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In vitro and field responses of various active ingredients to *Fusarium proliferatum* species which causes Fusarium root rot disease in Indian mulberry (*Morinda officinalis* How.) in Thai Nguyen

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Abstract:

***Fusarium proliferatum* has been identified as the causal pathogen of Fusarium root rot of Ba kich (*Morinda officinalis*) (FRRBK). No studies are available regarding the effectiveness of chemical treatments on the disease incidence of FRRBK. The efficacy of five active ingredients (metconazole, prochloraz, tebuconazole, kresoxim-methyl, and pyraclostrobin) from two chemical groups (demethylation inhibitors and quinone outside inhibitors) in reducing three isolates (BKVN, BKDT, and BKPL) of *F. proliferatum* mycelial growth was tested *in vitro*. The results indicate that only metconazole, prochloraz, and tebuconazole are highly effective in inhibiting the mycelial growth of *F. proliferatum*. These were selected for investigation of their efficacy with regard to the disease incidence of FRRBK in pot and in field conditions. Prochloraz and metconazole showed the highest efficacy and significantly suppressed the disease incidence of FRRBK in pot and in field conditions.**

Keywords: **Ba kich, demethylation inhibitors, *Fusarium proliferatum*, *Morinda officinalis*, quinone outside inhibitor.**

Classification number: **3.1**

Introduction

In Vietnam, Indian mulberry (*Morinda officinalis* How.) is locally known as 'Ba kich' and is widely grown in many mountainous provinces in the north of Vietnam. In Thai Nguyen province, Ba kich is mainly planted in Vo Nhai, Dai Tu, and Phu Luong districts for use in traditional medicine as it is rich in various medicinal compounds. Therefore, Ba kich is considered a cash crop, which brings substantial income for small households.

Fusarium root rot of Ba kich (FRRBK), caused by *Fusarium proliferatum*, is a widespread soilborne disease that causes serious damage and significant economic losses to Ba kich production in Vietnam in general and in Thai Nguyen in particular [1]. Several bio-products have been applied for the management of FRRBK in Thai Nguyen province. Tests of the efficacy of a new bio-product, MICROTECH-1$^{(NL)}$, were conducted in *in vitro*, pot, nursery, and field conditions. In *in vitro* antagonistic assay, MICROTECH-1$^{(NL)}$ significantly inhibited the mycelial growth of *F. proliferatum*. Double application of MICROTECH-1$^{(NL)}$ (applied both in the nursery and in the pot soil) significantly reduced disease incidence and markedly increased the number of plant-beneficial bacteria and actinomycota in rhizoplane Ba kich. In field conditions, double application of MICROTECH-1$^{(NL)}$ (both in nursery and in field soils) significantly decreased disease incidence compared to single application in either nursery or field.

However, bio-products usually act more slowly than chemical fungicides. Therefore, to immediately suppress the spread of the disease in nursery and field conditions and to maintain the plant's yield, chemical fungicides are the better choice. In addition, no fungicides have been identified and registered specifically to control FRRBK in Vietnam; therefore, the effect of five active ingredients (a.i.) that are either demethylation inhibitor (DMI) (metconazole, prochloraz and tebuconazole) or quinone outside inhibitor (QoI) (kresoxim-methyl and pyraclostrobin) fungicides on the growth of the causal pathogen *in vitro*, and their efficacy on reducing the disease incidence of FRRBK in pot and in field conditions are assessed.

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Materials and methods

Inoculum preparation

Three isolates of *F. proliferatum*, BKVN (Ba kich growing in Vo Nhai district), BKDT (Dai Tu district), and BKPL (Phu Luong district), which were previously identified as causal agents of FRRBK in Thai Nguyen province [1], were cultured on potato dextrose agar (PDA) plates for three days at 25^{0}C in dark conditions. A piece of PDA medium containing mycelium of each isolate was grown in potato dextrose broth at 25^{0}C with shaking at 120 rpm. After seven days of incubation, the harvested fungal suspensions were adjusted to 5×10^6 cfu/ml and used as inocula for further experiments.

Plants and a.i.

One-year old Ba kich plants that had previously been grown in small plastic pots (5×15×5 cm) were used. Five a.i. (Table 1) were applied in this study to evaluate their effects on the growth of *F. proliferatum* in *in vitro* condition; the highly effective a.i. were selected for their efficacy on the disease incidence of FRRBK in pot and in field conditions.

