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In vitro evaluation of minimum inhibitory concentration (mic) of fungicides against *Rhizoctonia solani* f. sp. *Sasakii* Exner causing banded leaf and sheath blight disease in maize

Sadhna Chauhan and Rajesh Pratap Singh

Abstract
Minimum inhibitory concentration (MIC) is defined as the minimum concentration of a compound that will result in the inhibition of growth of a microorganism. These are principally used to confirm resistance in chemical fungicides, but most often as a tool to determine the *in vitro* efficacy of new fungicides as well as antibiotics. In this study, four fungicides *viz*. Carbendazim 50% WP, Azoxystrobin 11% + Tebuconazole 18.30% SC, Tebuconazole 50% + Trifloxystrobin 25% WG (at 1, 2, 4, 6, 8 and 10 ppm concentration) and Azoxystrobin 18.2% + Difenoconazole 11.4% SC (at 10, 12, 14, 15, 20, 25 and 30 ppm concentration) were evaluated against *Rhizoctonia solani* causing banded leaf and sheath blight (BLSB) in maize for determining their minimum inhibitory concentrations (MICs) *in vitro* using poison food technique. All the tested fungicides exhibit variable response in inhibiting the mycelial growth of the fungus. Among all the tested MICs fungus proved highly sensitive to Carbendazim 50% WP at 6 ppm concentration, Azoxystrobin 11% + Tebuconazole 18.30% SC and Tebuconazole 50% + Trifloxystrobin 25% WG each at 10 ppm concentration and Azoxystrobin 18.2% + Difenoconazole 11.4% SC at 14 ppm concentration with 100% growth inhibition. Such studies can be helpful in exploring *R. solani* for determing its resistance development to specific fungicides and thereby devising suitable management strategy against BLSB in maize.

Keywords*:* Minimum inhibitory concentration (MIC), Fungicides and Rhizoctonia solani

1. Introduction
Maize is one of the most important versatile crops which is cultivated in different agro ecological systems all over the world. Among the various diseases being reported in maize, banded leaf and sheath blight (BLSB) incited by *Rhizoctonia solani* f. sp. *Sasakii* (teleomorph: *Thanatephorus cucumeris*) has become a severe and economically significant disease which occurs every year in moderate to severe form in several maize growing areas (Sharma *et al*., 2002) [11], During the year 1927, the disease was first reported from Sri Lanka by Bertus as a sclerotial disease **(**Singh & Shahi, 2012) [13, 13], According to some reports, this disease may cause upto 40.5 percent loss in grain yield under favorable weather conditions. Also, when disease severity level was 87.3 percent, the reduction in grain yield was found to be 31.9 percent in cultivars of maize. BLSB holds the potential to cause economic crop losses up to 100 per cent (Sharma *et al*., 2002a) [12], *R. Solani* is one of the most important soil-borne pathogens in the world, producing devastating illnesses in a wide range of hosts (Woodhall *et al*., 2007) [15], Pathogen can infect plants at any stage of their development i.e. right from seedling stage through maturity. The disease first occurs on maize plants in their pre-flowering stage (30-40 days old), but it can also be noticed on younger plants. The emergence of small patches discoloured with alternate and uneven dark bands is the first symptom. The symptoms appear first on the first and second leaf sheaths above ground, and then extend to the ear, resulting in ear rot. If infection occurs prior to ear emergence, the ear cannot develop if it develops they are malformed. If an infection develops after ear emergence, the stalk fiber at the tip darkens cakes up, and hardens, resulting in poor grain filling. The kernels become low in weight and lusterless if infection occurs after grain development. In humid conditions, the infection that starts on the lower leaf sheaths spreads to the top leaf sheaths, causing leaf sheath rot and entire leaf drying. In extreme cases, the majority of the leaves are blighted, and sclerotia grow on the lesions, which are easily separated.

