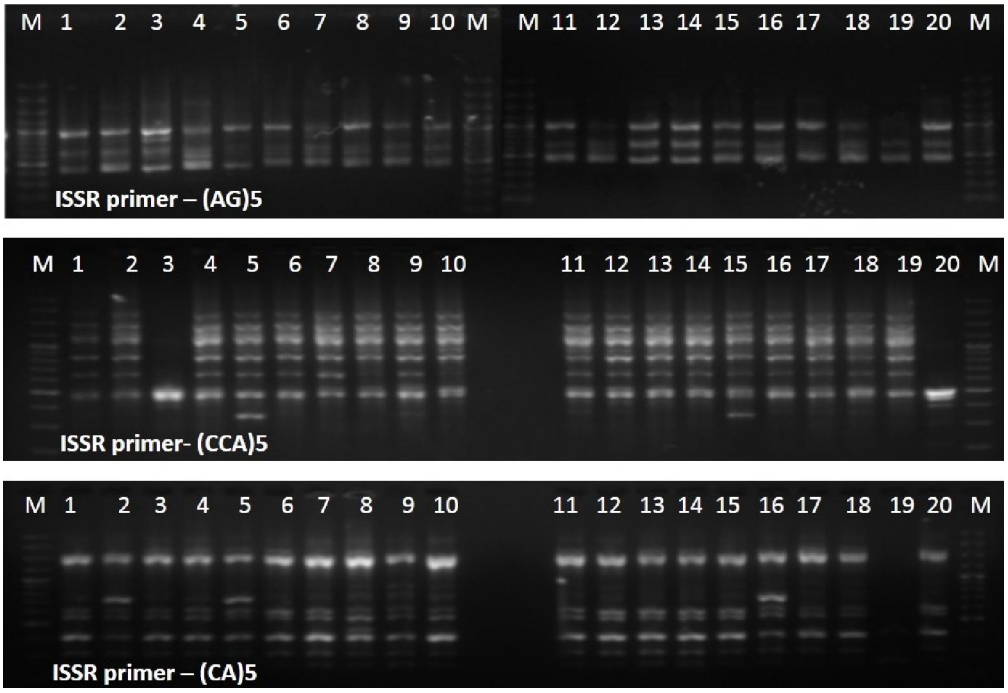


Plate 8: ISSR profiles of sensitive and resistant isolates of *Venturia inaequalis*

Chapter - 5

DISCUSSION

Apple scab is an economically important disease of apple, and its management relies upon 6 to 12 fungicidal sprays. The major fungicides presently in use for apple scab control in Kashmir include protectants (mancozeb, captan, zineb, dodine etc.) and curatives (DMIs and strobilurins). However, DMIs constitute the most important group because of their efficient control of other foliar diseases of apple as well. Such fungicides have been used increasingly for the past two decades to combat apple scab. Such a long history of fungicide use coupled with the reports of reduced sensitivity to fungicides in some orchards necessitated the study of development of fungicide resistance in *Venturia inaequalis*. The preliminary sensitivity studies of *V. inaequalis* populations at few locations to myclobutanil, hexaconazole and fenarimol was carried out few years back (Fatima, 2008; Kacho *et al.*, 2013). However, no information was available regarding the status of sensitivity of *V. inaequalis* to different fungicides from the other major apple growing districts of Kashmir. In order to devise resistance management strategies and increase the use-life of different fungicides, the present study was initiated to discern the sensitivity levels of *V. inaequalis* populations to different fungicides being used in Kashmir for the management of various apple diseases especially scab.

For any fungicide resistance monitoring programme, knowledge of baseline sensitivity holds the key. Baseline is a sensitivity profile of a target fungus constructed by using biological techniques to assess the response of unexposed fungal populations to the fungicide (Russell, 2004). Baseline doesn't represent a single data point but is constructed by sampling a number of individuals from a population, and establishing variability between them. The main aim of using baseline is to establish a reference point for fungal sensitivity to a fungicide to monitor the shifts in fungicide exposed populations from that of the baseline population (Koller *et al.*, 1999). Secondly, it is used to determine a

discriminatory dose of a fungicide to monitor the shifts in exposed populations and to classify the test samples as 'resistant' or 'sensitive'.