Table 1. Chemical active ingredients (a.i.) used in this study.

a.i.	Commercial name	a.i. tested (mg/l)	Group name	Mode of action
Metconazole	Workup 9 SL (Metconazole, min. 94%)	1-10-100	Demethylation inhibitors (DMI)	Sterol biosynthesis in membranes
Prochloraz	Agrivil 250EC (Prochloraz, min. 97%)	5-50-500	DMI	Sterol biosynthesis in membranes
Tebuconazole	Folicur 430SC (Tebuconazole, min. 95%)	3-30-300	DMI	Sterol biosynthesis in membranes
Kresoxim-methyl	1Sosim 300SC (Kresoxim-methyl, min. 95%)	10-100-1,000	Quinone outside inhibitor (QoI)	Inhibition of cell respiration (mitochondria). Inhibition of spore germination, germ tube elongation, mycelial growth and sporulation.
Pyraclostrobin	Headline 200FS (Pyraclostrobin)	10-100-1,000	QoI	Inhibition of cell respiration (mitochondria). Inhibition of spore germination, germ tube elongation, mycelial growth and sporulation.

Mycelial growth inhibition of F. proliferatum by different a.i.

The effect of different a.i. on the mycelial growth of *F. proliferatum* was determined by growing *F. proliferatum* on PDA plates (90 mm diameter). The experiment was replicated five times (one Pestri plate per replication) and the mean values were calculated. Each a.i. was separately applied to 100 ml of sterilised PDA medium at 60^{0}C before being divided equally into five Petri plates at the rates indicated in Table 1. Control plates were prepared by replacing a.i. with sterile distilled water. Mycelial discs of *F. proliferatum* of 5 mm diameter were transferred from the seven-day old growth colony to the PDA plates, tested, and incubated at 25±1^{0}C for seven days. Colony diameter growth was measured and recorded on the seventh day after treatment. The diameter of the *F. proliferatum* colony was measured in two directions at right angles to each other, and the average colony diameter was calculated. Measurement of the growth of *F. proliferatum* was undertaken at intervals of 24 hours for seven days. The inhibition percentage of each a.i. on the mycelial growth of *F. proliferatum* was determined on the seventh day of incubation following the formula $(1\text{-}C_n/C_o) \times 100$, where C_n is the average diameter of the *F. proliferatum* colony on the treatment plates, and C_o is the average diameter of *F. proliferatum* colony on the control plates.

Effect of a.i. on disease incidence of FRRBK in pot conditions

Based on the inhibition percentage (%) of each a.i. on the mycelial growth of *F. proliferatum*, three a.i. were selected and subjected to analysis of their effects on the disease incidence of FRRBK in pot conditions (20×40×20 cm). An eight-leaf (about one-year old) seedling was grown in each pot containing *F. proliferatum*-infested soil. The preventive and curative effects of each a.i. were determined according to following experiments: (1) the a.i. was drenched in the soil one week before pathogen inoculation, (2) the a.i. and the pathogen spores were applied simultaneously, and (3) the a.i. was drenched in the soil one week after pathogen inoculation. Spore suspensions of BKVN, BKDT and BKPL isolates were applied separately as drenches. These experiments were replicated three times using a completely randomised block design and 30 pots (30 plants) per treatment.

Disease severity was recorded on 0 to 3 visual scales as previously described by Trabelsi, et al. (2017) [2]. All infected plants in each treatment were recorded for disease incidence (%) using the following formula:

$$\text{Disease incidence (\%)} = \frac{(\sum \text{scale} \times \text{number of plants infected})}{(\text{highest scale} \times \text{total number of plants})} \times 100$$

Efficacy of a.i. on FRRBK in field conditions

Three a.i. were applied in field experiments in Vo Nhai district to determine their efficacy on FRRBK. The experiments were replicated three times with a completely randomised block design and a plot size of 24×6 m planted with 100 Ba kich plants per treatment. The efficacy (E) of the a.i. was calculated as follows:

$$E = 1 - \frac{T_a \times C_b}{T_b \times C_a} \times 100$$

where: E: the efficacy of the active ingredient (%); T_a: disease incidence in the treatment after applying the a.i.; T_b: disease incidence in the treatment before applying the a.i.; C_a: disease incidence in the control after application; and C_b: disease incidence in the control before application.

Data analysis

The collected data were summarized as mean ± standard deviation and analysed using Statistix 10 software.