After the infection has taken hold, it causes direct losses such as early death, stalk breakage, leaf destruction, leaf sheath Destruction and ear rot. (Rani, *et al*., 2013) [9], to manage the disease and maximise productivity, a solution-oriented strategy is essential to retain maize's economic status. Fungicides are a potentially valuable tool for controlling a disease when other methods have failed. The use of fungicides is a critical component of integrated disease management. Fungicides can affect or disrupt the pathogen's metabolic system (Bilgrami & Dube, 1976) [2, 2], the inherent toxicity and penetration of a fungicide determine its effectiveness. Because reliable sources of disease resistance are unavailable, chemical and biological control is necessary to avert crop losses. There is a lot more information available now on the long-term management of BLSB, which is typically treated with chemical applications. Because of the pervasiveness and indiscriminate use of chemical pesticides, as well as the development of resistance in pathogens to chemical fungicides, the use of botanicals and bio-control agents in the treatment of plant diseases is gaining ground (Rajput *et al*.,2016) [8], The control of BLSB has been accomplished using a variety of approaches. Cultural practices, chemical, and biological control are the most commonly employed ways for managing this disease. Furthermore, it is routine practise to apply fungicides on-farm at higher doses than are indicated for the particular crop. There have been various instances of work done on disease management, but given the injudicious use of chemicals and the current devastating situation of the diseases, it is necessary to develop a method that uses the minimum pesticides possible without jeopardizing agricultural output losses. The goal of this study is to evaluate the fungicides' minimum inhibitory concentration *in vitro* that have been demonstrated to be effective in treating the disease. The knowledge gained can be used to lessen the detrimental impact of fungicides on non-target species and the unwanted changes they cause in the environment by reducing the dose of the fungicide.

2. Material and Methods

The experiment was carried out in Soil borne Plant Pathogens and Fungicides Laboratory, Department of Plant Pathology, College of Agriculture, GBPUAT, Pantnagar, Uttarakhand.

2.1 Isolation Purification and Maintenance of Pathogen

Disease's typical symptoms were collected from Maize Pathology Block, Norman E. Borlaug Crop Research Centre, Pantnagar. Diseased tissues were trimmed into 2–3 mm pieces with a sterilised blade after being rinsed with clean and sterile water. These leaf pieces were surface sterilised for 1–2 minutes with sodium hypochlorite (1 percent), then rinsed three times with autoclaved distilled water. The diseased leaf pieces were surface sterilised, blotter dried, and then aseptically transferred to sterile Potato Dextrose Agar (PDA) media on Petri plates. Similarly, the same procedure was employed to isolate test pathogen from sclerotia. At a temperature of 28±1°C, inoculated tubes and Petri plates were incubated in a Biological Oxygen Demand (B.O.D.) incubator. After 48 hours of incubation, the mycelial growth thus obtained was transferred to culture/test tubes containing PDA for any further study. The pathogen was purified by using a hyphal tip/single spore isolation method. (Zhang *et al*., 2013) [16], Cultural and morphological parameters were used to identify the fungal culture. Slides were made and stained in lactophenol, and morphological properties of the fungus were evaluated under a compound microscope.

2.2 *In vitro* Evaluation of Minimum Inhibitory Concentration (MIC) of Fungicides against *R* Solani

2.2.1 Preparation of Fungicides Stock Solution

Each treatment's (fungicides) stock solution was made by dissolving a weighed amount in a measured volume of sterilised distilled water. The following formula was used to calculate the amount of stock solution to be added to the potato dextrose agar medium:

$C1V1 = C2V2$

Where,
C1= Concentration of stock solution (µg/ml)
C2=Desired concentration (µg/ml)
V1 = Volume (ml) of the stock solution to be added
V2= Measured volume (ml) of the PDA medium

2.2.2 Poisoned Food Technique

The poisoned food method described by Borum & Sinclair (1968) [3, 3], using potato dextrose agar medium was used to evaluate several fungicides *in vitro* to check the colony growth of the fungus *R. solani.* Carbendazim 50% WP, Azoxystrobin 11% + Tebuconazole 18.30% SC, Tebuconazole 50% + Trifloxystrobin 25% WG (at 1, 2, 4, 6, 8 and 10 ppm conc.) and Azoxystrobin 18.2% + Difenoconazole 11.4% SC (at 10, 12, 14, 15, 20, 25 and 30 ppm conc.) were tested against *R solani*, using the poison food technique to determine their minimum inhibitory doses (MICs) *in vitro* (Table 1).

