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Research Article

In vitro evaluation of different fungicides against *Rhizoctonia solani* and *Alternaria citri* infecting citrus

Susmita Sigdel[1*], Saurav Ranabhat[1], Srijana Bhandari[2], Prem Bahadur Magar[3], Jiban Shrestha[4] and Subash Subedi[5]

[1]Agriculture and Forestry University, Rampur, Chitwan, Nepal
[2]Warm Temperate Horticulture Center, Kirtipur, Kathmandu, Nepal
[3]Nepal Agricultural Research Council, National Plant Pathology Research Centre, Khumaltar, Lalitpur, Nepal
[4]Nepal Agricultural Research Council, National Plant Breeding and Genetics Research Centre, Khumaltar, Lalitpur, Nepal
[5]Nepal Agricultural Research Council, Oilseed Research Program, Nawalpur, Sarlahi, Nepal
*Corresponding author's email: susmitasigdel16@gmail.com
*ORCID: https://orcid.org/0000-0001-7573-4373

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ABSTRACT

Rhizoctonia solani and *Alternaria citri* are major plant pathogens of citrus, causing considerable production losses. Chemical fungicides are widely used for disease control. Using the food poisoning technique under in vitro conditions, an experiment was undertaken to investigate the efficacy of several fungicides against those pathogens. To evaluate the effect on *Rhizoctonia solani* mycelial growth, five different chemicals, viz. SAAF (Carbendazim 12% WP + Mancozeb 63% WP), Bavistin (Carbendazim 50% WP), VACOMIL PLUS (Metalaxyl 15% WP + Copper oxychloride 35% WP), and Raze (Copper oxychloride 50% WP) were used at 100 ppm and 200 ppm concentration each. Similar chemicals were used for *Alternaria citri* except for additional Mancozab (Mancozeb 75% WP). Mycelial growth inhibition was measured until the fungus nearly covered the plate in control. All fungicides reduced the fungal growth compared to control. After 96 hours of incubation with *Rhizoctonia solani*, maximum inhibition (100%) was achieved at both concentrations of Bavistin, followed by SAAF @ 200 ppm (97.59%) and SAAF @ 100 ppm (88.25%), whereas VACOMIL PLUS and Raze had the minimum effect on the mycelial growth. Similarly, after 8 days of incubation of *Alternaria citri*, SAAF @ 200 ppm showed the highest inhibition (70.86%), followed by SAAF @ 100 ppm (65.11%), Mancozab @ 200 ppm (64.39%), and Mancozab @ 100 ppm (47.48%), but the effect of Bavistin, Raze, and VACOMIL PLUS had the lowest impact. The chemical proven effective against the pathogens should be trialed in pot and field experiments for further verification.

Keywords: Food poisoning technique, fungicides, inhibition, pathogens

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INTRODUCTION

Rhizoctonia solani is a soil-borne necrotroph that produces a variety of symptoms in a wide range of host plants, including seed decay, damping-off, stem canker, root rot, fruit decay, crown rot, and leaf diseases (Menzies, 1970; Nagaraj *et al.,* 2017). The pathogen is classified as a basidiomycetes, which are septate multinucleate fungi that generate dark brown sclerotia in order to survive under unfavorable climate conditions (Ajayi-Oyetunde & Bradley, 2018). Aerial hyphae are used to identify the pathogen that has a septum in the branch near the constriction (Butler & Bracker, 2020).The susceptibility of the host plant is highly dependent on its stage in the life cycle, as this factor has a significant effect on disease progression in *Rhizoctonia solani*, so seedlings with main meristematic juvenile tissues are more vulnerable (Keijer, 1996). *Rhizoctonia solani* sclerotia are widely found in the soil, where they are attracted when growing plants generate chemical stimuli, allowing the pathogen to enter the epidermis via appressoria or other natural openings. As a result, the pathogen feeds on the host's nutrition, colonizes the host, and grows inside the dead tissue (Baker & Martinson, 2020; Keijer, 1996).