Results and discussion

Effect of different a.i. on the inhibition of the mycelial growth of F. proliferatum

In a dual-culture assay, the a.i. tested inhibited the growth of *F. proliferatum* BKVN, BKDT, and BKPL isolates by at least 48%; the highest values were obtained with prochloraz and metconazole. All DMI fungicides showed excellent activity in suppressing the mycelial growth of *F. proliferatum* up to seven days of incubation. In particular, metconazole and prochloraz completely suppressed colony growth of *F. proliferatum* at the concentrations of 10, 100 and 50, 500 mg/l, respectively. At 1 and 5 mg/l, the two a.i. inhibited up to more than 83-91% of the growth of *F. proliferatum* BKVN, BKDT, and BKPL isolates (Fig. 1). The application of another DMI fungicide, tebuconazole, also inhibited the mycelial growth of *F. proliferatum* BKVN, BKDT, and BKPL isolates by 70.38%, 71.80%, and 72.04%, respectively. The other two QoIs, kresoxim-methyl and pyraclotrobin, showed low inhibition (lower than 80% and 62%, respectively) (Fig. 1).

Effect of a.i. on the disease incidence of FRRBK in pot conditions

Treatment with a.i. one week before inoculation with *F. proliferatum* significantly reduced the disease incidence of FRRBK compared to the inoculated-untreated control. Of the a.i. tested, prochloraz and metconazole showed the highest suppression of disease incidence. The results indicated that with prochloraz treatments, disease incidence ranged from 2.59% for the BKDT isolate to 3.70% for the BKPL isolate. With metconazole treatments, disease incidence ranged from 4.07% for the BKVN isolate to 4.81% for the BKPL isolate. With the tebuconazole treatments, disease incidence ranged from 7.04% for the BKVN isolate to 9.26% for the BKDT isolate (Table 2).

Table 2. Disease incidence when Ba kich plants were treated with a.i. one week before the plants were inoculated with *F. proliferatum* spores.

a.i.	Disease incidence (%)		
	F. proliferatum BKVN	*F. proliferatum* BKDT	*F. proliferatum* BKPL
Metconazole	4.07^{c}	4.44^{c}	4.81^{c}
Prochloraz	3.33^{c}	2.59^{c}	3.70^{c}
Tebuconazole	7.04^{b}	9.26^{b}	8.15^{b}
Inoculated-untreated control	81.11^{a}	80.37^{a}	82.59^{a}
Non-inoculated-untreated control	0.00^{d}	0.00^{d}	0.00^{d}
p	<0.05	<0.05	<0.05
CV(%)	3.25	8.67	6.37
$LSD_{0.05}$	0.62	1.68	1.25

Note: values followed by a different letter(s) in the same column are significantly different (p<0.05).

