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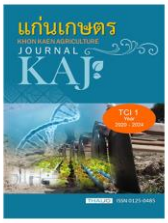

Quinone outside inhibitor fungicides combine with salicylhydroxamic acid to inhibit leaf spotting fungi from hydroponic lettuce for sensitivity *in vitro* test

Pornprapa Kongtragoul[1]*, and Anjana Junpatiw Ahuja[1]

[1] *Management Technology for Plant Production, Department of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus, Chumphon, Thailand*

ABSTRACT: Hydroponics is a popular alternative to conventional growing approaches in Thailand, and many farmers use this method, especially to grow lettuce. Several different types are common: green oak, red oak, butterhead, red coral, and frillice iceberg. However, outdoor hydroponics in tropical zone presents a problem for farmers due to the fungal disease known as leaf spot. Prevent treatments used for leaf spots include a class of fungicides categorized as quinone outside inhibitor (QoI) fungicides. The principal types are trifloxystrobin (TF), azoxystrobin (AZ), and pyraclostrobin (PR). This study visited commercial hydroponics operations and took 17 fungal pathogen isolates from lettuce plants exhibiting leaf spot disease. Among these isolates, there were 9, 5, 2, and 1 isolates of *Cercospora* sp., *Corynespora cassiicola*, *Curvularia* sp., and *Alternaria* sp., respectively. The inhibition of mycelial respiration on potato dextrose agar (PDA) as a consequence of the combined effect of TF, AZ, and PR with salicylhydroxamic acid (SHAM) were evaluated with the classification of the levels of sensitivity based on 50% effective concentrations (EC_{50}). The SHAM reaction demonstrated synergism in each of the tested fungicides with a synergy factor greater than 1. In the case of the two isolates of *C. cassiicola*, a high degree of resistance (HR) was reported with the isolated code as RO_PT012 to TR, AZ, and PY, and isolate RO_PT013 to AZ and TR, with an EC_{50} value above 100 mg/l. Furthermore, a majority of the fungal isolates were categorized as sensitive (S) to the QoI fungicides tested, having EC_{50} values lower than 10 mg/l. Therefore, it was concluded that SHAM showed a synergistic effect with the QoI fungicide, which was significant while assessing the level of QoI sensitivity under *in vitro* testing.

Keywords: alternative oxidase, fungicide resistance, leaf spot management, strobilurin fungicides

Introduction

In Thailand, hydroponics has provided a means of expanding vegetable production for salads, such as lettuce. The advantage of farmers is that crops grown using hydroponic methods gain higher prices than those grown in soil. Most commercial hydroponic operations involve temperate lettuce crops such as green and red oak, butterhead, or red coral (Wattanapreechanon and Sukprasert, 2012; Wattanapreechanon and Sukprasert, 2016). However, these temperate lettuce crops are susceptible to leaf spotting fungi when grown in hydroponics systems of Thailand (Koohakan *et al.*, 2008), although it tends to increase during the rainy season. Several fungi can cause leaf spot diseases, including *Alternaria* sp., *Cercospora* sp., *Corynespora* sp., *Curvularia* sp., and *Mycocentrospora* sp. (Koohakan et al., 2008; Chairin et al., 2017; O'Neill, 2019). It is a common practice to use foliar systemic fungicides such as methyl benzimidazole carbamates (MBC) and quinone outside inhibitors (QoI) when growing lettuce using