Alternaria citri is a citrus preharvest disease (Mohamed and Jiuxu, 2004). It causes stem-end browning, central axis rot, and fruit blemishes, and is a major cause of economic loss in citrus production, especially in humid areas (Lawrence *et al.*, 2013). *Alternaria citri*, an ascomycete fungal disease, also causes many pathogenic problems in the foliage, fruits, and general canopy of citrus, primarily fruit rotting in practically all citrus cultivars (Jaouad *et al.*, 2020). Spore chains are present, consisting of transverse and longitudinal septation and may or may not have beaks (Bliss & Fawcett, 1944). *Alternaria* species infection usually occurs after a period of prolonged rain, and the spores are in contact for roughly a week (Green *et al.*, 2001). The pathogen spreads through conidia, which can penetrate directly through natural openings or indirectly through the production of germ tubes, which later form appressoria, usually at the junction of epidermal cells (Allen *et al.*, 1983). The pathogen's penetration is accompanied by a chemical degradation of the surrounding host tissue, resulting in the formation of the lesion and subsequent blighting of the leaves (Allen *et al.*, 1983).

There are numerous management options available, ranging from preventive to curative, each with its own significance. Chemical control methods, for example, have long been used to control pathogens of the genus *Rhizoctonia* and *Alternaria*, despite their risk to human health but demonstrated efficacy when used properly (Ajayi-Oyetunde & Bradley, 2018; Farooq *et al.*, 2018; Karkee & Mandal, 2020; Pranaya *et al.*, 2020; Subedi, 2015). Despite the fact that both diseases cause harm to citrus, little study has been done to analyze the damage ratio and management aspects of the infection, with a focus on citrus cultivars. As a result, the current study examined the antifungal effects of various concentrations of commercial chemical fungicides against *Rhizoctonia solani* and *Alternaria citri* concerning in vitro growth inhibition to facilitate the recommendation of concentration for the field condition.

MATERIALS AND METHODS

Isolation of the pathogen

The root and shoot of diseased acid lime (*Citrus aurantifolia*) saplings were collected from the nursery of Warm Temperate Horticulture Center (WTHC), Kirtipur, Kathmandu, Nepal. These samples were examined for the presence of any pathogens at WTHC Pathology

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Laboratory. To eliminate soil particles, the root and shoot were rinsed under running water. The root pieces were surface sterilized for about 2 minutes in 2% NaOCl and the shoot portions were sterilized for about a minute in 1% NaOCl. After that, the sterilized parts were thoroughly rinsed three times with sterile distilled water. The treated root and shoot pieces were divided into six pieces per plate on autoclaved filter paper soaked in distilled water. For the next two days, all the plates were kept at 25±2 °C. After that, the pathogen was identified with the help of compound microscope on the basis of their peculiar character. The *Rhizoctonia solani* was identified on the basis of characteristic constriction in the hyphal branching point and the *Alternaria citri* was identified on the basis of pale colored conidiophore which were simple and sometimes branched sepatate with conidia that exist in solitary or in branched chains which was straight or slightly curved and isolation was done for making pure culture of those pathogens (Figure 1).

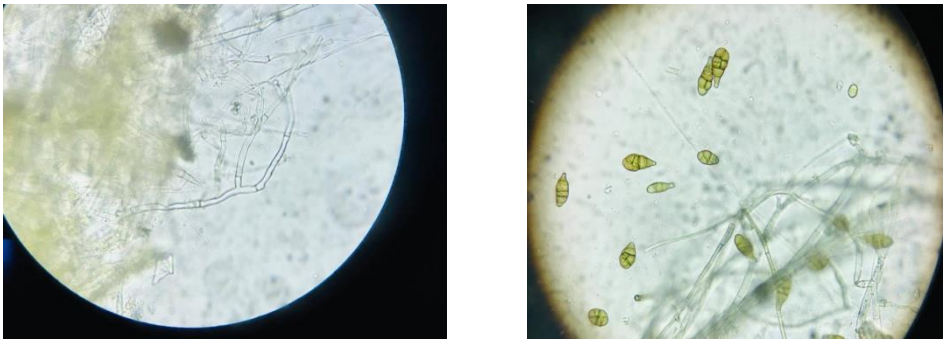

Figure1: Microscopic view of *Rhizoctonia solani* mycelia (Left) and Microscopic view of *Alternaria citri* conidia (Right)