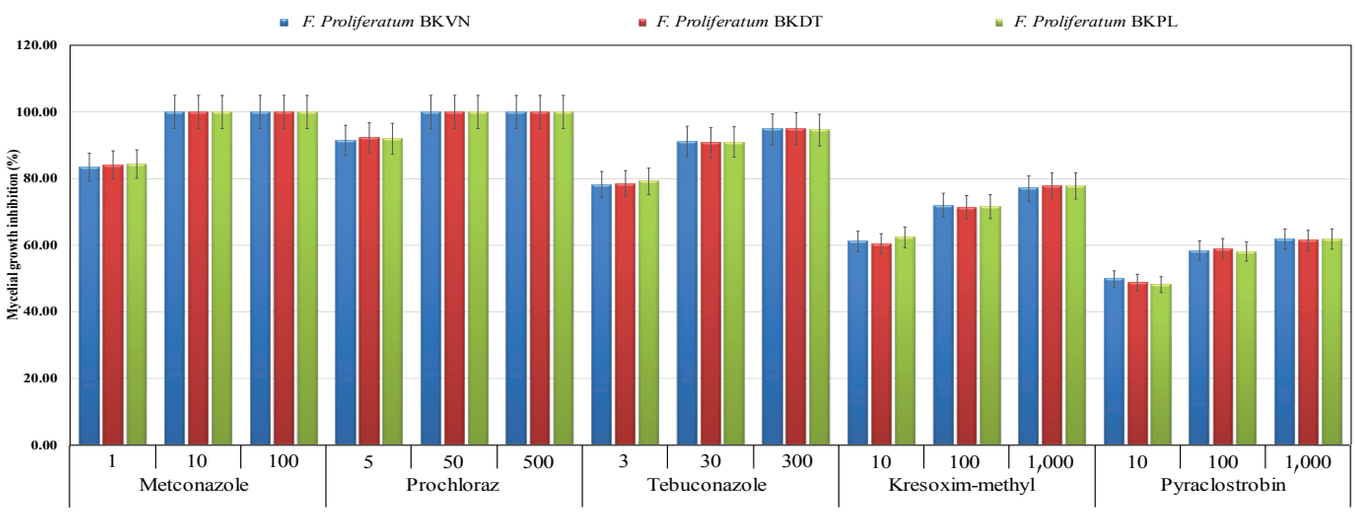

Fig. 1. The inhibition of the tested a.i. on mycelial growth of *F. prolifertum* isolates BKVN, BKDT, and BKPL. Three different concentrations of each a.i. (mg/l) were amended into PDA plates before the culturing of each isolate. Colony growth in diameter was measured and the inhibition values (%) were calculated.

The decrease of disease incidence when a.i. were applied simultaneously with inoculation by *F. proliferatum* spores showed similar trends, although the disease incidence was higher than when a.i. were applied one week before inoculation with *F. proliferatum*. Disease incidence in treatments with prochloraz or metconazole was statistically differentiated compared to treatments with tebuconazole and the inoculated-untreated control. Disease incidence with all a.i. ranged from 7.78% with prochloraz for the BKDT isolate to 13.70% with tebuconazole with BKVN isolate (Table 3).

Table 3. Disease incidence when Ba kich plants were treated with a.i. at the same time as the plants were inoculated with *F. proliferatum* spores.

a.i.	Disease incidence (%)		
	BKVN	***BKDT***	***BKPL***
Metconazole	9.26^{c}	9.63^{c}	10.74^{c}
Prochloraz	8.15^{c}	7.78^{c}	8.52^{c}
Tebuconazole	13.70^{b}	15.56^{b}	17.78^{b}
Inoculated-untreated control	81.11^{a}	80.37^{a}	82.59^{a}
Non-inoculated-untreated control	0.00^{d}	0.00^{d}	0.00^{d}
p	<0.05	<0.05	<0.05
CV(%)	4.18	12.74	6.12
$LSD_{0.05}$	0.74	2.28	1.14

Note: values followed by a different letter(s) in the same column are significantly different (p<0.05).

Treatment with a.i. one week after inoculation with *F. proliferatum* also reduced disease incidence and showed similar trends. For the treatment with prochloraz, disease incidence was 13.70% for the BKDT isolate and 14.81% for the BKVN isolate. For the treatments with metconazole, disease incidence ranged from 18.52% for the BKDT isolate to 20.74% for the BKPL isolate. Disease incidence was more than 20% and reached 23.33% with tebuconazole (Table 4).

Table 4. Disease incidence when Ba kich plants were treated with a.i. one week after the inoculation with *F. proliferatum* spores.

Active ingredient	Disease incidence (%)		
	BKVN	***BKDT***	***BKPL***
Metconazole	18.89^{c}	18.52^{bc}	20.74^{b}
Prochloraz	14.81^{d}	13.70^{c}	14.07^{c}
Tebuconazole	22.22^{b}	23.33^{b}	20.37^{b}
Inoculated-untreated control	81.11^{a}	80.37^{a}	82.59^{a}
Non-inoculated-untreated control	0.00^{e}	0.00^{d}	0.00^{d}
p	<0.05	<0.05	<0.05
CV(%)	2.52	10.89	5.41
$LSD_{0.05}$	0.55	2.35	1.24

Note: values followed by a different letter(s) in the same column are significantly different (p<0.05).

Efficacy of a.i. on FRRBK in field conditions

Double application of a.i. in the field significantly decreased disease incidence in comparison to the control. Among the three tested a.i., prochloraz showed the highest efficacy on FRRBK in the field condition. Its efficacy was 67.75% after one month of treatment, reached a maximum of more than 69% after 2-3 months after treatment, and started decreasing thereafter. Treatments with metconazole also significantly reduced the disease incidence of FRRBK, resulting in efficacy of 61.63% at one month after treatment; this reached a maximum of 65.66% at two months after treatment before gradually decreased thereafter. Tebuconazole also suppressed disease incidence of FRRBK in field conditions; however, its efficacy was lower than that of either prochloraz or metconazole (Table 5).

Table 5. Efficacy of different a.i. on FRRBK in field conditions.

