



Research Article

In Vitro Evaluation on Resistance of *Phytophthium vexans* (NG Isolate) Cultured from Sublethal Concentration against Several Fungicides

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ABSTRACT

Phytophthium vexans isolated from potatoes rhizospheres in Ngablak, Magelang, Central Java was reported to potentially cause potato tuber rot in *in vitro* test. Fungicides had been intensively applied in this area to control potato late blight, including mancozeb, dimethomorph, mixture of mancozeb and mefenoxam, and mixture of oxathiapiprolin and famoxadone. Experiment was conducted to observe the pathogen sensitivity against those fungicide and growing isolates in sublethal concentration to observe the resistance response. Based on Probit Analysis results, *P. vexans* were insensitive against dimethomorph at 50,000 ppm, but it was sensitive against mancozeb at 4,000 ppm, mixture of mancozeb and mefenoxam at 10 ppm, as well as mixture of oxathiapiprolin and famoxadone at 0.1 ppm. Mycelia were inhibited 100% after mancozeb treatment at recommended concentration. However, at sublethal concentration mycelial collony grew abnormally, which then were used for following experiment of resistance response. The effect of the sublethal application of mancozeb to *P. vexans* caused resistance to mancozeb itself and cross resistance against dimethomorph and mixture of oxathiapiprolin and famoxadone based on higher predicted EC50 and EC95 on the second experiment compared to first ones. Furthermore, the resistant *P. vexans* isolate was able to produce sporangium and chlamydospore.

Keywords: EC50; *Phytophthium vexans*; potato; resistance

INTRODUCTION

Phytophthium vexans (de Barry) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque is an Oomycetes member from genus *Phytophthium*. The sporangium appearance of this pathogen is simillar to *Phytophthora*, but the zoospores are released from vesicle which is similar to *Pythium* (Levesque & de Cock, 2008). There has been reports that *P. vexans* infect root and cause root rot on different host plants in several countries such as in Turkiye where *P. vexans* has been reported to attack kiwi tree (Polat *et al.*, 2017), ramie in China (Yu *et al.*, 2016), and durian in Indonesia (Santoso, 2016). UK CAB International in 1987, reported that *Phytophthium vexans* (Syn. *Pythium vexans*) first occurred in Sumatera and Sulawesi and potato was one of it host. The pathogen caused symptoms such as root rot, stunting, and plant death (Centre for Agriculture and Biosciences

International [CABI], 2019). Santika *et al.* (2021) also reported that NG isolates from potatoes rhizospheres in Ngablak, Magelang, Central Java Province was identified as *P. vexans* based on multi-gene analysis on ITS and LSU. It also reported that this oomycetes can cause tuber rot in potatoes with brown lesion symptom on potato tubers.

In Indonesia, small scale of potato farmers applies chemical pesticides to control plant disease especially potato late blight in Ngablak, Magelang, Central Java. Mixing several products of pesticide between different active ingredients are often done in this area. Some of the commonly used fungicides contained active ingredients of mancozeb, dimethomorph, mixture of oxathiapiprolin and famoxadone; or mixture of mancozeb and mefenoxam. This practice may lead to the emergence of pathogen resistance against chemical fungicides, especially fungicides

with single site effect and high-risk potency of fungal resistance (Fungicide Resistance Action Committee [FRAC], 2018). Resistances could happen due to the selection pressure in the field or gene mutation. Application of pesticides with multisite effect mixed with single site effect ones may reduce the risk of pathogen resistance in field (Sumardiyono, 2008). Amaradasa and Everhart (2016) used *Sclerotinia sclerotium* to study the effects of fungicide sublethal concentration under *in vitro* conditions. Results showed that eight isolates of *S. sclerotiorum* exposed to boscalid had higher EC50 values at the end of the experiment over 12 generations. More research is required to understand the effect of fungicides in sublethal concentration. This research is a continuous from the previous research by Santika *et al.* (2021) to investigate the sensitivity response and sublethal concentration effect of resistant *P. vexans* against several fungicides under *in vitro* conditions.