***In vitro* experiment**

In vitro evaluation of the fungicides were done to assess the suitability of different chemical fungicided that were being used in the field of WTHC to minimize the proliferation of the pathogen in citrus species. Also, the chemicals were selected on the basis of their proven efficacy. Using the poisoned food technique, the efficiency of four different fungicides against *Rhizoctonia solani* and five fungicides against *Alternaria citri* at two different concentrations were evaluated on PDA medium (Table 1 and Table 2). The concentration of 100 and 200 ppm was chosen keeping the current application rate i.e., 1000 ppm being followed in the field of WTHC. The 100 mL PDA was prepared in different volumetric flasks per the number of treatments, and then autoclaved for 15 minutes at 121 °C. PDA was put on laminar airflow chamber and UV light was turned on for about 15 minutes to do make sterilized environment and left it for cooling. An electronic weighing machine was used to measure the chemicals used in the treatment. The measured treatments were then added to the several labeled volumetric flasks, which were then transferred to 9 cm diameter petri plates. The PDA was solidified after 24 hours, and the laminar airflow was sterilized using UV light for 15 minutes. The inoculums were then extracted from a 7 day old pure culture of *Rhizoctonia solani* and *Alternaria citri* using a sterilized cork borer with 7 mm diameter, and an inoculation loop was employed to retain the inoculums in the center of the diameter marked on the petri plate. All of the petri plates were labeled before being wrapped with parafilm. Petri plates were kept at a temperature of 25±2 °C in an incubator. With four replications, the experiment was conducted in a completely randomized design. Due to its aggressive growth pattern, *Rhizoctonia solani* data was collected at 24 hours intervals for 6

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days, while *Alternaria citri* data was collected at 48 hours intervals for 8 days.

Table 1: Treatment details for the in vitro evaluation of *Rhizoctonia solani* during 2021 at WTHC, Kirtipur

SN	Commercial name	Chemical name	Active ingredients (a.i.)	Mode of action
1	SAAF	Carbendazim 12%+ Mancozeb 63%	75 % WP	Systemic+ Contact
2	Bavistin	Carbendazim 50%	50 % WP	Systemic
3	VACOMIL PLUS	Metalaxyl 15% + Copper oxychloride 35%	50 % WP	Systemic+ Contact
4	Raze	Copper oxychloride 50%	50 % WP	Contact
5	Control	Distilled Water		

Table 2: Treatment details for the in vitro evaluation of *Alternaria citri* during 2021 at WTHC, Kirtipur

SN	**Commercial name**	**Chemical name**	**Active ingredients (a.i.)**	**Mode of action**
1	SAAF	Carbendazim 12% + Mancozeb 63%	75 % WP	Systemic + contact
2	Bavistin	Carbendazim 50%	50 % WP	Systemic
3	VACOMIL PLUS	Metalaxyl 15% + Copper oxychloride 35%	50 % WP	Systemic + contact
4	Raze	Copper oxychloride 50%	50 % WP	Contact
5	Mancozab	Mancozeb	75% WP	Contact
6	Control	Distilled Water		

Growth Inhibition Test

For both pathogens, the growth of mycelium was assessed using a scale to measure the length of mycelium in each treatment. The percent growth inhibition of mycelia growth over control was calculated by using the formula given by Vincent (1947) (Eq. 1).

$$PGI = \frac{C-T}{C} \times 100 \quad \ldots\ldots. \quad (1)$$

where,
PGI = Percent Growth Inhibition
C = Average diameter of colony in control treatment
T = Average diameter of colony in fungicidal treatment

Statistical analysis

MS-Excel 2016 was used to enter the data. Statistical analysis was performed using R Studio version 4.0.3 (Agricolae package). The data was transformed into log10 (1+data) for normalization and 1 in each data set was added to remove potential infinity values from the log transformed data. The least significant difference (LSD) test was used to identify the significant differences between treatments at 5% level of significance (Gomez & Gomez, 1984).