* Corresponding author: pornprapa.ko@kmitl.ac.th

Received: date; February 2, 2021 Accepted: date; September 20, 2021 Published: date; February 5, 2022

hydroponic methods (O'Neill, 2019). Among the various types of antifungal treatments, QoI fungicides (azoxystrobin, trifloxystrobin, and pyraclostrobin) have broadspectrum effectiveness against each of the four fungal groups (Basidiomycota, Ascomycota, and Oomycota) which cause diseases in lettuce (O'Neill, 2019). Their fungicidal action is systemic, achieving translaminar, curative, preventative effects through inhibiting fungal respiration by disrupting the electron transport chain. This ensures that ATP synthesis cannot occur because the QoI fungicides will attach to the Qo site of Complex III inside the mitochondrion. Therefore, the ability of treated fungi respiratory will be severely reduced (Gisi et al., 2002). As a consequence of this activity at a single site, which is typical of QoI fungicides, it is relatively simple to address the issue of fungal sensitivity to the active ingredient through point mutations that take place at the target site in the *cytochrome b* (*cytb*) gene (Gisi et al., 2002; Fernández-Ortuño et al., 2008). Therefore, it is a case that the risk of resistance development to these QoI fungicides is quite high for several pathogens that are targeted. Some of the plant pathogens can also resist to QoI fungicides by inducing an alternative respiration pathway, enabling complexes III and IV to be bypassed within the mitochondrial respiration chain, the evidence for which lies in the presence of the respiratory enzyme alternative oxidase (Jin et al., 2009; Seyran et al., 2010). However, it remains the case that this alternative respiration phenomenon has only been seen within *in vitro* experiments. It is believed that this alternative respiration pathway does not naturally arise when fungi encounter QoI fungicides since the flavones produced by plants can inhibit the induction of alternative oxidase (AOX) inhibitors. There have been no reports to date explaining any fungus to use alternative respiration. Therefore, it is normal practice to employ alternative oxidase inhibitors in the medium, such as salicylhydroxamic acid (SHAM) to alternative respiration (Jin et al., 2009; Seyran et al., 2010). In earlier study, the response of *Mycosphaerella fijiensis* to QoI fungicides mixed with SHAM had a better inhibition on the mycelial growth (Sierotaki et al. 2000). This study aimed to (i) collect and isolate the fungal pathogens responsible for leaf spot diseases in hydroponically grown lettuce, (ii) evaluate the sensitivity of the fungal pathogens against azoxystrobin, pyraclostrobin, and trifloxystrobin using mycelial growth assays. (iii) the toxic effects of azoxystrobin, pyraclostrobin, and trifloxystrobin in combination with the AOX inhibitor SHAM on leaf spotting fungi.

Materials and Methods

Fungicides

Commercial formulation of azoxystrobin (AZ; 25% a.i.), pyraclostrobin (PY; 25% a.i.), and trifloxystrobin (TR; 50% a.i.) were used in this study. Salicylhydroxamic acid (SHAM) was purchased from Sigma-Aldrich, Germany.

Isolation of leaf spotting fungi

The samples of hydroponically grown lettuce leaves exhibiting leaf spot symptoms were collected from commercial varieties in outdoor farms located in the districts of Pathio (red oak (RO) and green oak (GO)) and Sawi (green oak (GO), cos (Co), butter head (Bh), and frillice iceberg (Fi)), Chumphon province, Thailand in 2018.

The infected leaf tissue was employed at the Plant Disease Clinic, King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus, Thailand, to isolate the fungal pathogens. The technique involved slicing out a small portion of the leaf, which showed signs of leaf spot infection. The surface of leaf tissue was then sterilized for 2 minutes using sodium hypochlorite (10% Clorox®, USA) before washing sterilized distilled water and

drying using a blotting approach with sterile paper towels under a laminar airflow hood. Once dried, the collected tissue samples were pasted on the plates of water agar for incubation at room temperature. Colony growth in the culture was observed and recorded every day. Hyphal tips were transferred to a potato dextrose agar (PDA) (Difco, USA). This study of fungicide sensitivity used the pure culture from each isolate.