In the present study, the baseline sensitivity of *V. inaequalis* to dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole was established and the respective discriminatory doses determined. ED_{50} value of dodine for *V. inaequalis* baseline isolates varied from 0.020 to 0.457 µg ml^{-1} with a mean value of 0.14µg ml^{-1}. The mean value closely resembles that of baseline sensitivity of New York orchard (ED_{50} value of 0.17 µg ml^{-1}), but the range of values was broader (0.024 to 1.20 µg ml^{-1}) than that observed in the present study. This may be due to the fact that the sample size in present study was comparatively smaller providing the likelihood of exclusion of the isolates having higher ED_{50} values. In the present study, the mean baseline ED_{50} value of hexaconazole was 0.036µg ml^{-1}. Fatima (2008) reported that ED_{50} values for *V. inaequalis* populations from Shalimar, Zakura and Kunnil were 0.0061, 0.0233 and 0.0110 µg ml^{-1}, respectively. The discrepancy between ED_{50} values for the isolates from different locations is attributed to difference in population composition (Xu *et al.*, 2010). The ED_{50} values of myclobutanil for *V. inaequalis* baseline isolates recorded in the present studies varied from 0.016 to 0.501 µg ml^{-1} with a mean value of 0.17 µg ml^{-1} which was very close to that (0.07 µg ml^{-1}) obtained by Koller *et al.* (1991) in New York using 300 isolates; the slight variation in the values can be attributed to variation in sample size. The ED_{50} values of difenoconazole for *V. inaequalis* baseline isolates observed in the present study varied from 0.002 to 0.137 µg ml^{-1} with mean value of 0.02 µg ml^{-1}. It is in close resemblance with the value (0.017 µg ml^{-1}) determined for 50 Chilean baseline isolates by Jose Luis Henriquez *et al.* (2011). However, the mean ED_{50} value was considerably lower in the studies conducted by Villani *et al.* (2015). It indicates that the baseline sensitivity of the pathogen may vary from region to region. The mean baseline ED_{50} value of difenoconazole in this study also departed from the value (0.09 µg ml^{-1}) reported by Kunz *et al.* (1997) which is due to the fact that they used *in vivo*

assays and then carried out microscopic evaluation of conidiophore development instead of *in vitro* mycelial growth assays. The ED_{50} values of *V. inaequalis* baseline isolates to flusilazole ranged from 0.002 to 0.079 µg ml^{-1} with mean value of 0.022 µg ml^{-1} which was close to the ED_{50} value of 0.008 µg ml^{-1} determined in New York (Koller *et al.*, 1991; Smith *et al.*, 1991). The baseline sensitivity values derived from 30 baseline isolates in this study closely resemble the values determined by Koller *et al.* (1991) for 300 baseline isolates, indicating that the isolates collected and assayed truly represented the baseline population. However, much higher ED_{50} value of 0.09 µg ml^{-1} for flusilazole was reported by Thind *et al.* (1986) from France.

Apart from ED_{50} values, ED_{90} and the MIC values of different fungicides for baseline isolates were also determined. ED_{90} values for dodine ranged from 3.1 to 31.6 µg ml^{-1} and the MIC values ranged from 7.0 to 79.4 µg ml^{-1}. For hexaconazole, ED_{90} values varied from 0.1 to 5.0 µg ml^{-1} and the MIC values ranged from 1.0 to 14.0 µg ml^{-1}. Similarly, ED_{90} values varied from 1.5 to 39.8 µg ml^{-1}, 0.4 to 4.7 µg $ml^{-1}$and 0.5 to 3.9 µg ml^{-1}, respectively in case of myclobutanil, difenoconazole and flusilazole. The MIC values for myclobutanil, difenoconazole and flusilazole ranged from 4.7 to 100.0 µg ml^{-1}, 1.9 to 11.2 µg ml^{-1} and 1.9 to 10.0 µg ml^{-1}, respectively. However, ED_{50} values are considered more precise than ED_{90} and MIC values (Brent, 1992; Smith *et al.*, 1991), and were therefore considered for subsequent experiments.

To draw the overall inferences regarding the sensitivity of *V. inaequalis* baseline population, frequency distributions of ED_{50} values were constructed, and the mean ED_{50} values and the resistance factors for different fungicides were calculated. The frequency distributions of ED_{50} values of each fungicide were lognormal as the curves were skewed. The curves indicated that there are many smaller values (lower ED_{50} values) and fewer larger values (higher ED_{50} values); hence, less sensitive isolates had very low frequency. Therefore, lognormal distribution of ED_{50} values is one of the characteristic features of baseline