RESULTS AND DISCUSSION

In vitro evaluation of fungicides against *Rhizoctonia solani*

Rhizoctonia solani mycelial growth and percentage growth inhibition in comparison to control after 24, 48, 72, and 96 hours of incubation were presented in Table 3 and Table 4

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respectively. In comparison to the untreated control, all the chemical fungicides tested

considerably reduced the pathogen's growth. The treatment differed significantly ($p<0.01$) in reducing the mycelium growth on all days of observation. After 24 hours of incubation, SAAF @ 200 ppm, Bavistin @ 100 ppm and 200 ppm; all showed 100 % mycelail growth inhibition while SAAF @ 100 ppm showed 89.7 % only. However, both concentrations of VACOMIL PLUS and Raze reduce growth at a statistically lower rate, with the control showing the least growth inhibition. In 48 and 72 hours, similar result was achieved. Petri plates in the control treatments were fully covered with mycelium after 96 hours of incubation. Meanwhile, Bavistin @ 100 ppm and 200 ppm completely inhibited *Rhizoctonia solani* mycelium growth in all obervations which was further illustrated by Figure 2.

Table 3: Effect of different doses of fungicides on radial mycelia growth of Rhizoctonia solani at WTHC, Kirtipur

S.N.	Treatment	Radial growth (cm)			
		24 hrs	48 hrs	72 hrs	96 hrs
1	SAAF @ 100 ppm	0.25 (0.0975^{f})	0.525 (0.1825^{f})	0.675 (0.2250^{e})	0.975 (0.295^{c})
2	SAAF @ 200 ppm	0.000 (0.0000^{g})	0.050 (0.0200^{g})	0.1 (0.0400^{f})	0.200 (0.075^{d})
3	Bavistin @ 100 ppm	0.000 (0.0000^{g})	0.000 (0.0000^{g})	0.000 (0.0000^{g})	0.000 (0.000^{e})
4	Bavistin @ 200 ppm	0.000 (0.0000^{g})	0.000 (0.000^{g})	0.00 (0.000^{g})	0.000 (0.000^{e})
5	VACOMIL PLUS @ 100 ppm	1.100 (0.3200^{d})	2.825^{d} (0.5825^{d})	4.725 (0.7550^{c})	6.350 (0.865^{b})
6	VACOMIL PLUS @ 200 ppm	0.875 (0.2725^{e})	2.350 (0.5250^{e})	4.125 (0.7100^{d})	5.625 (0.820^{b})
7	Raze @ 100 ppm	1.750 (0.4400^{b})	4.950 (0.7750^{b})	7.200 (0.9125^{ab})	8.300 (0.970^{a})
8	Raze @ 200 ppm	1.475 (0.3925^{c})	3.975 (0.6975^{c})	6.575 (0.8800^{b})	7.650 (0.935^{a})
9	Control	2.425 (0.5325^{a})	6.300 (0.8650^{a})	7.725 (0.9375^{a})	8.300 (0.097^{a})
	Grand mean	0.228333	0.40527	0.4956	0.5478
	SEm±	0.002723	0.002528	0.003837	0.005217
	CV (%)	7.156263	3.7420	4.6456	5.714
	LSD (0.05)	0.02384	0.02213	0.03359	0.04568
	F-test	***	***	***	***