The sensitivity of lettuce leaf spotting fungi to QoI fungicides

Mycelial growth assays were used to assess fungicide sensitivity by poisoned food technique. The diluted suspensions of TR, AZ, and PY were prepared in sterile distilled water and added to PDA after autoclaving at the final concentration of 0, 1, 10, and 100 mg (a.i.)/l. Seventeen isolates of lettuce leaf spotting fungi were grown for 3-7 days on PDA plates at 25°C, before the mycelial discs of diameter 5 mm were cut from the margins of the growing colony and placed on PDA modified with each of the various fungicide concentrations with four replications. The percentage of mycelial growth inhibition achieved by each of the fungicide treatments were determined using the formula given as: [(colony diameter mean on the control medium – colony diameter mean on the medium with fungicide)/(colony diameter mean on the control medium) × 100] (Kongtragoul et al., 2011). The mycelial growth inhibition values as percentages were plotted as probits versus the $\log_{10}$ of the fungicide concentration before linear regression (Y=a+bX) was conducted. The regression equation was used to appraise a 50% effective concentration (EC_{50}) of each fungicide to inhibit the mycelial growth of each isolate. The EC_{50} values were used to recognize three categories for sensitivity assays. The isolates with EC_{50} values < 10 mg/l were considered sensitive (**S**); isolates with EC_{50} values of 10 to 100 mg/l were classified as intermediately resistant (IR), and isolates with EC_{50} values greater than 100 mg/l were considered highly resistant (HR) (Torres-Calzada et al. 2015).

The effect of QoI fungicide toxicity with salicylhydroxamic acid on mycelia growth

Seventeen isolates were tested using TR, AZ, and PY, with the AOX inhibitor SHAM to determine the synergistic effects. The TR, AZ, and PY were prepared in sterile distilled water and added to the medium after autoclaving at the final concentration of 0, 1, 10, and 100 mg (a.i.)/l. SHAM was dissolved in methanol and added to TR, AZ, and PY amended PDA. The test for synergistic effects was performed both with and without 100 mg/l of SHAM (Kongtragoul et al., 2020). The fungal isolates were grown for 3-7 days on PDA plates at 25°C, before the mycelial discs of diameter 5 mm were cut from the margins of the growing colony and placed on PDA modified with each of the various fungicide concentrations both with SHAM or without before testing was performed with four replications. The colony diameter was then observed and recorded after 7-14 days of incubation at 25°C. At that point, the formula was given as follows used to determine the mycelial growth percentage [(colony diameter mean on the medium with treatment)/(colony diameter mean on the control medium) × 100]. It was determined the synergy factor (SF) using mycelial growth percentage without SHAM, divided by the percentage of mycelial growth with SHAM, as first explained by Zhang et al. (2011). The experiment was arranged in factorial in CRD with 4 replications. Data were subjected to Statistix 8 analytical software. Mean of treatment was compared by least significant difference (LSD) at $P \leq 0.01$.

Results and Discussion

Fungal pathogens obtained from leaf spot in hydroponically grown lettuce

There were 17 isolates of leaf spotting fungi **Table 1**. Nine isolates of *Cercospora* sp. (GO_08, GO_09, GO_12, Co_43, Co_46, Bh_32, Bh_36, Fi_49, Fi_53) were isolated from Sawi farm. Eight isolates were isolated from Pathio farm, including five isolates (RO_PT012, RO_PT013, RO_PT015, RO_PT018, GO_PT003) of *Corynespora cassiicola*, two isolates (GO_PT005, GO_PT003) of *Curvularia* sp., and one isolate (GO_PT006) of *Alternaria* sp. It has previously been reported that in Thailand, leaf spot is among the most serious lettuce diseases affecting the hydroponics sector (Koohakan et al., 2008). The pathogens responsible for the leaf spots in this study were similar to those identified in earlier works examining lettuce grown in similar conditions in Thailand, with those cited including *Cercospora* sp. (Koohakan et al., 2008) and also *C. cassiicola* (Chairin et al., 2017). Moreover, we found that *Cercospora* sp. infected all commercial varieties (GO, Co, Bh, Fi) in Sawi farm. This result was similar to Koohakan et al. (2008) which reported that this pathogen could infect various lettuce varieties, including oak leaf, head, and romaine lettuce. In addition, Chairin et al. (2017) reported that *C. cassiicola* was the causal agent of leaf spot in hydroponically grown lettuce in southern Thailand. Many farmers in Thailand use multi-fungicides to address leaf spots, so it is important to understand and consider fungicide sensitivity to effectively manage the disease.

