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Baseline Sensitivity Profiling of *Alternaria mali* Populations to Tebuconazole in Apple (*Malus sp.*), a Demethylation Inhibitor Fungicide

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Abstract

In Jammu and Kashmir, apple farming has evolved into an industry, contributing around 65% of the country's total production and serving as a cornerstone of the state's economy. However, several diseases caused by fungal pathogens significantly reduce crop yield and quality. Demethylation inhibitor fungicides viz., tebuconazole, difenoconazole, myclobutanil, hexaconazole

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and flusilazole are a key group of systemic fungicides recommended for use on apples in the valley to manage Alternaria mali by inhibiting the synthesis of ergosterol, a critical component of cell and mitochondrial membranes in the pathogen. However, the possibility of these fungicides to rapidly acquire resistance and the recent finding of decreased Tebuconazole sensitivity in Alternaria mali emphasizes the need for Alternaria mali to be tested against tebuconazole in the the valley. Thirty baseline isolates of Alternaria mali were tested at six different concentrations of tebuconazole (0.0001, 0.001, 0.01, 0.1, 1.0, and 10 µg/ml) using mycelial growth assays. BP isolates exhibited high relative growth at lower fungicide doses (65.9–95.9% at 0.0001 µg ml^{-1}), which declined progressively with increasing concentration, resulting in strong growth suppression at 10 µg ml^{-1} (1.89–34.9%). The mean EC50 value for Tebuconazole was determined to be 0.15µg/ml, and a dose of 0.20 µg/ml was chosen as the discriminatory dose for assessment of resistance in exposed population.

Keywords: Alternaria mali; baseline; demethylation; resistance development; tebuconazole.

1. Introduction

Apple (*Malus* spp., family Rosaceae) is the most widely cultivated temperate fruit globally.

India ranks 5th in apple production, with Jammu and Kashmir (J&K) contributing approximately 85% of the country's output (Anonymous, 2023a; Anonymous, 2023b). The productivity in Kashmir reached 12.47 tonnes per hectare in 2021, highlighting the region’s significance in national apple cultivation. Apples are rich in antioxidants, flavonoids, dietary fiber, and essential nutrients. Apple production and quality are significantly affected by fungal, bacterial, and viral diseases, with fungi being the most common cause (Grove et al., 2003; Nabi et al., 2020). However, apple production in J&K faces substantial losses due to various fungal diseases, among which Alternaria leaf blotch, caused by *Alternaria mali*, has emerged as a major threat. Once considered a minor disease, *Alternaria mali* gained prominence after a devastating epidemic in 2013, which caused yield losses of 40-60%. The disease is now recognized as a major foliar concern, particularly under changing climate conditions and intensive agrochemical use (Shahzad et al., 2002; Kishore and Sharma, 2005). Symptoms appear by mid-May as light brown leaf spots that coalesce into greyish lesions (Shahzad, 2003), causing defoliation, reduced tree vigor, and lower yields (Sharma, 2000). Mature fruit show sunken brown spots (Persley & Horlock, 2009) and the pathogen's conidia are ovoid with short, swollen beaks and transverse septa (Roberts, 1924). Effective management of *A. mali* primarily depends on the timely application of synthetic fungicides. Among these, tebuconazole, a systemic fungicide from the demethylation inhibitor (DMI) group, has been extensively recommended in apple orchards of Kashmir, to inhibits fungal ergosterol biosynthesis by targeting the C14-demethylase enzyme in the sterol biosynthesis pathway, ultimately leading to cell membrane disruption and fungal death. It exhibits both preventive and curative activity and is commonly applied during key infection periods. Despite its efficacy, the repeated use of tebuconazole poses a risk for the selection of resistant strains. The Fungicide Resistance Action Committee (FRAC) classifies DMIs, including tebuconazole, as moderate- to high-risk fungicides in terms of resistance development. Evidence of reduced sensitivity to DMI fungicides, including difenoconazole and myclobutanil, has already been reported in *Alternaria mali* populations from apple orchards in Kashmir (Fatima and Qazi, 2008; Nabi et al., 2022). However, to date, no comprehensive data exist on the sensitivity of *Alternaria mali* to tebuconazole, especially in the context of resistance development following prolonged exposure. This knowledge gap is significant, especially given the widespread and repeated use of tebuconazole in Kashmir’s orchards. To ensure the continued efficacy of DMI fungicides and support resistance management, baseline sensitivity studies and resistance monitoring using *In-vitro* approaches are essential. *In-vitro* assays such as mycelial growth inhibition provide quantitative insights into fungicide effectiveness. In this context, the present study evaluates the sensitivity of *Alternaria mali* to tebuconazole under *In-vitro* conditions to determine whether resistance is emerging in apple-growing regions of Kashmir.