*Values are means of three replications. Data was transformed using log10 (1+data), the presented means are original and values inside the parentheses indicate transformed data and ANOVA was conducted on transformed data. The mean values in the same column with same letter are not statistically significantly different ($p<0.05$). hrs = hours after incubation, SEm± = standard error of mean, CV= coefficient of variation, LSD = least significant differences. *** = significant at 0.001 level of significance.*

In vitro evaluation of fungicides against Alternaria citri

The efficiency of various fungicides against Alternaria citri was given in Table 5 and Table 6. In all days of observation, all the tested fungicides showed significant inhibition when compared to the untreated control at $p<0.01$ level of significance. After two days of incubation, Mancozab @ 200 ppm recorded the highest inhibition (81.36 %) which was statistically similar to Mancozab @ 100 ppm (77.97 %), SAAF @ 100 ppm (79.66 %), and

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SAAF @ 200 ppm (83.05 %) whereas VACOMIL PLUS, Raze and Bavistin showed significantly less inhibition. After four days of incubation, the highest inhibition (79.02 %)

was obtained in SAAF @ 200 ppm followed by SAAF @ 100 ppm (74.83 %), Mancozab @ 200 ppm (72.03 %), Mancozab @ 100 ppm (69.23 %) whereas VACOMIL PLUS, Raze and Bavistin showed very low inhibition. A similar result was obtained in the 6th and 8th days of incubation until the petri plates of untreated control were fully covered with mycelium of Alternaria citri. The result of this experiment was further illustrated by Figure 3.

Table 4: Effect of different doses of fungicides on percentage growth inhibition of Rhizoctonia solani at WTHC, Kirtipur

S.N.	Treatment	Growth inhibition (%)			
		24 hrs	48 hrs	72 hrs	96 hrs
1	SAAF @ 100 ppm	89.70	91.67	91.27	88.25
2	SAAF @ 200 ppm	100.00	99.20	98.71	97.59
3	Bavistin @ 100 ppm	100.00	100.00	100.00	100.00
4	Bavistin @ 200 ppm	100.00	100.00	100.00	100.00
5	VACOMIL PLUS @ 100 ppm	54.70	55.16	38.87	23.49
6	VACOMIL PLUS @ 200 ppm	64.00	62.70	46.64	32.22
7	Raze @ 100 ppm	34.20	29.37	16.24	0.00
8	Raze @ 200 ppm	39.30	36.90	14.94	7.80