population (Smith *et al.*, 1991). The mean ED_{50} values of flusilazole and difenoconazole were lower than that for hexaconazole which was in turn lower than that for myclobutanil and dodine indicating that flusilazole and difenoconazole have the highest intrinsic activity followed by hexaconazole, dodine and myclobutanil. Despite having similar mode of action, difference in intrinsic activities of different DMIs may be due to the fact that individual molecules have different chemical formulae and structures. Therefore, they have different physico-chemical properties and dose response curves. Previous reports have also illustrated the fact that DMIs vary in their intrinsic activity against *V. inaequalis* and other pathogens as well (Koller *et al.*, 1991; Elcock *et al.*, 2000; Wong and Midland, 2007). The lower values of resistance factors were observed in the present study for myclobutanil (3.3), dodine (3.3) and flusilazole (3.5), whereas comparatively higher values were observed for difenoconazole (6.9) and hexaconazole (4.7). The fungicides with higher values of resistance factors are indicative of their wider range (greater difference between mean and the highest ED_{50} value) of sensitivities and vice-versa. Therefore, resistance build up in pathogen isolates is evidently rapid for dodine, myclobutanil and flusilazole, and more gradual for difenoconazole and hexaconazole. This is because the proportion of less sensitive individuals shifting at a given time of selection pressure beyond a threshold level might be higher for a fungicide with narrow sensitivity distribution (Koller *et al.*, 1991). However, the impact of resistance factors is hard to predict especially in baseline populations with less sample size as individuals with higher ED_{50} values may exist in a population but are too infrequent to be identified in a small sample size.

To monitor the shift in sensitivity of fungicide-exposed populations of *V. inaequalis*, relative mycelial growth assays using discriminatory doses is a simplified test (Koller *et al.*, 1991). Discriminatory dose is a dose rate of a particular fungicide, determined based on baseline sensitivity, and helps in categorising pathogen response to a fungicide as ‘sensitive’ or ‘resistant’.

Therefore, the discriminatory dose for each fungicide was determined by selecting a dose slightly higher than the corresponding mean ED_{50} value. The discriminatory doses taken for the present studies for dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole were 0.20, 0.04, 0.20, 0.03 and 0.03 μg ml^{-1}, respectively. Subsequently, sensitivities in fungicide-exposed orchard populations of *V. inaequalis* to different fungicides were monitored by testing the pathogen isolates at a single discriminatory dose for each fungicide. Varied response in terms of relative growth of the pathogen isolates to different fungicides at respective discriminatory doses was recorded to ascertain shift in their sensitivities, if any. The frequency distributions of sensitivities of fungicide-exposed orchard populations of the pathogen to dodine from district Baramulla and Shopian differed from that of baseline population, and the frequencies of less sensitive isolates had increased. The shift is obvious, as dodine has been in use continuously for at least 10 years in the orchards monitored. The shift in dodine sensitivity, however, had been much more in New York (Koller *et al.*, 1999), where it served as an exclusive fungicide for scab control for more than ten years. The use of dodine in alternation with other fungicides in Kashmir valley might have compensated for the risk of practical resistance development. Furthermore, orchardists in Kashmir employ a single spray of dodine at pink bud stage, when the disease pressure in field is very less. Hence, lesser proportion of population is exposed to this fungicide. Several researchers have also reported reduced dodine sensitivity in dodine sprayed orchards as compared to baseline populations in New York, Canada and Poland (Ross and Newbery, 1977; Koller *et al.*, 1999; Jobin and Carisse, 2007; Broniarek-Niemiec, and Bielenin, 2008; Msezka *et al.*, 2008), thereby corroborating the data generated in the present investigation.

The frequency distribution of the sensitivities exhibited by the pathogen isolates exposed to hexaconazole from both the districts did not differ much from that exhibited by the isolates of the baseline population. Fatima (2008) also found similar distribution patterns in baseline and exposed orchard populations, and only

a small proportion (2-6%) of the isolates had RG values greater than 80, indicating thereby that hexaconazole must be providing an efficient scab control inspite of its continued and widespread use over the years. There are no such reports of resistance build-up in *V. inaequalis* to hexaconazole in particular. It indicates that resistance mechanism in *V. inaequalis* to hexaconazole may differ from that for other DMIs and the isolates with such resistance mechanism are less frequent in *V. inaequalis* populations in Kashmir valley.