Table 1: Nomenclature of fungicides used in the study

S. No.	Trade name	Common name	Chemical name	Chemical group
1	Carbendazim 50% WP	Bavistin	Methyl (1H -1,3 benzimidazol -2 yl) carbamate	Benzimidazole
2	Azoxystrobin 11% + Tebuconazole 18.30% SC	Custodia	Methyl (2E) -2- (2-{[6-(2-cyanophenoxy)pyrimidin-4-yl]oxy}phenyl)-3-methoxyprop-2-enoate+ (RS)- 1-(4-Chlorophenyl)- 4,4-dimethyl-3-(1H, 1,2,4-triazol-1-ylmethyl)pentan- 3-ol	Strobilurins and Triazoles
3	Tebuconazole 50% + Trifloxystrobin 25% WG	Nativo	(RS)- 1-(4-Chlorophenyl)- 4,4-dimethyl-3-(1H, 1,2,4-triazol-1-ylmethyl)pentan-3-ol + methyl (E)-methoxyimino-{(E)-α-[1-(α,α,α-trifluoro-m-tolyl)ethylideneaminooxy]-o-tolyl}acetate	Triazoles and Strobilurins
4	Azoxystrobin 18.2% + Difenoconazole 11.4% SC	Amistar Top	Methyl (2E) -2- (2-{[6-(2-cyanophenoxy)pyrimidin-4-yl]oxy}phenyl)-3-methoxyprop-2-enoate + 1-((2-(2-Chloro-4-(4-chlorophenoxy)phenyl)-4-methyl-1,3-dioxolan-2-	Strobilurins and Triazoles

Aseptically, 20 ml of potato dextrose medium poisoned with varied amounts of different fungicides was placed into each Petri plate. Three replications were kept for each fungicide concentration. After the medium had solidified, each plate was inoculated in the centre with a 5 mm disc cut off the edge of a 3 days old *R. solani* culture using a sterile sharp cork borer. As a control, petri plates without fungicide inoculated with *R. solani* were used. At 30±1 °C, all the inoculated plates were incubated. Data for radial growth was taken after 7 days, whereas data for the number of sclerotia produced, days taken for sclerotia development, and total weight of sclerotia were taken starting on the day of sclerotia formation and observed for up to 30 days as required.

After inoculation, radial growth was recorded using a measuring scale. The percentage radial growth inhibition above control was computed using the formula below (Mc kinney, 1923).

$$I = \frac{C-T}{C} \times 100$$

Where,
I = Per cent Inhibition
C = Colony diameter in control
T = Colony diameter in treatment

3. Statistical Analysis: The data were statistically analyzed with Completely Randomized Block Design (CRD), two way ANOVA and the treatments were compared by the mean of critical differences (CD) at 5% level of significance.

4. Results

In this study, fungicides were evaluated for their effect on mycelial growth and sclerotial parameters of *R. solani* to identify best effective MICs for respective fungicides used. Four selected fungicides are known to be effective against the test fungus. The fungicides showed variable response in inhibiting the colony growth. Carbendazim 50%WP proved to be the best fungicides giving 100 per cent growth inhibition at MIC of 6 ppm however at 4 ppm inspite of mycelial growth 6.67 mm, sclerotia formation was completely checked.

Table 2: Effect of different fungicides on mycelial growth and Sclerotal parameters of *R. solani*