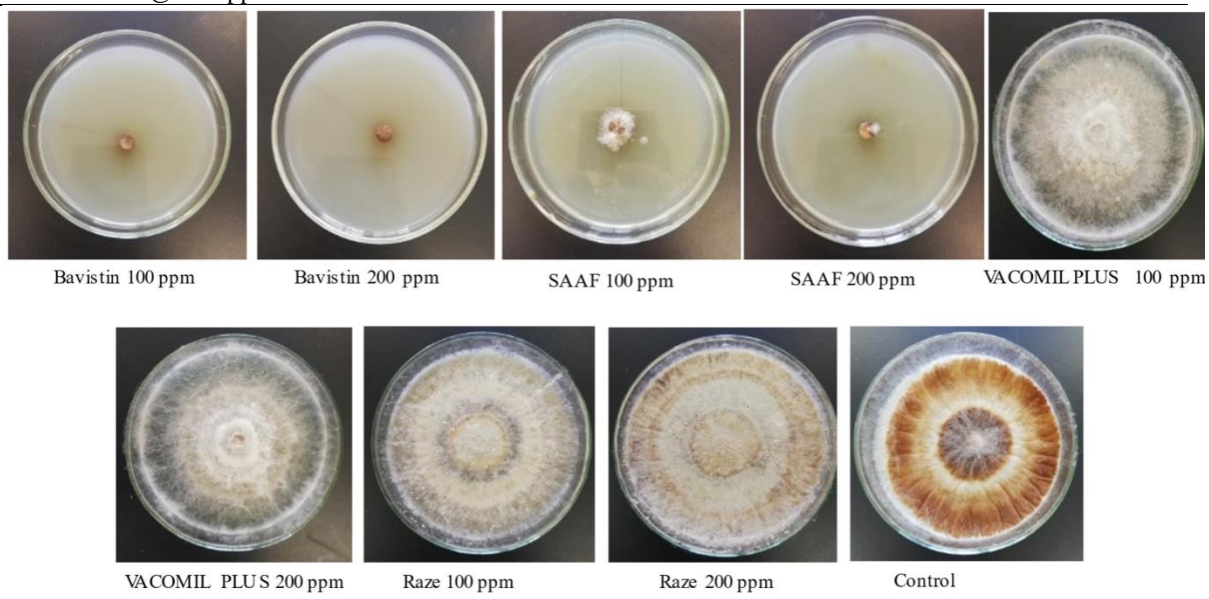

Figure 2: Growth of Rhizoctonia solani in different chemicals at WTHC, Kirtipur

Rhizoctonia solani mycelial growth was severely suppressed when grown in carbendazim at concentrations of 100 and 200 ppm. Kumar *et al.* (2017), Devi *et al.* (2008) and Rajendra prasad *et al.* (2017) who found complete fungal growth suppression in carbendazim and also stated the mode of action to be addressed, are all in agreement with this finding. McMahan *et al.* (2001) speculated that carbendazim works by interfering with fungal growth by altering tubulin function, hence preventing mitosis and cell division. Furthermore, mancozeb disrupts vital life functions such as lipid metabolism, respiration, and even adenosine triphosphate production by interacting with and inactivating the sulfhydryl groups of amino acids and enzymes present within the fungal cell (Tomlin, 2003). In the poisoned food bioassay, SAAF

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at both concentrations (100 and 200 ppm) inhibited *Rhizoctonia solani* mycelium growth by more than 85 %.

Table 5: Effect of different doses of fungicides on radial mycelial growth of *Alternaria citri* at WTHC, Kirtipur

S.N.	Treatment	Radial growth (cm)			
		Day 2	Day 4	Day 6	Day 8
1	Bavistin @ 100 ppm	1.100 (0.3200^{b})	2.875 (0.5850^{b})	4.175 (0.7125^{bc})	5.375 (0.805^{cd})
2	Bavistin @ 200 ppm	0.800 (0.2550^{c})	2.725 (0.57^{b})	3.825 (0.6800^{c})	4..725 (0.755^{d})
3	VACOMIL PLUS @ 100 ppm	1.050 (0.3100^{b})	2.925 (0.5925^{b})	4.450 (0.7350^{b})	6.075 (0.850^{abc})
4	VACOMIL PLUS @ 200 ppm	1.000 (0.3000^{bc})	2.850 (0.5850^{b})	4.350 (0.7250^{bc})	5.900 (0.8400^{bc})
5	Raze @ 100 ppm	1.450 (0.3875^{a})	3.550 (0.6580^{a})	5.175 (0.7906^{a})	6.550 (0.8779^{ab})
6	Raze @ 200 ppm	1.125 (0.3250^{b})	2.950 (0.5950^{b})	4.200 (0.7150^{bc})	5.875 (0.8375^{bc})
7	Mancozab @ 100 ppm	0.325 (0.1200^{d})	1.100 (0.320^{c})	2.375 (0.5250^{d})	3.650 (0.5350^{f})
8	Mancozab @ 200 ppm	0.275 (0.1025^{d})	1.000 (0.300^{c})	1.725 (0.4325^{e})	2.475 (0.6650^{e})
9	SAAF @ 100 ppm	0.300 (0.1125^{d})	0.900 (0.2800^{cd})	1.650 (0.4225^{ef})	2.425 (0.5350^{f})
10	SAAF @ 200 ppm	0.250 (0.0950^{d})	0.750 (0.2425^{d})	1.400 (0.3775^{f})	2.020 (0.480^{f})
11	Control	1.475 (0.3925^{a})	3.575 (0.6575^{a})	5.200 (0.7625^{a})	6.950 (0.900^{a})
	Grand Mean	0.2472	0.4893	0.6279	0.7345
	SEm±	0.005649	0.004267	0.004885	0.005864
	CV (%)	15.1533	5.7839	5.160	5.2954
	LSD (0.05)	0.05411	0.0408	0.042272	0.05617
	F-test	***	***	***	***