A major shift towards reduced sensitivity in *V. inaequalis* populations to myclobutanil was observed in both the districts during the present study. Myclobutanil has been used in selected orchards for about 5-15 years, an adequate period for evolution of increased frequencies of less sensitive isolates. Among all the DMIs, major shift in myclobutanil sensitivity and widespread myclobutanil resistance development in *V. inaequalis* as reported during the present study has also been reported in various other countries (Koller *et al.*, 1997; Pfeufer, 2010; Beresford *et al.*, 2012; Villani *et al.*, 2015). It is, therefore, speculated that the resistance mechanism against myclobutanil is different from that against other DMIs (Villani *et al.*, 2015), and the isolates with such resistance mechanism are more frequent in natural populations of *V. inaequalis*.

The frequency distributions of difenoconazole sensitivity of fungicide-exposed orchard populations of *V. inaequalis* differed slightly form that of baseline population in both the districts. Difenoconazole is being used for apple scab control for about more than eight years in Kashmir. However, the shift in sensitivity to difenoconazole is less compared to myclobutanil. The scarcity of less sensitive isolates in *V. inaequalis* baseline population in Kashmir valley, as reflected by lesser value of resistance factor may have contributed to delayed resistance build-up in case of difenoconazole as has also been argued by Villani *et al.* (2015).

The frequency distributions of flusilazole sensitivity of fungicide-exposed *V. inaequalis* orchard populations from both the districts showed an evident shift

from that of baseline population. A marked shift in flusilazole sensitivity was observed compared to other DMIs except for myclobutanil where the shift was more pronounced. Flusilazole has been introduced in apple scab spray schedule for about four or five years back. However, the recorded shift in sensitivity in a short period might have occurred due to the reason that DMI applications (myclobutanil in particular) over the years predisposed *V. inaequalis* isolates to preferential selection for resistance to another fungicide (Koller and Wilcox, 2001). In other words, the the isolates less sensitive to myclobutanil are more prone to develop resistance to other fungicides such as flusilazole. This phenomenon occurs due to complete or partial cross-resistance among fungicides, which is in turn possible due to similarity in resistance mechanisms for different fungicides (Koller and Wilcox, 2001).

In order to assess the frequency of resistant isolates in *V. inaequalis* populations in Kashmir, the isolates from both the districts were classified into three categories *viz.*, 'sensitive', 'shifted' and 'resistant' to different fungicides as per the criteria adopted by Chapman *et al.*(2011), and the results revealed that the number of dodine-'resistant' isolates was neglegible. Although, the mode of action of dodine is not well established, it is a multi-site inhibitor having less chances of resistance development (FRAC, 2017). Maximum number of 'resistant' isolates was observed for myclobutanil (35.5%) followed by that for flusilazole (19%), though the number of 'resistant' isolates were comparatively less in case of flusilazole. The isolates 'resistant' to difenoconazole and hexaconazole comprised only a small percentage of the total population. Various researchers have reported widespread resistance in *V. inaequalis* populations to myclobutanil as compared to other DMIs from various countries (Koller *et al.*, 1997; Jobin and Carisse, 2007; Pfeufer and Ngugi, 2010; Villani *et al.*, 2015). Resistance build-up to this fungicide in *V. inaequalis* populations in Kashmir can be explained by the extent of myclobutanil usage, as it has been available for scab control in Kashmir valley for the last 15 years or more. Despite similar modes of

action of DMIs, preponderant resistance to myclobutanil may be due to the phenomenon of occurrence of differential resistance mechanisms (Villani *et al.*, 2015). Development of resistance to myclobutanil is supposed to have different mechanism than that to difenoconazole (Villani *et al.*, 2016). Despite recent introduction into the scab control spray schedule, flusilazole-'resistant' isolates comprised greater percentage of the total population than did the difenoconazole and hexaconazole-'resistant' isolates. It may be due to the fact that flusilazole may have partial cross-resistance to myclobutanil (Jobin and Carisse, 2007), and that myclobutanil 'resistant' isolates were pre-disposed to flusilazole resistance. Hence, it can be concluded that different resistance mechanisms operate in *V. inaequalis* against different DMIs.

As far as district-wise distribution of isolate categories is concerned, the isolates sensitive to dodine were compared to those for other fungicides, the sensitive isolates comprising 38 and 48 per cent of the total population from district Baramulla and Shopian, respectively; none of the isolates was found 'resistant' to dodine in district Baramulla and only a single isolate was dodine-'resistant' in district Shopian. The development of resistance to dodine has been reported from various countries (Bakker, 1999; Koller *et al.*, 1999; Meszka, and Bielenin, 2001; Broniarek-Niemiec and Bielenin, 2008; Carisse and Jobin, 2010) where dodine served as an exclusive option for apple scab control. In the present study, percentage of dodine 'resistant' isolates was negligible despite the fact that the fungicide is being used for more than 20 years. Firstly, the population composition of *V. inaequalis* in Kashmir valley may differ from that of other countries. Secondly, dodine is used in alternation with other fungicides and usually sprayed during early growth stages (pink bud stage), resulting in a small proportion of population being subjected to selection pressure. Chapman *et al.* (2011) also found that dodine 'resistant' isolates comprise small percentage of population in Indiana compared to the other states like Michigan and New York.