MATERIALS AND METHODS

P. vexans Culture Preparation

In this study, NG isolate was used from the collection of the Laboratory of Plant Disease, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. This isolate has been confirmed as *P. vexans* with accession number registered in GeneBank: MW898226 and MW911663 for ITS and LSU gene markers. The isolates were subcultured on PDA for five days at 18°C in dark condition and isolate were used for further study.

P. vexans Sensitivity Response against Fungicides

P. vexans sensitivity response against several fungicides were conducted under *in vitro* tests. The medium for pesticide treatment consists of 9 mL of PDA, 1 mL of fungicide for each concentration, and 200 µL Rifampicin in 1000 ppm. While 1 mL of sterile aquadest was used for control. Then medium mixture was poured into a petri dish and kept to solidified for several minutes. Four commercial fungicides were used to predict EC50, EC95 and later compared to recommended field concentrations of each fungicide. Baseline concentration was the field recommended concentration of each fungicide, which were: Acrobat®50 WP (a.i.: dimethomorph): 625 ppm;

Dithane®M45 (a.i.: mancozeb): 4,800 ppm; Ridomil Gold®MZ2/64 WG (a.i.: mancozeb and mefenoxam): 5,000 ppm; Zorvec Encantia®330SE (a.i.: oxathiapiprolin and famoxadone): 1200 ppm. Preliminary research was conducted to obtain concentrations for predicted EC50 based on the baseline concentration, as follow:

- 1) Acrobat®50 WP (a.i.: dimethomorph/DM) 5,000 ppm; 6,250 ppm; 12,500 ppm; 25,000 ppm; and 50,000 ppm.
- 2) Dithane®M45 (a.i.: mancozeb/MZ) 250 ppm; 500 ppm; 1,000 ppm; 2,000 ppm; and 4,000 ppm.
- 3) Ridomil Gold® MZ2/64 WG (a.i.: mancozeb/MZ and mefenoxam/MFX) 1.25 ppm; 2.5 ppm; 5 ppm; and 10 ppm.
- 4) Zorvec Encantia®330SE (a.i.: oxathiapiprolin/OXTP and famoxadone/FMX) 0.00032 ppm; 0.0016 ppm; 0.008 ppm; 0.04 ppm; and 0.1 ppm.
- 5) Control (no fungicide treatment).

Mycelial discs of *P. vexans* with 0.5 cm diameter were bored and placed in the center of PDA medium mixed with respective fungicide concentrations. Culture incubation was conducted in a growth chamber at temperature of 18 °C and dark condition. Mycelial inhibition of *P. vexans* were measured by its diagonal length and was calculated using the following formula according to Senhaji *et al.* (2013):

$$MI = \frac{(a - b)}{a} \times 100\%$$

MI = Mycelial Inhibition (%), a = Colony Diameter of *P. vexans* in control treatment, b = Colony diameter of *P. vexans* in fungicide treatment.

The EC50 obtained from this experiment was compared to the field recommended concentration of each fungicide. The fungicide treatments with similar EC95 and recommended concentrations (named as X concentration) were marked and fungal culture growth in the sublethal concentration from this fungicide (½ X concentration) was used for further experiment.

Resistance Response Experiment of *P. vexans* Growing from Sublethal Concentration

The selected isolate of *P. vexans* from sublethal concentration (½ X concentration from the 1st experiment) of mancozeb fungicide was then used for resistance testing. The experiment protocols and

concentrations were similar to previous sensitivity response test of *P. vexans*. Predicted EC50 and EC95 from this second experiment were measured and compared to those in the 1st experiment. The higher EC50 and EC95 in the second experiment was considered as the resistant potency of *P. vexans* after sublethal concentration treatment.