*Values are means of three replications. Data was transformed using log10 (1+data), the presented means are original and values inside the parentheses indicate transformed data and ANOVA was conducted on transformed data. The mean values in the same column with same letter are not statistically significantly different ($p<0.05$). hrs = hours after incubation, SEm± = standard error of mean, CV= coefficient of variation, LSD = least significant differences. *** = significant at 0.001 level of significance.*

Table 6 : Effect of different doses of fungicides on growth inhibition of *Alternaria citri* at WTHC, Kirtipur

S.N.	Treatment	Growth inhibition (%)			
		Day 2	Day 4	Day 6	Day 8
1	Bavistin @ 100 ppm	25.42	19.58	19.71	22.06
2	Bavistin @ 200 ppm	45.76	23.78	26.44	32.01
3	VACOMIL PLUS @ 100 ppm	28.81	18.18	14.42	12.59
4	VACOMIL PLUS @ 200 ppm	32.20	20.28	16.35	15.11
5	Raze @ 100 ppm	1.69	2.70	4.50	5.75
6	Raze @ 200 ppm	23.73	17.48	19.23	15.47
7	Mancozab @ 100 ppm	77.97	69.23	54.33	47.48
8	Mancozab @ 200 ppm	81.36	72.03	66.82	64.39
9	SAAF @ 100 ppm	79.66	74.83	68.27	65.11
10	SAAF @ 200 ppm	83.05	79.02	73.08	70.86

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The findings are consistent with those of Srinivas *et al.* (2014) who found that carbendazim + mancozeb at 0.1% was effective in preventing fungus growth among fourteen fungicides tested. Furthermore, SAAF (carbendazim 12.25% + mancozeb 74.12%) and carbendazim at lower concentrations suppressed fungus growth in research by Dutta and Kalha (2011), and both the contact and systemic modes of action worked to decrease pathogen growth. Furthermore, the results of this experiment are consistent with those of Karkee and Mandal (2020), who found that at 100 ppm concentrations of SAAF and carbendazim, fungal growth was completely inhibited.