Active ingredient	DI (%) before treatment	1 MAT		2 MAT		3 MAT		4 MAT		5 MAT	
		DI (%)	*Efficacy (%)*	*DI (%)*	*Efficacy (%)*	*DI (%)*	*Efficacy (%)*	*DI (%)*	*Efficacy (%)*	*DI (%)*	*Efficacy (%)*
Metconazole	6.56	10.33	61.63^{b}	12.67	65.66^{a}	16.33	61.88^{ab}	23.00	57.98^{ab}	33.89	53.87^{b}
Prochloraz	6.22	8.33	67.75^{a}	10.78	69.81^{a}	12.56	69.87^{a}	18.33	64.71^{a}	27.78	60.43^{a}
Tebuconazole	6.33	11.44	56.01^{c}	15.00	58.38^{b}	17.78	58.08^{b}	23.56	53.90^{b}	33.78	52.10^{b}
Control	6.44	26.56	-	36.78	-	43.44	-	54.00	-	72.22	-
p			<0.05		<0.05		<0.05		<0.05		<0.05
CV(%)			4.95		6.10		9.91		10.64		7.81
$LSD_{0.05}$			1.87		2.41		3.84		3.83		2.65

Note: DI: disease incidence, MAT: month after treatment. Values followed by a different letter(s) in the same column are significantly different (p<0.05).

F. proliferatum has been reported as the causal pathogen of FRRBK in Vietnam [1]. To investigate the effects of different fungicides on *F. proliferatum*, DMI (metconazole, prochloraz, and tebuconazole) and QoI (kresoxim-methyl and pyraclostrobin) fungicides were evaluated in this study. Metconazole, prochloraz, and tebuconazole are sterol demethylation inhibitors that inhibit the C-14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of the ergosterol of fungi [3]. Kresoxim-methyl and pyraclostrobin are QoIs that inhibit mitochondrial respiration by binding to cytochrome *c* oxidoreductase, leading to an energy deficiency due to a lack of Adenosine triphosphate (ATP) [4].

Our study reveals that DMI fungicides are more effective in inhibiting the mycelial growth of *F. proliferatum* than are QoI fungicides (Fig. 1). Among the tested a.i., prochloraz and metconazole were the most effective fungicides for inhibiting mycelial growth of *F. proliferatum*. Tebuconazole, another DMI fungicide, exhibited lower levels of inhibitory effects on the growth of *F. proliferatum*. The two QoI fungicides, kresoxim-methyl and pyraclostrobin, were the least effective (Fig. 1). Our results suggest that *F. proliferatum* is more sensitive to prochloraz and metconazole than to tubecunazole, kresoxim-methyl, and pyraclostrobin. The most effective fungicide was prochloraz, followed by metconazole and tebuconazole, among the a.i. tested for the inhibition of mycelial growth of *F. proliferatum.* In pot and field conditions, prochloraz had the highest efficacy in suppressing disease incidence in comparison with other fungicides and the control.

Previous studies indicate that DMI fungicides effectively control different diseases caused by *Fusarium*, such as *F. graminearum*, which causes Fusarium head blight in wheat [5], *F. oxysporum*, which causes Fusarium wilt in bananas [6], *F. subglutinans* and *F. temperatum*, which causes maize stalk rot [7], *F. proliferatum*, which causes Fusarium bulb rot in garlic [8], and *F. proliferatum*, which contaminates cereals [9]. Other studies have also revealed the greater efficacy of DMI fungicides on *Fusarium* species than the other groups of fungicides. Among those groups, prochloraz and tebuconazole have been proven to be more effective against *Fusarium* spp. than kresoxim-methyl [10, 11]. *Fusarium* species were more sensitive to fungicides belonging to the DMI group but were intrinsically resistant to complex III respiration inhibitors [12].

Our results show the high effectiveness of DMI fungicides (prochloraz, metconazole, and tebuconazole) in inhibiting mycelial growth of *F. proliferatum*, although tebuconazole failed to control FRRBK in field conditions (its efficacy was as low as 58%), suggesting that there were some factors might affect the efficacy of a.i. in field conditions. These are pathogen inocula in soil, fungicide application, soil temperature and relative humidity, and soil fertility [9, 13, 14].

Conclusions

Of the tested a.i., prochloraz and metconazole showed the highest efficacy in inhibiting the growth of *F. proliferatum* in *in vitro* conditions, and significantly suppressed disease incidence of FRRBK in pot and in field conditions.

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

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