Table 1 List of fungal pathogens isolated from leaf spot of lettuce grown in hydroponics

Location	Host	Isolate code	Pathogen
Pathio	Red oak	RO_PT012	*Corynespora cassiicola*
		RO_PT013	*C. cassiicola*
		RO_PT015	*C. cassiicola*
		RO_PT018	*C. cassiicola*
	Green oak	GO_PT003	*C. cassiicola*
		GO_PT005	*Curvularia* sp.
		GO_PT006	*Alternaria* sp.
		GO_PT008	*Curvularia* sp.
Sawi		GO_08	*Cercospora* sp.
		GO_09	*Cercospora* sp.
		GO_12	*Cercospora* sp.
	Cos	Co_43	*Cercospora* sp.
		Co_46	*Cercospora* sp.
	Butter head	Bh_32	*Cercospora* sp.
		Bh_36	*Cercospora* sp.
	Frillice iceberg	Fi_49	*Cercospora* sp.
		Fi_53	*Cercospora* sp.

Leaf spotting fungi sensitivity to QoI fungicides

Table 2 presents the *in vitro* fungicide sensitivity recorded when lettuce leaf spotting fungi were exposed to TR, AZ, and PY. For all of TR, AZ, and PY with RO_PT012, and TR and AZ with RO_PT013, the EC_{50} values as *C. cassiicola* exceeded 100 mg/l, while these particular isolates were deemed to be HR. One further isolate, RO_PT015, was considered IR when exposed to TR with an EC_{50} value of 30.03 mg/l. A majority of the isolated samples of leaf spotting fungi showed greater sensitivity when exposed to the QoI fungicides; the EC_{50} values did not exceed 10 mg/l, and the scores were 94.12% for PY, 88.24% in the case of AZ, and 82.36% for TR. Previously, QoI fungicides were used in hydroponics farms to control leaf spot disease. Therefore, this has resulted in a high risk of resistance development. This type of QoI resistance was observed in earlier studies, such as the work of Corio-Costet et al. (2011) on *Plasmopara viticola* in grapevines, Zhang et al. (2011), who studied *Botrytis cinereal* in vegetables, Vaghefi et al. (2016) who investigated *Cercospora beticola* in table beet, and Teramoto et al. (2017) who focused on *C. cassiocola* in soybeans. To control diseases effectively, it is necessary to understand the fungicide sensitivity in the relevant pathogen population. Given the factors described above, it should be reconsidered whether QoI fungicides are suitable to manage leaf spot disease in lettuce. Lettuce growers should reduce the frequency of fungicide applications, rotate with fungicides carrying different modes of action, and/or mix with multi-site contact fungicides (Asim et al., 2019). Currently, there are several reports on effective agents for controlling plant diseases. For example, Li and Zou 2017 reported that salicylic acid and calcium ion presents a complex mode of action against *Botrytis cinerea* in tomato by disease-resistant induction. Moreover, growers should introduce integrated approaches with cultural practices or biological control. Further studies are necessary to monitor the fungicide resistance in a wider range.