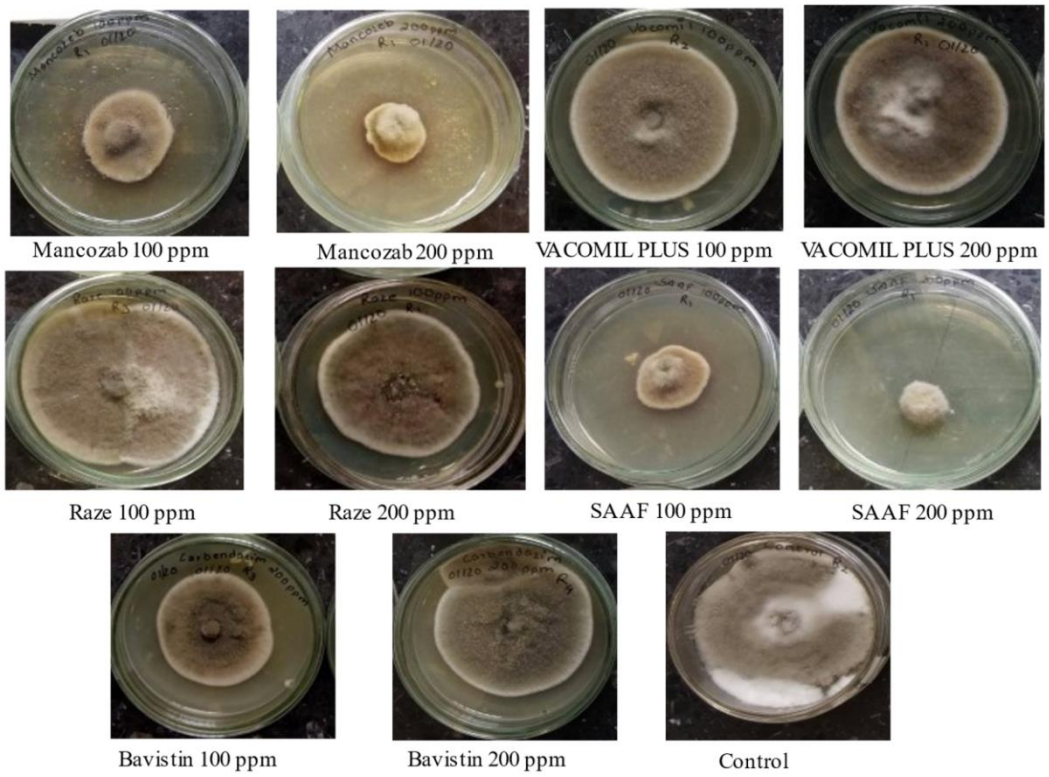

Figure 3: Growth of *Alternaria citri* in different chemicals at WTHC, Kirtipur

SAAF @ 200 ppm (carbendazim 12% + mancozeb 63%) inhibited *Alternaria citri* growth the most (70.86%), followed by SAAF @ 100 ppm (65.11%) and mancozeb 200 ppm (64.39%).

In a study conducted by Farooq *et al.* (2018), mancozeb was found to have 75.66% and 80.22% growth inhibition on the mycelial growth of *Alternaria citri* in 100 and 200 ppm concentrations, respectively. According to Pranaya *et al.* (2020), there was a relatively significant decrease in *Alternaria* sp. colony growth as the chemical concentration was increased. At varying concentrations of the chemical, SAAF (carbendazim + mancozeb) and mancozeb have been shown to totally inhibit the development of *Alternaria*sp (Prasad *et al.*, 2018). In this study, SAAF (carbendazim + mancozeb) was found to have higher growth inhibition than mancozeb alone, which is consistent with the findings of Pranaya *et al.* (2020), who observed 86.1% and 96.9% inhibition in mancozeb and carbendazim + mancozeb, respectively.

Furthermore, the findings of this study agree with those of Rani *et al.*, (2018), who found that

SAAF (carbendazim + mancozeb) inhibits growth by 67.59% and 71.92% at 0.1% and 0.2% of concentration, respectively, and mancozeb inhibits growth by 58.57% and 70.98% at 0.1% and 0.2% of concentration, respectively. According to Datar (1996), mancozeb works against *Alternaria* sp. by interfering with the activity of several hormones such as Indole-3-Butyric Acid or Naphthalic Acid, altering critical plant growth and development processes. A low percent inhibition of mycelial growth using carbendazim has also been reported by Vanitha *et al.* (2013). Gaikwad (2000) found that mancozeb and carbendazim work together to suppress *Alternaria* species. The management of both pathogens necessitates a thorough examination of various aspects of the pathogen, such as its mode of action, proliferation, and mode of survival.

CONCLUSION

Several fungicides were shown to be effective in controlling *Rhizoctonia solani* and *Alternaria citri* in this investigation, but their efficacy varied. The application of Bavistin at 100 ppm and 200 ppm was found most effective for the suppression of mycelium growth of *R. solani* and SAAF @ 200 ppm against *A. citri*. Therefore, these fungicides should be tested under *in vivo* conditions for further verification and to find out the degree of control over the pathogens.

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Author's contribution

SS, NP, SR: Acquisition of data; analysis and interpretation of the data; writing the paper.
SB: Provision of research facility; writing manuscript
PBM, JS and SS: Data analysis, critical revision and approval of the final manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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