S. NO.	Fungicide concentration (µg/ml)	Fungicide/ Treatment	Colony diameter*(mm)	Inhibition %	Dsf *(days)	Nsf* (no.)	Total weight* (mg)
1	1 ppm	T1 Carbendazim 50% WP	84.33	6.30	3.00	72.67	232.29
		T2 Azoxystrobin 11% + Tebuconazole 18.30% SC (29.3% SC)	13.33	85.19	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	9.50	89.44	10.33	17.67	43.26
		Control	90.00	0.00	3.00	85.33	336.40
2	2 ppm	T1 Carbendazim 50% WP	28.00	68.89	5.00	66.00	198.46
		T2 Azoxystrobin 11% + Tebuconazole 18.30% SC (29.3% SC)	11.17	87.59	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	8.50	90.56	12.67	13.00	22.64
		Control	90.00	0.00	3.00	85.33	336.40
3	4 ppm	T1 Carbendazim 50% WP	6.67	92.59	0.00	0.00	0.00
		T2 Azoxystrobin 11% + Tebuconazole 18.30% SC (29.3% SC)	10.50	88.33	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	7.50	91.67	0.00	0.00	0.00
		Control	90.00	0.00	3.00	85.33	336.40
4	6 ppm	T1 Carbendazim 50% WP	0.00	100.00	0.00	0.00	0.00
		T2 Azoxystrobin 11% + Tebuconazole 18.30% SC (29.3% SC)	9.50	89.44	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	6.50	92.78	0.00	0.00	0.00
		Control	90.00	0.00	3.00	85.33	336.40
5	8 ppm	T1 Carbendazim 50% WP	0.00	100.00	0.00	0.00	0.00
		T2 Azoxystrobin 11% + Tebuconazole 18.30% SC (29.3% SC)	6.33	92.96	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	6.00	93.33	0.00	0.00	0.00
		Control	90.00	0.00	3.00	85.33	336.40
6	10 ppm	T1 Carbendazim 50% WP	0.00	100.00	0.00	0.00	0.00
		T2 Azoxystrobin 11% + Tebuconazole 18.30 SC (29.3% SC)	0.00	100.00	0.00	0.00	0.00
		T3 Tebuconazole 50% + Trifloxystrobin 25% WG (75% WG)	0.00	100.00	0.00	0.00	0.00
		Control	90.00	0.00	3.00	85.33	336.40
C.D. (p=0.05) C.V.			0.433 0.846	- -	0.273 8.163	2.353 5.744	6.762 4.366

* All values are mean of three replications
Nsf= No.of sclerotia formed per petridish; Dsf= Days taken to form sclerot

The maximum colony diameter was obtained at Carbendazim 50%WP 1 ppm concentration i.e. 84.33 mm with inhibition percentage of 6.33 and it took 3 days to form sclerotia with 72.67 and 232.9 which are the no. of sclerotia and total weight of sclerotia respectively. In case of Azoxystrobin 11% + Tebuconazole 18.30% SC, inhibition percent of mycelial growth varies from 85.19 per cent to 100 per cent at 1 ppm and 10 ppm respectively. Similarly, with treatment of Tebuconazole 50% + Trifloxystrobin 25% WG, inhibition percent of mycelial growth varies from 89.44 per cent to 100 per cent at 1 ppm and 10 ppm respectively (Table 2 and Fig 1).