Analysis Data

Mycelial inhibition and pesticide treatment of *P. vexans* were used for Probit Analysis using SAS JMP Software for Windows with alpha 5% for Predicted EC50 and EC95. The output from Predicted EC50 and EC95 was visualized by sigmoid graph. If the Predicted EC50 and EC95 from culture growth from sublethal concentration (2nd experiment) were on the right side (higher) than the Predicted EC50 and EC95 from sensitivity test (1st experiment), it was considered as potentially resistance for *P. vexans*.

Morphological Observation

Morphological observation was done under microscopic observation and colony pattern on *P. vexans*. A small cut of *P. vexans* mycelial discs from PDA were placed on a glass slide and dyed using lactophenol cotton blue. Observation was conducted using microscope Olympus X32 in 1,000 \times magnification. Hypae, sporangium and chlamydospore measurements were done on 30 sample sights (Rudsari *et al.*, 2015). Colony pattern and diameter measurement were conducted on the 5th day. Colony of *P. vexans* with stellate pattern were categorized as normal and abnormal ones had circular or imperfect stellate pattern.

RESULTS AND DISCUSSION

Sensitivity Response of *P. vexans* against Fungicides

The first experiment was conducted to observe the sensitivity response of pathogen against tested fungicides. Mycelium diameter of untreated control reached 9 cm at 5 days (Table 1). Data showed that *P. vexans* were not sensitive to dimethomorph (DM). The results showed that DM at 50,000 ppm only inhibit 64% of mycelial growth. Predicted EC50 and EC95 of DM was far above the suggested concentration at 625 ppm (Table 2). On the contrary, *P. vexans* was sensitive against mancozeb (MZ), mixture of mancozeb and mfenoxam (MZ+MFX),

and mixture of oxathiapiprolin and famoxadone (OXPT+FMX). This was showed by the high percentage of mycelial inhibition on the gradient concentration of treatments. The mixture of OXPT+FMX suppressed the mycelial growth by 51% at 0.1 ppm. This results was followed by MZ+MFX by 81.56% mycelia inhibition at 10 ppm concentration. Predicted EC50 and EC95 of the OXPT+FMX and MZ+MFX was below the recommended field concentration which was 1,200 ppm and 5,000 ppm. Meanwhile, MZ caused 100% mycelial growth inhibition at 4,000 ppm. Therefore, it was pointed as the X concentration, while at 2000 ppm the mycelial inhibition was 45.44%. Isolate of *P. vexans* at 2,000 ppm ($\frac{1}{2}$ X) was cultured for 7 days and was monitored for the resistance response, which gave the following results.

P. vexans Resistance Response from Sublethal Concentration

P. vexans from sublethal concentration of MZ showed various resistance response to different fungicides treatments. The responses still showed that *P. vexans* was not affected by DM treatments. The predicted EC50 and EC95 on the second experiment was higher compared to the 1st experiment (Figure 1.A). The *P. vexans* resistance to DM was presumably affected by continuous and intensive application of this active ingredient, both as a solo treatment or in a mixture with other fungicides active ingredients. The first application of dimethomorph to control *Plasmopara viticola* in France used EC95 in range 0.25–1.15 ppm and after 10 years the EC95 significantly increased to 1–30 ppm (Corio-Costet, 2011).

The resistance of *P. vexans* was also shown on the second experiment against MZ (Figure 1.B) and OXPT+FMX (Figure 1.D). Predicted EC95 of MZ at the 2nd experiment was 19,936 ppm and it was four times higher compared to the predicted EC95 at the 1st experiment. This result showed that although MZ works as a multisite effect fungicide, it was possible to cause pathogen resistance when sublethal concentration of fungicide were applied. Malandakris *et al.* (2015) reported that *Alternaria* in tomato field in Greece were insensitive against mancozeb due to resistance due to long-term use of this active ingredient. While on OXPT+FMX,