In case of hexaconazole, most of the pathogen isolates were identified as

'sensitive' and few as 'resistant'. Despite the use of hexaconazole over years in time and space, resistance build-up in the population has progressed at slower rate. Hence, hexaconzole can still provide an optimum level of disease control. The percentage of 'resistant' isolates was slightly greater in district Baramulla (12%) compared to that in district Shopian (8%), probably due to differences in population composition (Xu *et al.*, 2010).

A greater percentage of isolates was found 'resistant' to myclobutanil in both the districts and most of the isolates were identified as 'shifted'. Myclobutanil resistance in *V. inaequalis* is prevalent in various other apple growing regions like Michigan, Indiana, Virginia, Nova Scotia, Pennsylvania and Quebec (Koller *et al.*, 1997; Jobin and Carisse, 2007; Marine *et al.*, 2007; Chapman *et al.*, 2011; Pfeufer and Ngugi, 2012) as well, and even practical resistance from various orchards has been reported for myclobutanil (Villani *et al.*, 2015). Pfeufer (2010) opines that the fungicide is one of the oldest DMI used to manage apple scab, and hence the populations are well adapted to this molecule. However, the fungicide has not been in use in Kashmir over wider areas and for longer periods as compared to other DMIs especially hexaconazole. It is therefore evident that the molecule has inherent attributes to easily lead to resistance development in the pathogen isolates. The fungicide may therefore, be used with caution and its use reduced for a certain period to avoid the development of practical resistance in Kashmir valley.

In case of difenoconazole, 'resistant' isolates comprised only 7 and 8 per cent of the Baramulla and Shopian populations, respectively. Shift in sensitivity to difenoconazole has been reported previously, but practical resistance to difenoconazole is not widespread (Kunz *et al.*, 1997; Villani *et al.*, 2015). Therefore, difenoconazole provides an optimum control of apple scab even after continued use of several years. Hence, its use can be continued in future also.

For flusilazole, most of the pathogen isolates were identified as 'shifted' and smaller percentage found 'resistant' (16% from district Baramulla and 20%

from district Shopian). Resistance development against flusilazole has been reported previously elsewhere as well (Stevic *et al.*, 2010); however, the resistance to the fungicide in pathogen populations in Kashmir is comparatively less, probably because the fungicide has been introduced in apple scab spray schedule recently and has not, therefore, been in use for such a considerable period as to pave way for evolution of resistant isolates of the pathogen.

Distribution of 'sensitive', 'shifted' and 'resistant' isolates to different fungicides varied amongst orchards as well. Dodine resistance was negligible in both the districts; however, the 'shifted' isolates ranged from 10 to 90 per cent in orchards of district Baramulla and 10 to 100 per cent in district Shopian. Hexaconazole-'resistant' isolates ranged from 0 to 20 and 0 to 40 per cent, respectively in orchards of baramulla and Shopian. Likewise, myclobutanil-'resistant' isolates ranged from 10-80 per cent in fungicide-exposed orchards of both the districts. Difenoconazole-'resistant' isolates of *V. inaequalis* were less prevalent (0-20%) in orchards of both the districts, whereas isolates 'resistant' to flusilazole ranged from 0 to 60 and 0 to 40 per cent in orchards of district Baramulla and Shopian, respectively. The variation in the number of 'resistant', 'shifted' and sensitive isolates in the *V. inaequalis* populations of different orchards is because of the variation in continuous use of fungicides in these orchards and the level of adherence to the recommended spray schedule. The orchards which are continuously sprayed with particular fungicide for a considerable number of years, and where the sprays are made two or more times in a season without alternation with other fungicides are likely to lead to development of 'resistant' or 'shifted' isolates. Pfeufer and Ngugi (2012) also reported that management factors have a significant impact on the occurrence of the 'resistant' isolates.