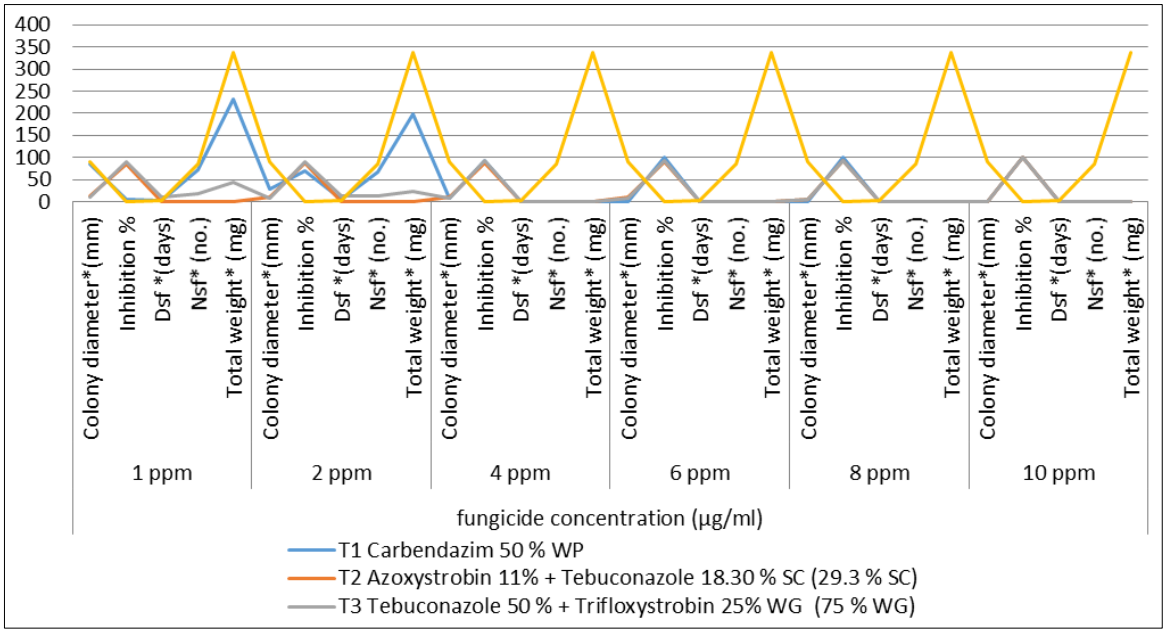

Fig 1: Effect of different fungicides on mycelial growth and sclerotial parameters of *R.* solani

In case, among all the MICs of Azoxystrobin 18.2% + Difenoconazole 11.4% SC, at 10 ppm concentration, mycelial growth was observed to be 6.5 mm with inhibition percentage of 92.78 per cent (Fig 2). There was no sclerotia formation in this fungicide at any of the concentration levels of Azoxystrobin 11% + Tebuconazole 18.30% SC and Azoxystrobin 18.2% + Difenoconazole 11.4% SC. All the fungicides showed variable response in inhibiting the colony growth of the pathogen. Among all the tested MICs fungus proved highly sensitive to Carbendazim 50% WP at 6 ppm concentration, Azoxystrobin 11% + Tebuconazole 18.30% SC and ebuconazole 50% + Trifloxystrobin 25% WG each at 10 ppm concentration and Azoxystrobin 18.2% + Difenoconazole 11.4% SC at 14 ppm concentration with 100% growth inhibition.

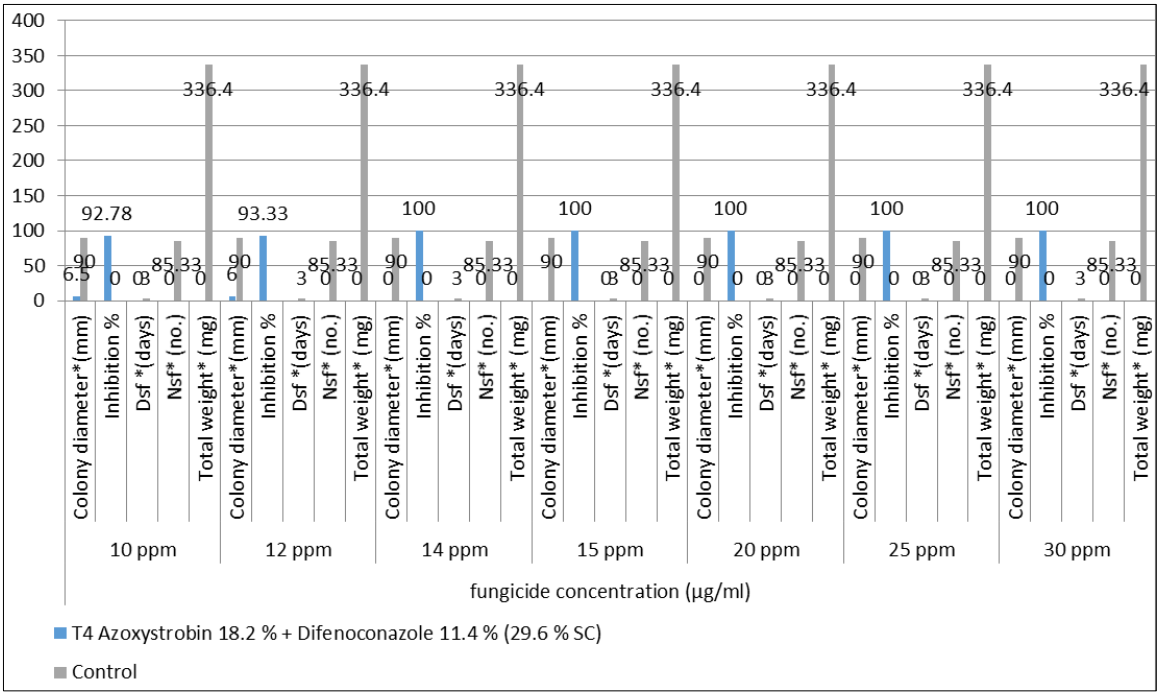

Fig 2: Effect of fungicide Azoxystrobin 18.2% + Difenoconazole 11.4% (29.6% SC) on mycelial growth and sclerotial parameters of *R. sola*