Table 1. Colony diameter, mycelium inhibition and colony mycelium pattern of *Phytopythium vexans* treated with several fungicides on the 5th day after inoculation

Active ingredient	Concentration (ppm)	Colony diameter (cm)		Mycelium Inhibition (%)		Colony Mycelium Pattern	
		1 st test	2 nd test	1 st test	2 nd test	1 st test	2 nd test
DM	5	7.81	7.81	13.22	54.11	Normal	Abnormal
	6.25	8.19	8.19	9.03	13.56	Normal	Normal
	12.5	5.8	5.8	35.56	22.67	Normal	Normal
	25	6.34	6.34	29.56	35.56	Normal	Normal
	50	3.2	3.2	64.03	37.22	Abnormal	Normal
MZ	250	9	8.6	0	4.44	Normal	Abnormal
	500	8.1	6.88	10	23.56	Normal	Abnormal
	1	7.36	5.91	18.22	34.33	Normal	Abnormal
	2	4.91	4.38	45.44	51.33	Abnormal	Abnormal
	4	0	2.8	100	68.89	(-)	Abnormal
MZ+MFX	1.25	6.3	3.46	30	61.56	Abnormal	Abnormal
	2.5	5.48	2.09	39.11	76.78	Abnormal	Abnormal
	5	3.1	1.24	65.56	86.22	Abnormal	Abnormal
	10	1.6	0	81.56	100	Abnormal	-
OXPT+FMX	0.00032	8.66	9	4	0	Normal	Normal
	0.0016	8.26	8.63	8	4.11	Normal	Normal
	0.008	8.48	8.74	6	2.89	Normal	Normal
	0.04	6.2	7.91	31	12.11	Abnormal	Abnormal
	0.1	4.4	5.11	51	43.22	Abnormal	Abnormal
Control	0	9	9	0	0	Normal	Normal

Notes: Normal = mycelium pattern was stellate, Abnormal = mycelium pattern was imperfect stellate or circular and (-) = no mycelial growth, 1st test = sensitivity response of *P. vexans* against fungicides, 2nd test = resistance response experiment of *P. vexans* from sublethal concentration.

Table 2. Predicted EC 50 and EC 95 from two test experiments

Active ingredient	Recommended concentration (ppm)	EC	Value of Predicted EC (ppm)	
			1 st test	2 nd test
DM	625	50	34,818.00	93,642.00
		95	471,353.00	7,177,18
MZ	4.8	50	1,662.85	1,856.36
		95	5,038.86	19,936.04
MZ+MFX	5	50	2.99	0.95
		95	29.98	7.43
OXPT+FMX	1.2	50	0.15	0.24
		95	19.96	8.74

Notes: 1st test = *Phytopythium vexans* sensitivity response against several fungicides, 2nd test = resistance response experiment of *P. vexans* from sublethal concentrations.

the insensitive isolate of *P. vexans* had the EC50 value two times higher than the sensitive *P. vexans* isolates. OXPT and FMX were fungicides with single site effect, but different points of target. OXPT inhibits the oxysterol binding protein homologue and FMX as Quinone outside Inhibitors (QoI) in mitochondria. Both of the active ingredients in-

cluding OXPT+FMX has high risks for their potential which caused resistance on their targets (FRAC, 2020).

Different results were shown by cultures grown on MZ+MFX because the predicted EC50 and EC95 moved to the left of the sigmoid curve (Figure 1.C). It means that there was no increment of fungicide