2. Materials and Methods

2.1 Collection of Diseased Samples and Isolation of *Alternaria mali* Isolates

2.1.1 *In vitro* Baseline Sensitivity Assessment

The baseline population, consisting of thirty isolates of *Alternaria mali*, was collected from the Pattan region in Baramulla district (Table 1). This area, located at the top of the terrain, had never been exposed to fungicides due to its unique geographical features. To confirm the pathogenicity of the baseline isolates of *Alternaria mali*, Koch postulates was employed by detached leaf inoculation method under aseptic lab conditions using spore suspension solution (Plate 1). These isolates were evaluated against DMI fungicide, Tebuconazole using *in-vitro* assays to assess effects on mycelial growth. The baseline sensitivity levels of the pathogen populations were evaluated at six concentrations *viz.,* 10.0, 1.0, 0.1, 0.01, 0.001 and 0.0001 µg ml^{-1} of this fungicide using poison food technique (Nene and Thapliyal, 2002). Baseline sensitivity levels of the pathogen (*Alternaria mali*) to Tebuconazole was determined using conventional mycelial growth assays. Tebuconazole was dissolved in water and dispensed into lukewarm sterilized PDA. A 0.1% stock solution (1000 µg/ml) was prepared and the required test concentrations were obtained by serial dilution. Mycelial discs of 5mm diameter of each pathogen isolate were placed at the centre of Petri plates containing PDA medium amended with required fungicide concentrations with three replications and control plates (without fungicide) for each isolate using completely randomised design. Inoculated Petri-plates were incubated at 25ºC and the radial growth was measured after 5 days.

2.1.2 Data Analysis

Percent relative growth (RG) for each isolate was calculated according to Chapman *et al*. (2011) using the following formula

RG (%) = (Mean radial growth on fungicide-amended medium / Mean radial growth on unamended medium) × 100

EC_{50} values were calculated using linear regression analysis of dose-response data.

Table 1. Details of the Isolates of *Alternaria mali* isolates collected from baseline orchards of district Baramullah (Pattan)

District	Location (Village)	Latitude (N)	Longitude (E)	Isolate code	No. of isolates
Baramulla	(Pattan)	34.161°	74.556°	BP1-BP30	30

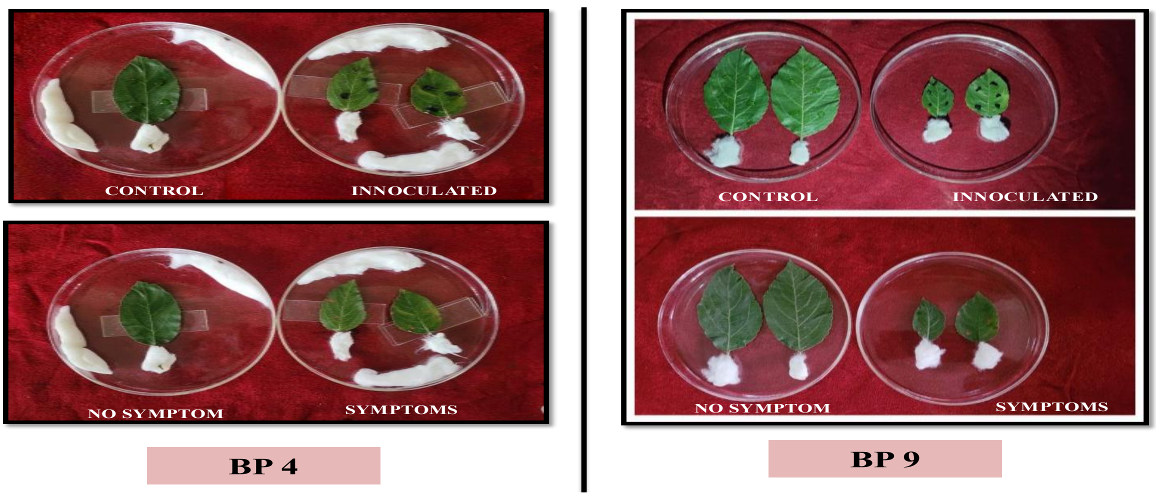

Plate 1. Pathogenicity Assessment of Alternaria mali isolates on Apple Leaves (Detached Leaf Assay)

