



# 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 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<sup>-1</sup>), which declined progressively with increasing concentration, resulting in strong growth suppression at 10 µg ml<sup>-1</sup> (1.89–34.9%). The mean EC<sub>50</sub> 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 inhibit 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  $\mu\text{g ml}^{-1}$  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  $\mu\text{g/ml}$ ) 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 25°C and the radial growth was measured after 5 days.

#### 2.1.2 Data Analysis

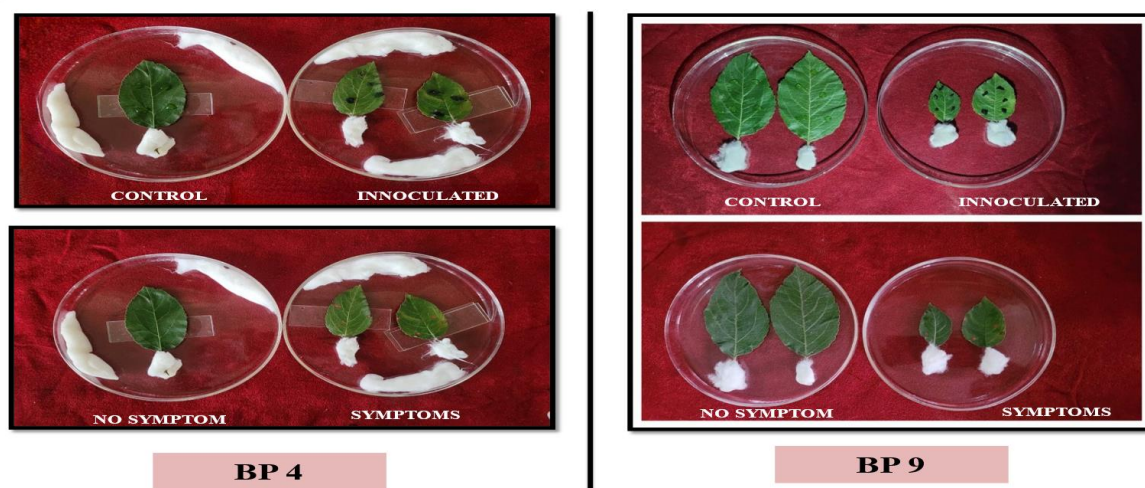
Percent relative growth (RG) for each isolate was calculated according to Chapman *et al.* (2011) using the following formula

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

EC<sub>50</sub> 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<sub>50</sub> 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<sup>-1</sup> and from 53.7% (BP9) to 88.7% (BP6) at 0.001 µg ml<sup>-1</sup>. Relative growth declined progressively with increasing concentration, varying from 47.6% (BP9) to 83.8% (BP24) at 0.01 µg ml<sup>-1</sup> and from 37.8% (BP20) to 79.6% (BP12) at 0.1 µg ml<sup>-1</sup>. At 1 µg ml<sup>-1</sup>, growth was markedly reduced (12.5% in BP22 to 50.9% in BP11), while severe inhibition was observed at 10 µg ml<sup>-1</sup> (1.89% in BP5 to 34.9% in BP8) (Table 2). The EC<sub>50</sub> values ranged from 0.017 to 0.371 µg ml<sup>-1</sup>, with the lowest value (0.017 µg ml<sup>-1</sup>)

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

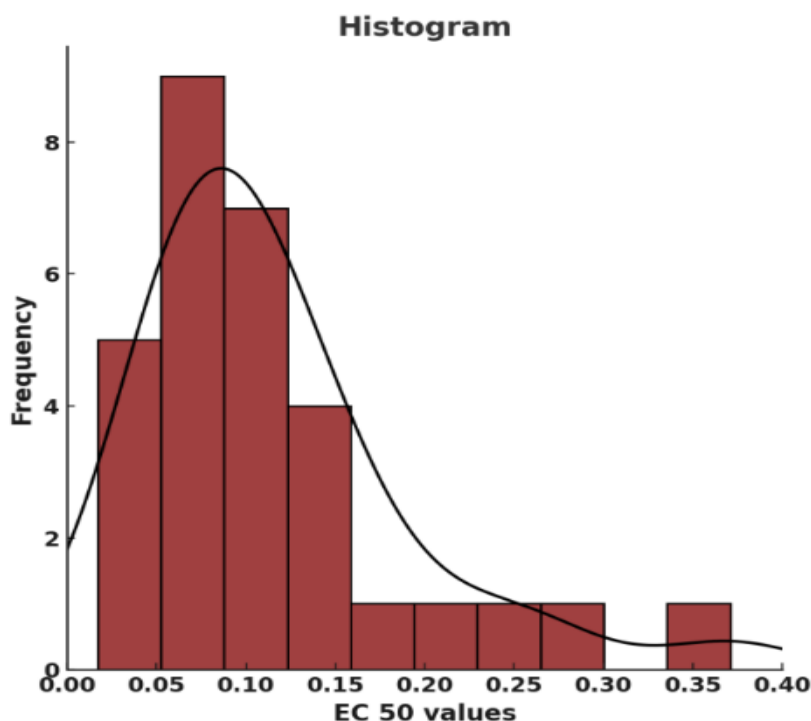
Isolates	Percent relative growth at different concentrations (RG)						
	Conc. (µg ml <sup>-1</sup> )	10	1	0.1	0.01	0.001	0.0001
BP1		31.03	41.38	58.62	67.24	75.86	93.1
BP2		15.6	24.4	42.2	77.8	86.7	95.6
BP3		7.7	28.2	74.4	79.5	84.6	94.9
BP4		2.2	41.3	50.0	63.0	82.6	87.0
BP5		1.89	49.06	73.58	81.13	88.68	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<sub>50</sub> values for thirty baseline isolates of *Alternaria mali* against Tebuconazole**

Isolate	EC <sub>50</sub> (µg ml <sup>-1</sup> )	Isolate	EC <sub>50</sub> (µg ml <sup>-1</sup> )
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<sup>-1</sup>) for isolate BP11 (Table 3; Fig. 1). The mean EC<sub>50</sub> value was 0.15 µg ml<sup>-1</sup>, which is

comparable to the mean ED<sub>50</sub> value of 0.141 µg ml<sup>-1</sup> reported by Mubashir et al. (2023) in apple for 30 baseline isolates of *Venturia inaequalis*

against the DMI fungicide difenoconazole. However, the mean EC<sub>50</sub> obtained in the present study was slightly higher than the value of 0.07 µg ml<sup>-1</sup> 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<sub>50</sub> value of 0.02 µg ml<sup>-1</sup> for difenoconazole against 30 baseline isolates of *V. inaequalis* of apple. A concentration of 0.20 µg ml<sup>-1</sup> 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<sup>-1</sup>) and decreased progressively with increasing concentration, resulting in severe growth inhibition at 10 µg ml<sup>-1</sup> (1.89–34.9%). EC<sub>50</sub> values ranged from 0.017 to 0.371 µg ml<sup>-1</sup> with a mean of 0.15 µg ml<sup>-1</sup> 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<sup>-1</sup> 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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