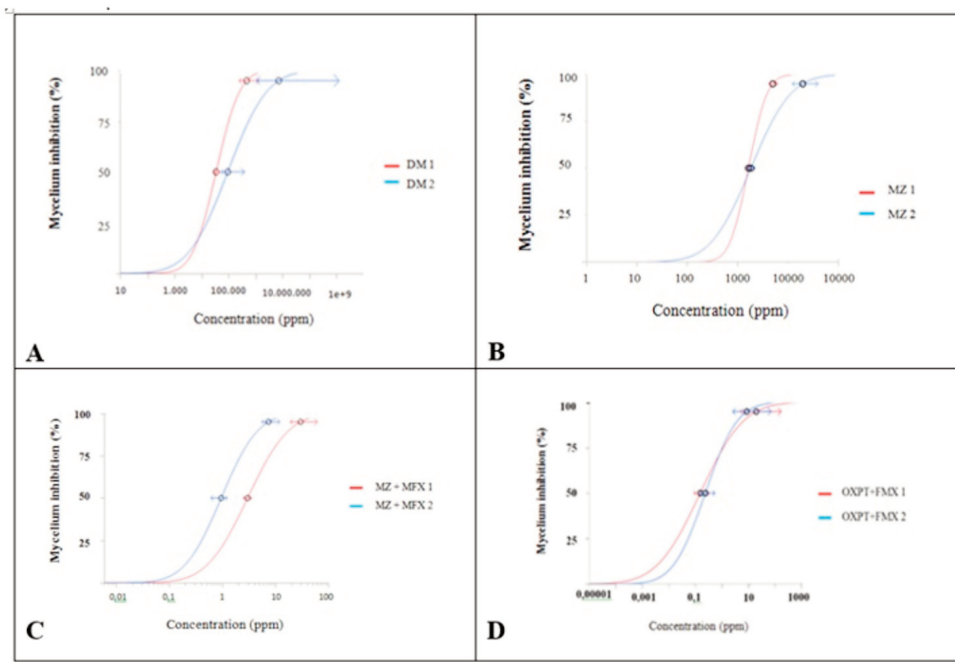


Figure 1. Probit analysis for EC50 and EC95 from food poison technique on the first test (red line) and on the second test (blue line) on different active ingredients (A) DM, (B) MZ, (C) MZ + MFX and (D) OXPT + FMX; 1 = sensitivity response of *P. vexans* against fungicides, 2 = resistant response experiment of *P. vexans* growing from sublethal concentration

concentration required to inhibit 50 or 95% of mycelial growth for *P. vexans* treated with sublethal concentration of MZ. However why the predicted EC50 and 95 of MZ+MFX against *P. vexans* was slighter compared to the recommended fungicide concentration, and how the predicted EC50 and 95 at the 2nd experiment decreased was still unclearly understood.

Morphological Observation

Normal *P. vexans* (control) had the hyphae size that ranged from 3.36–4.60 μm and 30-day-old isolate which was incubated at 25°C produced chlamydo-spore, sporangium (Figure 3.A and B) and stellate pattern (Figure 2.A). The morphology of culture grown at sublethal concentration of MZ showed abnormal hyphae structure by producing inflamed hyphae (Figure 3.D). The abnormal *P. vexans* colony showed circular pattern of culture colony instead of stellate (Figure 2. C). Empty sporangium were observed in the culture of 2nd experiment on MZ (Figure 3.E). This empty sporangium occurred because mancozeb consist of manganese and zinc as the main components to control *Phytophthora* by inhibiting the expression levels of the genes *csn4* and *csn7* which are responsible for sporangiogenesis

and zoosporangiogenesis of *P. nicotianae* (Luo *et al.*, 2020).

P. vexans produced both normal and abnormal sporangium when treated with DM under in vitro conditions. The normal sporangium had an ovoid shape with size length of 19.67 and width 13.54 μm . The abnormal sporangium was completed with long discharge tube which is grown from the middle part of the sporangium. The size of sporangium length was 21.94 μm and width was 10.32 μm (Figure 3.C). Until now, there were no reports regarding sporangium abnormalities due to the misuse of dimetomorph. However, based on research by Kuhn *et al.* (1991), the application of dimetomorph to *Phytophthora* were associated with extensive proliferation and aberrant deposition of cell-wall material. There was no abnormality on hyphae and collony pattern as stellate except for 50,000 ppm on sensitive response test and 5,000 ppm on resistance response. This case was also reported in Sweden where four isolate of *P. infestans* were insensitive to propamocarb HCl and sporulating ability was affected at 1000 ppm treatment. (Mazáková *et al.*, 2011).