3. Results and Discussion

Apple diseases, including those caused by *Alternaria mali*, are major threats to apple orchards and typically require 6-12 fungicide sprays per season for its management. In Kashmir, control of this disease depends mainly on protectant fungicides such as mancozeb, captan and dodine, along with curative fungicides, particularly DMIs and strobilurins. Among these, DMIs are widely used because of their broad-spectrum efficacy against several apple foliar diseases. Their intensive and prolonged use over the past two decades, together with reports of reduced field sensitivity, has raised concerns about the possible development of fungicide resistance in *Alternaria mali*. Therefore, the present study was undertaken to assess the baseline sensitivity of 30 *Alternaria mali* isolates and to calculate the mean ED_{50} value for determining a discriminatory dose for resistance monitoring in exposed populations. In the present study, baseline isolates of *Alternaria mali* exhibited variable sensitivity to tebuconazole across different concentrations (Table 2). Thirty baseline isolates exhibited high relative growth at lower fungicide concentrations, ranging from 65.9% (BP9) to 95.9% (BP12) at 0.0001 μg ml^{-1} and from 53.7% (BP9) to 88.7% (BP6) at 0.001 μg ml^{-1}. Relative growth declined progressively with increasing concentration, varying from 47.6% (BP9) to 83.8% (BP24) at 0.01 μg ml^{-1} and from 37.8% (BP20) to 79.6% (BP12) at 0.1 μg ml^{-1}. At 1 μg ml^{-1}, growth was markedly reduced (12.5% in BP22 to 50.9% in BP11), while severe inhibition was observed at 10 μg ml^{-1} (1.89% in BP5 to 34.9% in BP8) (Table 2). The EC_{50} values ranged from 0.017 to 0.371 μg ml^{-1}, with the lowest value (0.017 μg ml^{-1})

Table 2. *In vitro* response of thirty *Alternaria mali* isolates to six concentrations of tebuconazole

Isolates / Conc. (μg ml^{-1})	Percent relative growth at different concentrations (RG)					
	10	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
BP1	31.03	41.38	58.62	67.24	75.86	93.1
BP2	15.6	24.4	42.2	77.8	86.7	95.6
BP3	7.7	28.2	74.4	79.5	84.6	94.9
BP4	2.2	41.3	50.0	63.0	82.6	87.0
BP5	1.89	49.06	73.58	81.13	88.68	94.34
BP6	12.7	36.6	54.9	64.8	67.6	88.7
BP7	5.3	15.8	44.7	55.3	63.2	89.5
BP8	34.9	34.9	53.5	65.1	72.1	81.4
BP9	17.1	26.8	42.7	47.6	53.7	65.9
BP10	11.8	14.7	61.8	64.7	79.4	91.2
BP11	21.1	50.9	64.9	75.4	77.2	91.2
BP12	8.2	24.5	79.6	81.6	87.8	95.9
BP13	18.8	40.6	53.1	68.8	78.1	84.4
BP14	25.5	30.9	50.9	63.6	76.4	87.3
BP15	12.1	33.3	51.5	78.8	87.9	93.9
BP16	21.7	46.7	53.3	65.0	68.3	73.3
BP17	8.0	16.0	52.0	72.0	80.0	92.0
BP18	16.7	26.2	52.4	61.9	76.2	85.7
BP19	19.1	25.5	63.8	66.0	68.1	78.7
BP20	8.1	29.7	37.8	78.4	83.8	91.9
BP21	5.1	30.8	64.1	74.4	76.9	89.7
BP22	7.5	12.5	42.5	77.5	85.0	87.5
BP23	11.9	26.2	59.5	69.0	76.2	85.7
BP24	8.1	24.3	75.7	83.8	86.5	89.2
BP25	6.3	29.2	60.4	70.8	79.2	91.7
BP26	13.7	43.1	52.9	62.7	78.4	92.2
BP27	25.6	37.2	53.5	65.1	81.4	86.0
BP28	28.3	35.8	45.3	52.8	60.4	92.5
BP29	10.2	44.9	55.1	65.3	73.5	83.7
BP30	18.8	40.6	53.1	68.8	78.1	84.4

Table 3. EC_{50} values for thirty baseline isolates of *Alternaria mali* against Tebuconazole

Isolate	EC_{50} (µg ml^{-1})	Isolate	EC_{50} (µg ml^{-1})
BP1	0.268	**BP16**	0.098
BP2	0.087	**BP17**	0.048
BP3	0.145	**BP18**	0.052
BP4	0.059	**BP19**	0.062
BP5	0.239	**BP20**	0.104
BP6	0.064	**BP21**	0.083
BP7	0.017	**BP22**	0.041
BP8	0.154	**BP23**	0.065
BP9	0.198	**BP24**	0.143
BP10	0.143	**BP25**	0.075
BP11	0.371	**BP26**	0.099
BP12	0.165	**BP27**	0.104
BP13	0.115	**BP28**	0.052
BP14	0.087	**BP29**	0.081
BP15	0.115	**BP30**	0.115