Table 2 The sensitivity of leaf spotting fungi to azoxystrobin, pyraclostrobin, and trifloxystrobin with salicylhydroxamic acid and phenotype classification

Isolate code	Pathogen	Azoxystrobin		Pyraclostrobin		Trifloxystrobin	
		EC_{50}	Sensitivity	EC_{50}	Sensitivity	EC_{50}	Sensitivity
RO_PT012	*C. cassiicola*	>100	HR	>100	HR	>100	HR
RO_PT013	*C. cassiicola*	>100	HR	2.20	S	>100	HR
RO_PT015	*C. cassiicola*	<1	S	<1	S	30.03	IR
RO_PT018	*C. cassiicola*	<1	S	<1	S	<1	S
GO_PT003	*C. cassiicola*	<1	S	<1	S	<1	S
GO_PT005	*Curvularia* sp.	<1	S	<1	S	<1	S
GO_PT006	*Alternaria* sp.	<1	S	<1	S	<1	S
GO_PT008	*Curvularia* sp.	<1	S	<1	S	<1	S
GO_08	*Cercospora* sp.	<1	S	<1	S	<1	S
GO_09	*Cercospora* sp.	<1	S	<1	S	<1	S
GO_12	*Cercospora* sp.	<1	S	<1	S	<1	S
Co_43	*Cercospora* sp.	<1	S	<1	S	<1	S
Co_46	*Cercospora* sp.	<1	S	<1	S	<1	S
BH_32	*Cercospora* sp.	<1	S	<1	S	<1	S
BH_36	*Cercospora* sp.	<1	S	<1	S	<1	S
Fi_49	*Cercospora* sp.	<1	S	<1	S	<1	S
Fi_53	*Cercospora* sp.	<1	S	<1	S	<1	S
	S% (no.)	88.24% (15)		94.12% (16)		82.36% (14)	
	IR% (no.)	-- (0)		-- (0)		5.88% (1)	
	HR% (no.)	11.76% (2)		5.88% (1)		11.76% (2)	

S = sensitive to fungicide (EC_{50}< 10 mg/l); IR = intermediately resistant to fungicide (EC_{50} 10 to 100 mg/l); and HR = highly resistant (EC_{50}> 100 mg/l) (15)

The synergy of QoI fungicides combined with salicylhydroxamic acid on leaf spotting fungi

The synergistic effects using TR, AZ, and PY, both with and without SHAM was present have (**Table 3**), when used as an inhibitory AOX for *in vitro* assay. The isolate, fungicide type, concentration, and interaction between them significantly influenced on SF. It was found that the addition of SHAM to TR, AZ, and PY at concentrations of 100 mg/l and to TR at 10 mg/l provided complete inhibition of *Cercospora* sp. in nine of the tested isolates (GO_08, GO_09, GO_12, Co_43, Co_46, BH_32, Fi_49, Fi 53), while AZ, PY, and TR at 1 mg/l provided inhibition in three isolates (GO_08, GO_12, Co_43), three isolates (GO_12, Co_43, Co_46), and six isolates (GO_08, GO_12, Co_43, Co_46, BH_32, BH_36) respectively. Meanwhile, five isolates were inhibited by AZ and PY at 10 mg/l. These results were achieved with a synergy factor greater than 1 (**Table 3**). Furthermore, when SHAM at any concentration was added to TR, AZ, and PY, all tested fungal pathogens demonstrated increased mycelial growth inhibition, indicating the possibility that these pathogens might be using the alternative respiration pathway to counteract the mycelial growth inhibition with results from the activity of QoI fungicides (Zhang et al., 2011). In addition, synergistic effects have been found for SHAM with trifloxystrobin on *Mycosphaeralla fijiensis* (Sierotaki et al., 2000). However, Li et al. (2005) found no synergistic effects for SHAM with azoxystrobin for the mycelial growth inhibition of *Colletotrichum capsicum*, while

to the contrary, Jin et al. 2009 reported improved mycelial growth inhibition for azoxystrobin combined with SHAM upon *C. capsici*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Magnaporthe grisea* than was the case when azoxystrobin was applied alone. It must be considered that alternative respiration will be presented differently in the context of different fungi types, varying SHAM concentrations, and alternative AOX inhibitors (Seyran et al., 2010; Bradley and Pedersen, 2011). For this reason, it is necessary to add an AOX inhibitor to the QoI fungicide when conducting *in vitro* assays.