Cross-resistance across different fungicides limits growers' chemical options for pathogen control, and results in control failures with all the fungicides having similar mode of action or resistance mechanism. All the pathogen isolates,

therefore, were assessed for cross-resistance. About 42 per cent of 'resistant' isolates were 'resistant' to myclobutanil only. High level of cross-resistance was found between myclobutanil and flusilazole with 21 per cent of isolates simultaneously 'resistant' to both the fungicides. Jobin and Carisse (2007) has also reported cross-resistance between myclobutanil and flusilazole in case of *V. inaequalis*, thereby concurred the findings of the present study. Cross-'resistant' isolates among other set of fungicide combinations were comparatively less. Despite similar mode of action, variation in cross-resistance patterns among DMIs is probably due to differential resistance mechanism for different molecules. Villani *et al.* (2016) reported that over expression of *CYP51A1* gene is responsible for difenoconazole resistance in *V. inaequalis*, but this mechanism is not a primary genetic determinant of myclobutanil resistance, thus providing an evidence of differential resistance mechanism(s) among DMIs against *V. inaequalis*. Furthermore, cross-resistance patterns among DMIs are not consistent but vary with respect to different pathogens (Reynolds *et al*., 1997; Karaoglandis and Thanassoulopoulos, 2003; Mavroedi and Shaw, 2005).The present studies thus implicate that the myclobutanil and flusilazole may have similar resistance mechanism and other DMI's i.e., hexaconazole and difenoconazole have different resistance mechanisms. Hence, cross-resistance patterns among all sets of DMIs in case of *V. inaequalis* need to be further investigated to devise strategies that would hinder resistance development in the pathogen populations.

Conventional mycelial growth assays are believed to be labour-intensive and time-consuming; however molecular biological techniques can help in rapid detection of resistant strains, and would provide better understanding of the underlying resistance mechanism. In order to discriminate 'resistant' isolates from the 'sensitive' ones, PCR based RAPD and ISSR markers were used as a tool. Studies with PCR based RAPD molecular markers revealed that 'resistant' bulk and 'sensitive' bulk samples could be differentiated with only two RAPD primers (P16 and P17); rest of the primers could not differentiate them. Therefore, the

band that differentiates 'resistant' bulk from that of 'sensitive' bulk can be eluted and sequenced to characterize it. Although, analysis based on ISSR markers could group the 'sensitive' and 'resistant' isolates separately to some extent, these markers didn't yield a band that could differentiate between 'resistant' and sensitive isolates. Hence, these are not suitable markers to differentiate between DMI 'sensitive' and 'resistant' isolates. Different mechanisms have been proposed for development of DMI resistance in various pathogens (Delye *et al.*, 1997; Delye *et al.*, 1998; Hamamoto *et al.*, 2000 and Schnabel and Jones, 2001). These include mutations within the target *CYP51A1* gene (Albertini *et al.*, 2003) and over expression of *CYP51A1* gene (Hamamoto *et al.*, 2000; Schnabel and Jones, 2001; Villani *et al.*, 2016). Over-expression of energy dependant ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters encoding drug efflux pumps is another mechanism (De Ward *et al.*, 1987). In *V. inaequalis*, over-expression of *CYP51A1* gene is reported as a mechanism for development of resistance to difenoconazole (Villani *et al.*, 2016). Therefore, a focussed study on characterisation and level of expression of *CYP51A1* gene in 'resistant' isolates is essentially required to unravel the facts. Moreover, the membrane transport system responsible for the active exclusion of fungicides from fungal cells has as well been described as a potential mechanism that could confer multi-fungicide resistance in pathogen isolates (De Waard *et al.*, 1996; De Waard, 1997). Therefore, studies oriented towards the transporters encoding efflux pumps are necessary to characterise the isolates showing resistance (multiple resistance) to different fungicides.

Chapter - 6

SUMMARY AND CONCLUSION

Apple scab caused by *Venturia inaequalis* is one of the major diseases of apple and a major threat to horticulture industry in Jammu and Kashmir since 1973. Reliance for its management on various protectant and curative fungicides during the past few decades has created an apprehension that resistance to different fungicides might have developed in *V. inaequalis* populations in Kashmir valley. Indiscriminate use and the reports of reduced efficacy of different fungicides necessitated the monitoring of fungicide resistance in *V. inaequalis* populations to various fungicides namely dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole in Kashmir valley to modify the apple scab spray schedule and to develop resistance management strategies to increase the durability of management capsules.