Table 4. Baseline sensitivity characteristics of thirty *Alternaria mali* isolates against Tebuconazole

Fungicide	Min EC 50 (µg/ml)	Max EC 50 (µg/ml)	Mean EC 50 (µg/ml)	Discriminatory Dose (µg/ml)
Tebuconazole	0.017	0.371	0.15	0.20

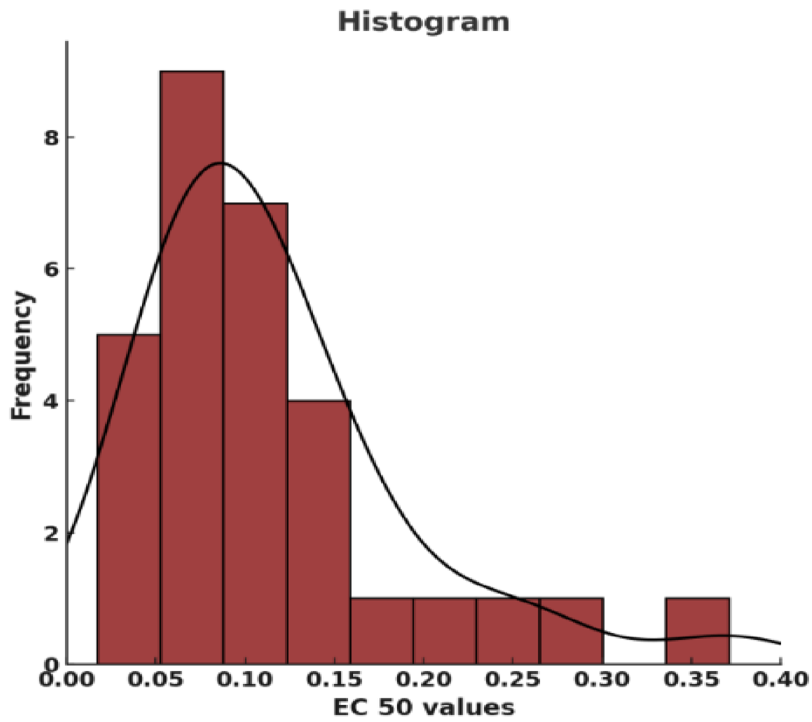

Fig. 1. Histogram showing diverse responses of Alternaria mali baseline isolates to various concentrations of tebuconazole

recorded for isolate BP7 and the highest (0.371 µg ml^{-1}) for isolate BP11 (Table 3; Fig. 1). The mean EC_{50} value was 0.15 µg ml^{-1}, which is comparable to the mean ED_{50} value of 0.141 µg ml^{-1} reported by Mubashir et al. (2023) in apple for 30 baseline isolates of *Venturia inaequalis* against the DMI fungicide difenoconazole. However, the mean EC_{50} obtained in the present study was slightly higher than the value of 0.07 µg ml⁻¹ reported by Koller et al. (1991) in apple for 300 baseline isolates of *V. inaequalis* from New York, which may be attributed to the large temporal gap between the two studies (1991-2025). Similarly, Nabi et al. (2022) reported a mean EC_{50} value of 0.02 µg ml^{-1} for difenoconazole against 30 baseline isolates of *V. inaequalis* of apple. A concentration of 0.20 µg ml^{-1} was selected as the discriminatory dose for assessing resistance in the exposed population (Table 4).

4. Conclusion

The study determined the baseline sensitivity of *Alternaria mali* to tebuconazole using thirty representative isolates. Relative growth of Baseline isolates was highest at lower fungicide doses (65.9–95.9% at 0.0001 µg ml^{-1}) and decreased progressively with increasing concentration, resulting in severe growth inhibition at 10 µg ml^{-1} (1.89–34.9%). EC_{50} values ranged from 0.017 to 0.371 µg ml^{-1} with a mean of 0.15 µg ml^{-1} indicating variability in sensitivity within the population. These results suggest that tebuconazole remains effective against *Alternaria mali* under current conditions in Kashmir. A discriminatory concentration of 0.20 µg ml^{-1} was established for resistance monitoring. This benchmark will aid in detecting shifts in sensitivity in field populations and supports the judicious use of DMI fungicides within integrated disease management programs to delay resistance development.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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Competing Interests

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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