Table 3 The synergy factor of leaf spotting fungi to azoxystrobin, pyraclostrobin, and trifloxystrobin combine with salicylhydroxamic acid

Isolates code	Pathogen	Synergy factor (SF)[1]								
		Azoxystrobin			Pyraclostrobin			Trifloxystrobin		
		1	10	100	1	10	100	1	10	100
RO_PT012	*C. cassiicola*	1.01	1.14	1.37	1.10	1.01	1.10	1.11	1.11	1.14
RO_PT013	*C. cassiicola*	1.20	1.25	1.29	1.12	1.35	1.83	1.37	1.24	1.25
RO_PT015	*C. cassiicola*	1.37	1.52	1.20	1.30	1.29	1.24	1.06	1.16	1.16
RO_PT018	*C. cassiicola*	1.74	3.16	5.24	1.39	1.67	1.55	2.06	1.75	1.18
GO_PT003	*C. cassiicola*	1.42	1.39	1.12	1.24	1.56	1.19	1.43	1.39	1.13
GO_PT005	*Curvularia* sp.	1.75	1.87	1.83	1.41	1.79	1.83	1.52	1.47	1.55
GO_PT006	*Alternaria* sp.	1.74	1.91	1.79	1.65	1.32	1.11	1.43	1.62	1.64
GO_PT008	*Curvularia* sp.	2.06	2.04	2.02	1.33	1.36	2.07	1.83	2.00	2.01
GO_08	*Cercospora* sp.	CI[2]	CI	CI	7.19	12.70	CI	CI	CI	CI
GO_09	*Cercospora* sp.	12.50	CI	CI	2.79	7.05	CI	12.54	CI	CI
GO_12	*Cercospora* sp.	CI	CI	CI	CI	CI	CI	CI	CI	CI
Co_43	*Cercospora* sp.	CI	CI	CI	CI	CI	CI	CI	CI	CI
Co_46	*Cercospora* sp.	7.23	CI	CI	CI	CI	CI	CI	CI	CI
BH_32	*Cercospora* sp.	5.01	9.05	CI	2.89	4.29	CI	7.01	CI	CI
BH_36	*Cercospora* sp.	4.67	11.25	CI	7.36	CI	CI	CI	CI	CI
Fi_49	*Cercospora* sp.	5.57	5.52	CI	2.48	CI	CI	CI	CI	CI
Fi_53	*Cercospora* sp.	7.58	9.45	CI	5.58	7.44	CI	16.69	CI	CI
$LSD_{0.01}$ Isolate (A)						0.0617				
$LSD_{0.01}$ Fungicides (B)						0.0259				
$LSD_{0.01}$ Concentration (C)						0.0259				
$LSD_{0.01}$ A*B						0.1068				
$LSD_{0.01}$ A*C						0.1068				
$LSD_{0.01}$ B*C						0.0449				
$LSD_{0.01}$ A*B*C						0.1850				

[1]Synergy factor (SF) values were calculated according to the formula: SF = percentage of mycelial growth without SHAM/ percentage of mycelial growth with SHAM

[2]CI = Completely inhibition

Conclusion and Suggestion

Four genera of lettuce leaf spotting fungi, *Cercospora* sp., *C. cassiicola*, *Curvularia* sp., and *Alternaria* sp. were obtained from hydroponically grown lettuce farms in Chumphon province, Thailand. This study found that it is possible for the QoI fungicides TF, AZ, and PY to restrict the mycelial growth against all the tested fungi, while SHAM is able to demonstrate synergistic effects in combination with various QoI fungicides. Furthermore, among the 17 isolates, two isolates of *C. cassiicola* exhibited a high level of resistance to QoI fungicides, presenting EC_{50} values above 100 mg/l. However, it is also well-understood that QoI resistance has occurred in Thailand.

Acknowledgments

This study was supported by the Department of Agricultural Technology lab, King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus, Thailand.

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