Thirty isolates of *V. inaequalis* were collected from an orchard in Baramulla never exposed to fungicides and other 200 isolates from fungicide-exposed orchards of district Baramulla and Shopian (10 isolates per orchard from 10 orchards from each district). The history of fungicide usage for each orchard obtained from respective orchardists revealed that fungicides namely bitertanol, captan, mancozeb, zineb, carbendazim, dodine, hexaconazole, myclobutanil, difenoconazole, flusilazole, fenarimol and propioneb were commonly used in both the districts. The total number of fungicidal sprays in a single growing season varied from 5 to 10 in both the districts. Only three orchardists from Baramulla and two from Shopian followed recommended schedule of fungicide application. The number of years of continuous use of fungicides varied from 5 to 15 in case of Baramulla, and 5 to 14 in Shopian. Moreover, orchardists experienced diminished level of disease control in orchards having prolonged fungicide usage.

In order to monitor sensitivity levels in fungicide-exposed orchard populations, baseline sensitivity of *V. inaequalis* (with 30 baseline isolates) was

established for different fungicides, by testing the isolates separately at seven concentrations of these fungicides. The ED_{50}, ED_{90} and MIC values of dodine for *V. inaequalis* baseline isolates ranged from 0.020 to 0.457, 3.1 to 31.6 and 7.0 to 79.4 μg ml^{-1}, respectively. In case of hexaconazole, ED_{50} values ranged from 0.001 to 0.169 μg ml^{-1}, ED_{90} values varied from 0.1 to 5.0 μg ml^{-1} and MIC values varied from 1.0 to 14.0 μg ml^{-1}. The ED_{50}, ED_{90} and MIC values of myclobutanil for *V. inaequalis* baseline isolates ranged from 0.016 to 0.501, 1.5 to 39.8 and 4.7 to 100.0 μg ml^{-1}, respectively. In case of difenoconazole, ED_{50} values for *V. inaequalis* baseline isolates ranged from 0.002 to 0.137 μg ml^{-1}, ED_{90} values from 0.4 to 4.7 μg ml^{-1} and MIC values from 1.9 to11.2 μg ml^{-1}. The ED_{50}, ED_{90} and MIC values of flusilazole for *V. inaequalis* baseline isolates ranged from 0.002 to 0.079, 0.5 to 3.9 and 1.9 to 10.0 μg ml^{-1}, respectively. The frequency distributions of ED_{50} values of all the fungicides were log normal and the mean ED_{50} values of dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole were 0.14 μg ml^{-1}, 0.036 μg ml^{-1} 0.17 μg ml^{-1}, 0.02μg ml^{-1} and 0.022 μg ml^{-1}, respectively. The resistance factors of 3.3, 4.7, 3.3, 6.9 and 3.5 were recorded for dodine, hexaconazole myclobutanil, difenoconazole, flusilazole, respectively. Based on their respective mean ED_{50} values, the discriminatory doses of 0.20, 0.04, 0.20, 0.03 and 0.03 μg ml^{-1} were selected for dodine, hexaconazole, myclobutanil, difenoconazole and flusilazole, respectively.

To monitor the shift in fungicide sensitivity fungicide-exposed *V. inaequalis* orchard populations of both the districts, all the pathogen isolates were tested for their sensitivities at respective discriminatory doses of different fungicides, and the frequency distributions of relative growth (RG) values of fungicide-exposed *V. inaequalis* orchard isolates compared to those of the baseline isolates. A major shift in myclobutanil sensitivity followed by flusilazole sensitivity was observed in fungicide-exposed pathogen populations in both the districts. However, the frequency distribution of RG values of the fungicide-

exposed populations of *V. inaequalis* exhibited slight variation from that of the baseline population in case of dodine, hexaconazole and difenoconazole.

All the two hundred *V. inaequalis* isolates collected from the fungicide-exposed orchards were classified as 'sensitive', 'shifted' and 'resistant' based on RG values (Chapman *et al.*, 2011). The highest proportion of isolates (35.5%) exhibited 'resistant' response to myclobutanil followed by that (19.5%) for flusilazole. However, minimum proportion of isolates (0.5%) showed 'resistant' response to dodine.

Resistance of *V. inaequalis* isolates to different fungicides was detected at varying frequencies in both the districts. The resistance to myclobutanil was predominant in both the districts and the isolates exhibiting 'resistant' response comprised 32 and 39 per cent of the total population in district Baramulla and Shopian, respectively. After myclobutanil, greater proportion of isolates exhibited 'resistant' response to flusilazole and such isolates comprised 17 and 22 per cent of the total population, respectively, in district Baramulla and Shopian. However dodine-'resistant' isolates were negligible in both the districts and such isolates comprised only 1 per cent of the total population in district Shopian.