5. Discussion

The fungicide Carbendazim have its active ingredient as Methyl 1-2 Benz imidazole Carbamate, and belongs to the Benz imidazole category of fungicides (MBC). It has a systemic nature and is easily absorbed to reach the required location within the plant. It inhibits mitosis in fungal hyphae, and there is a strong link between *in vitro* efficacy and fungal disease development. These fungicides generate MBC when treated with water. They have a wide range of fungi toxic action against members of the Fungi Imperfect class. They've been shown to be quite efficient against *R. Solani* when studied *in vitro* and *in vivo*. Tebuconazole and Difenoconazole are two of the most regularly used triazoles in the Sterol biosynthesis inhibitors (SBIs) series of fungicides. They work by inhibiting sterol (ergo sterol) biosynthesis, which is a major component of Asco and Basidiomycotina cell membranes. Because of their mechanism of action, the fungicides Azoxystrobin and Trifloxystrobin are classified as strobilurins, sometimes known as QoIs (quinol oxidation inhibitors). They prevent fungal mitochondria from producing ATP by blocking electron transport at the quinol oxidation site (the QO site) in the cytochrome bc1 complex. Trifloxystrobin moves exclusively translaminarly, whereas Azoxystrobin moves both translaminarly and systemically.

Previously, the use of fungicides was limited, but foliar fungicides have become more common in maize production over the last decade. Several fungicides, including carbendazim, benodanil, and validamycin, have been proven to be efficacious *in vitro* and *in vivo* against this pathogen (Ahuja and Payak, 1986) [1, 1], They found that validamycin A, aureofungin, carbendazim, benodamil, dichlroline, thiobendazole and thiophanate methyl were effective against *R.solani* isolates. Bavistin, Rhizolex, and Thiophanate M, three commonly used fungicides, have exhibited complete control of *R. solani* f. sp. *Sasakii* mycelial growth with 100 percent inhibition (Sharma *et al*., 2002a) [12], Saxena (2002) [10], assessed the efficacy of propiconazole (0.1 percent) and carbendazim (0.05 percent) and found that propiconazole was more successful when sprayed at the 30th or 40th day after planting, with the second spray done 10 days later. The usage of foliar fungicides in the corn belt of America has expanded in recent years, owing to the alleged yield gain linked with quinine outside inhibitors (Qois) such as strobilurins (Bradley, 2012) [4], Validamycin was shown to be the most effective in controlling the spread of banded leaf and sheath blight in maize (Dalmacio *et al*., 1990) [5],

Carbendazim 50 per cent WP was the most effective treatment for the sclerotial state of the disease, with 87 percent disease control, followed by Brestan 60 WP (fentin) and Calixin 75 EC (tridemorph), with 77 and 74 percent disease control, respectively (Sinha, 1992). Carbendazim was tested *in vitro* and *in vivo*, and it was observed that at 1 ppm, carbendazim totally suppressed the growth of R. solani mycelia. Carbendazim was able to reduce per cent disease intensity whether given as a soil drench or foliar spray. When sprayed 24 hours before inoculation, the fungicide demonstrated improved efficacy (Meena *et al*., 2003) [7], Sundravadana *et al*., (2007) [14], reported that azoxystrobin showed complete inhibition of mycelial development at 1, 2 and 4 ppm after an *in vitro* experiment.

6. Conclusion

All of the fungicides had an overall inhibitory effect, and colony growth slowed as the fungicidal concentration increased. Fungus was highly sensitive to all four tested fungicides, with 100 percent suppression, when the efficacy and cumulative performance were considered. Carbendazim 50% WP proved to be the best fungicides giving 100 per cent growth inhibition at MIC of 6 ppm. The effectiveness of fungicides in the laboratory versus in the field is determined by their *in vitro* efficiency at low, cost-effective dosages, as well as their efficient and quick delivery to the infection site. Fungi can acquire resistance as a result of indiscriminate or inappropriate use. The use of MICs for four different fungicides can help standardise the appropriate dosage when designing a fungicidal spraying schedule.

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