P. vexans treated with OXPT+FMX at 0.1 ppm concentration showed inflamed hyphae. The width

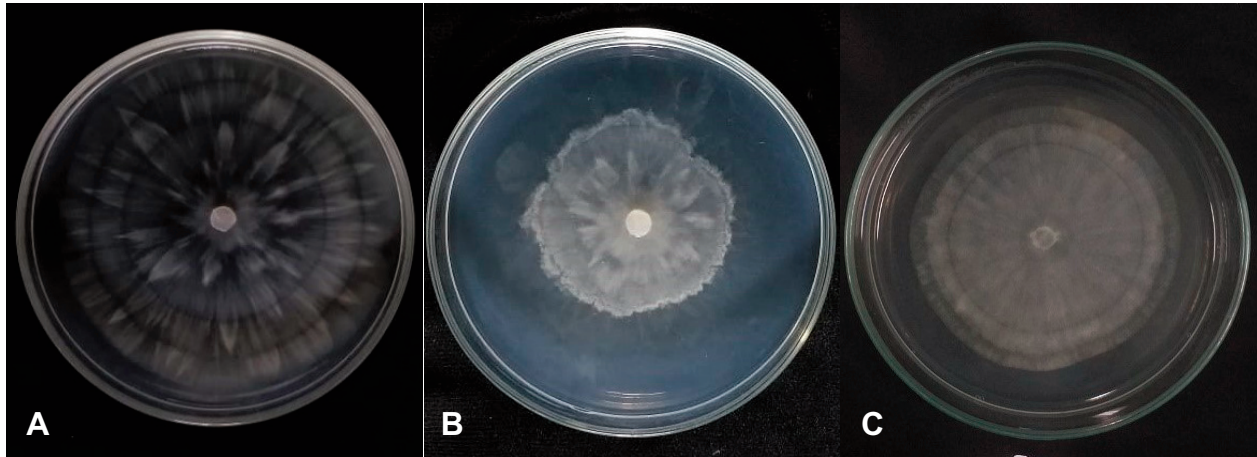


Figure 2. Collony pattern of *Phytophthium vexans* (A) stellate pattern as normal, (B) imperfect stellate and (C) circular stellate