The pathogen isolates from various orchards varied in their responses to different fungicides. In district Baramulla, none of the isolates exhibited 'resistant' response to dodine, whereas a single isolate exhibiting 'resistant' response was observed in orchard S_2 of district Shopian. In case of hexaconazole, the highest proportion (40%) of 'resistant' isolates was found in orchard B_7 of district Baramulla, and hexaconazole-'resistant' isolates comprised 20 per cent of the total isolates in orchards S_1 and S_6 of district Shopian. Resistance to myclobutanil in *V. inaequlais* isolates was prevalent in both the districts. In district Baramulla, the highest percentage of myclobutanil-'resistant' isolates (80%) was observed in orchard B_2, and 70 per cent of myclobutanil-'resistant' isolates was found in orchard S_3 of district Shopian. Difenoconazole resistance was less prevalent among orchards in both the districts. In district Baramulla, the

highest percentage (20%) of difenoconazole-'resistant' isolates was observed in orchards B_6 and B_7, whereas 20 per cent of the 'resistant' isolates was observed in orchard S_6 of district Shopian. In case of flusilazole, the highest percentage (60%) of 'resistant' isolates was observed in orchard B_7 of district Baramulla, whereas the highest proportion (40%) of flusilazole-'resistant' isolates was found in orchards S_3, S_5 and S_6 of district Shopian.

All the *V. inaequalis* isolates collected from district Baramulla and Shopian when assessed for cross-resistance revealed a high level of cross resistance between myclobutanil and flusilazole, with 21% of isolates 'resistant' to both the fungicides, whereas cross-'resistant' isolates among other set of fungicide combinations were comparatively less. However, 42 per cent of the total 'resistant' isolates were resistant to myclobutanil only.

Molecular analysis of the selected 'resistant' and 'sensitive' isolates of *V. inaequalis* using 46 RAPD primers yielded a single locus difference between 'resistant' and 'sensitive' bulks with two primers (P16 and P17) only. However, genotyping of selected sensitive and resistant isolates with ISSR markers yielded two clusters (Cluster I and II) and one independent lineage at 84 per cent similarity. Cluster I comprised of 17 isolates and cluster II comprised of two isolates, whereas the isolate Bp5 formed an independent lineage. Cluster I was further divided into four sub-clusters *viz.,* cluster Ia, Ib, Ic and Id in which resistant isolates were grouped together and sensitive isolates were grouped separately; however, no major separate clusters for 'resistant' and 'sensitive' isolates were observed.

It can thus be concluded from the present study that the fungicides like flusilazole and difenoconazole have the highest intrinsic activity than hexaconazole, myclobutanil and dodine, and the discriminatory doses of the test fungicides determined on the basis of baseline sensitivity in this study could be used in future fungicide resistance monitoring programmes. Shift in sensitivity in *V. inaequalis* populations was greater in case of myclobutanil followed by

flusilazole; therefore, myclobutanil usage must be limited or reduced for a certain period. Based on orchard-wise distribution of resistant isolates, fungicide management practices have a significant impact on resistance build up in pathogen populations. Moreover, the highest degree of cross resistance was observed between myclobutanil and flusilazole, providing an evidence of similar resistance mechanism(s); hence flusilazole should be used with caution in orchards already resistant to myclobutanil. Lack of cross resistance among all the DMIs is advantageous as the problem of resistance build up doesn't take place simultaneously with the whole class of fungicides. Molecular analysis implicates that the locus difference found with two RAPD primers needs to be characterised further; however ISSR markers are not suitable to discriminate sensitive isolates from the resistant ones. Therefore, studies on characterisation of *CYP51A1* gene in resistant isolates, *CYP51A1* gene expression and over-expression of efflux transporters need to be undertaken to determine the possible resistance mechanism(s) for different fungicides.

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*Original not seen

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Pathology

CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Prof. M. Y. Ghani, Professor (Retired) Plant Pathology, SKUAST-Kashmir during Viva-Voce examination held on 01-03-2018 have been incorporated in the manuscript entitled **"Monitoring fungicide sensitivity and resistance in *Venturia inaequalis* (Cke.) Wint. Causing apple (*Malus* × *domestica*) scab in Kashmir"** submitted by **Ms. Asha Nabi (Regd. No. 2013-479-D).**

Prof. Mushtaq Ahmad
Chairman
Advisory Committee