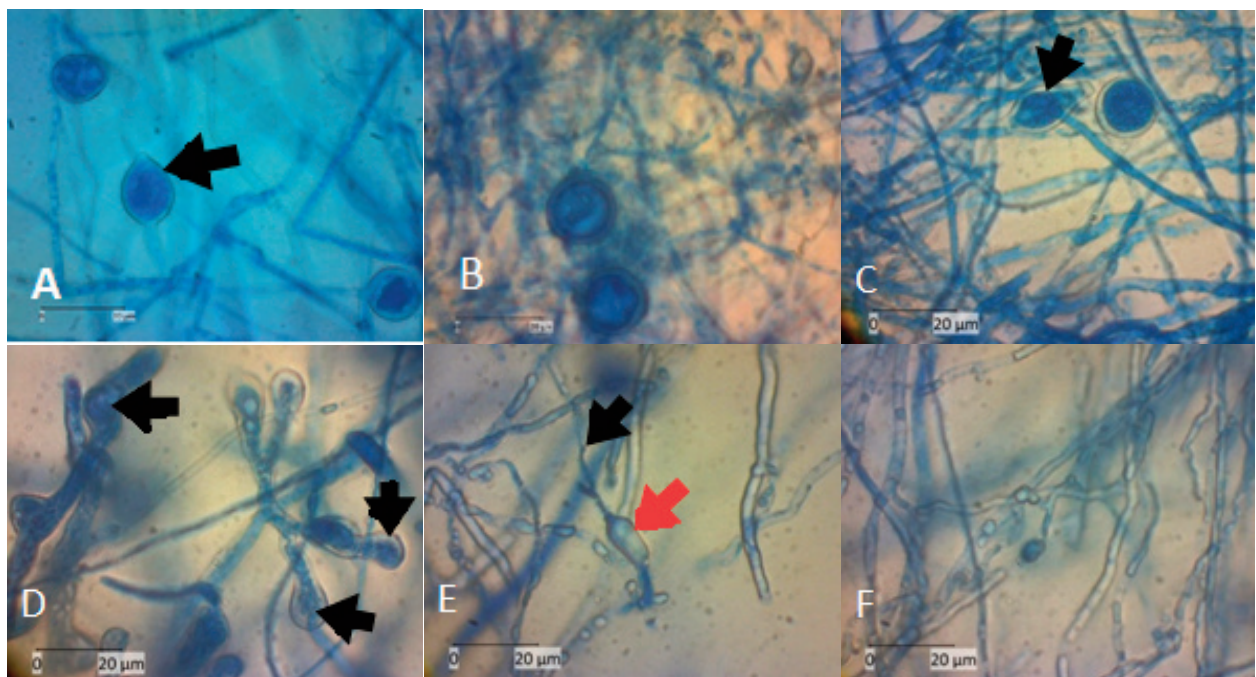


Figure 3. Morphology of *Phytophthium vexans* under microscope; *P. vexans* isolates in control with (A) ovoid shaped sporangium with papillate (→) and (B) chlamydospore; *P. vexans* isolates treated with fungicides, (C) abnormal sporangium completed with long discharge tube (→) (D) abnormal inflated hyphae (→), (E) empty sporangium (→) and abnormal hyphae lysis (→) (F) abnormal hyphae lysis

of hyphae was 5.69–8.97 µm, which was wider than the hyphal size from control (untreated) treatment. Chlamydospore had diameter of 14.02 µm, but sporangium was not found. In the resistance assessment of oxathiapiprolin against *Phytophthora capsisi* by Miao *et al.* (2016), HNJJ10-mutants isolates had significantly fewer number of sporangia under *in vitro* test and failed to produce any sporangia on detached bell pepper leaves compared to the parental isolates. Based on research conducted by Cohen *et*

al. (2018), oxathiapiprolin inhibited zoospore release from sporangium and stopped the growth of mycelium *P. infestans*. Meanwhile, famoxadone has a specific region site in cytochrome-b to inhibit the respiration in mitochondria (FRAC, 2018). Based on this experiment, OXPT+FMX inhibited mycelium growth and sporangium production. However, its resistance potency should be considered, as the predicted EC₅₀ at the 2nd experiment was higher than it was at the 1st experiment.

The mixture of MZ+MFX inhibited the mycelium growth of *P. vexans* by not producing any chlamydospore and sporangium. The hyphae became abnormal, and the width was shorter than control (untreated) treatment with average width of 1.4–2.70 μm (Figure 3.F). The colony pattern were abnormal with imperfect stellate pattern (Figure 2.B). The morphology changes in colonies because of the presence of mancozeb might be caused by the presence of both manganese and zinc that act effectively on the mycelium (Manalu *et al.*, 2020).

CONCLUSION

Based on this study, *P. vexans* was sensitive to mancozeb at recommended field concentrations. However, when sublethal concentration was applied, pathogen resistance occurred indicated by increasing EC50 and EC95 prediction of similar or other tested fungicides (mixture of oxathiapiprolin and famoxadone, and dimethomorph). Abnormal microscopic morphologies were different depending on fungicide treatment and included several characteristics, such as empty sporangium on mancozeb, inflamed hyphae on mancozeb and mixture of oxathiapiprolin and famoxadone, abnormal long discharge tube on dimethomorph, the lysis of hyphae on mixture of mancozeb and mefenoxam.

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