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Effect of salicylhydroxamic acid on mycelial growth and baseline sensitivity to azoxystrobin in *Phytophthora infestans* causing potato late blight in Thailand

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Abstract Late blight of potato caused by *Phytophthora infestans* is proved to be an aggressive pathogen. Azoxystrobin, a broad spectrum quinone outside inhibitor (QoI), has been used in potato cultivation, but not directly recommended to use to control late blight disease. In this study, a suitable method to determine the sensitivity of *P. infestans* against azoxystrobin and azoxystrobin sensitivity to *P. infestans* population was recorded in Chiang Rai and Chiang Mai, Thailand. The toxicity of SHAM was proved a nessary of SHAM addition to artificial media for an efficient determination of azoxystrobin sensitivity. Increased of SHAM concentration led increased mycelial inhibition. SHAM at concentration of 5 to 10 µg/ml showed less effect to mycelia growth of six representative isolates with means of 5.33 to 12.06% inhibition. Both concentrations of SHAM were applied with azoxystrobin for pre-*in vitro* sensitivity determination. Effective concentration for 50% inhibition (EC_{50}) of azoxystrobin without SHAM was 0.0873 µg/ml, and the sensitivity were significantly increased in azoxystrobin amended with SHAM at 5 and 10 µg/ml for 55.34 and 70.90%, respectively. SHAM at 5 µg/ml was used for *in vitro* sensitivity assay to azoxystrobin. The thirty-six isolates of *P. infestans* were determined their azoxystrobin sensitivities based on EC_{50} evaluation. The mean EC_{50} value of isolate was 0.0531 µg/ml and ranged from 0.0005 to 0.4415 µg/ml indicating that azoxystrobin was very effective to control the mycelial growth of *P. infestans* isolated from Chiang Rai and Chiang Mai, Thailand.

Keywords: Late blight, *Phytophthora infestans*, SHAM, Azoxystrobin, Sensitivity

Introduction

Potato production has dramatically increased in recent years in Thailand, especially in northern region. Potato was cultivated in either double cropping highland zone or single winter crop after rice cultivation in lowland regions (Kittipadakul *et al.*, 2016). Potato plant is damaged by several phytopathogens

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and insect pests. Late blight of potato, caused by *Phytophthora infestans* (Mont.) de Bary, is considered as an important disease world wild including Thailand. This disease causes potato yield loss by destroying the stems, leaves and tubers. In favored environment for disease development, the disease severely epidemic and cause yield loss up to 100% (Möller *et al*., 2007; Chawdappa *et al*., 2015). To protect the crop from late blight disease, fungicide application is the most common practice in potato fields' world wild (Lal *et al*., 2018).

Azoxystrobin, a member of quinone outside inhibitor (QoI) fungicide, is known as a broad-spectrum fungicide to control several pathogens among *Ascomycetes, Basidiomycetes* and *Oomycetes* (Bartlett *et al*., 2002). The molecular mechanism of this fungicide is a block of electron flow in mitochondrial respiration chain by interfere the electron transfer at outer quinol oxidation site (Qo) of cytochrome bc1 complex. This interference results a lack of ATP in cells (Becker *et al*., 1981; Bartlett *et al*., 2002; Gisi *et al*., 2002; Sierotzki, 2015). Although QoI fungicides has high efficiency, an appearance of QoI fungicide-resistant isolates have been continually reported among several pathogens (Heaney *et al*., 2000; Vincelli and Dixon, 2002; Mc Grath and Shishkoff, 2003; Gullino *et al*., 2004; Sierotzki *et al*., 2005; Chen *et al*., 2007; Banno *et al*., 2009; Yamada and Sonoda, 2012; Hausladen *et al*., 2015; Ma *et al*., 2018). In addition to the mutation on cytochrome b gene (*cytb*) commonly at G143A, F129L and G137R, activation of alternative respiration pathway in mitochondria by expression of alternative oxidase (AOX) is considered as another resistant mechanism which might occurred when the fungi face to QoI fungicides (Fernández-Ortuño *et al*., 2008). This resistant pathway gives only 40% of energy to fungi comparing with that from mitochondrial oxidative phosphorylation. Thus, fungal developments depending on high energy during plant infection cannot be completed. Moreover, this alternative respiration pathway was inhibited by plant antioxidants such as flavones (Wood and Hollomon, 2003). Consequently, the function of AOX is not directly important for determine the QoI fungicides sensitivity *in planta*. In case of QoI fungicides sensitivity evaluation *in vitro*, salicylhydroxamic acid (SHAM) is usually recommended to amend in media as alternative respiration suppresser to avoid an appearance of escape pathway for the effect of QoI inhibition by the fungicides, however, the dose of SHAM is not same in different pathogens. (Vincelli and Dixon, 2002; Avila-adame *et al*., 2003; Wood and Hollomon, 2003; Pasche *et al*., 2004; Malandrakis *et al*., 2006; Markoglou *et al*., 2006; Rebollar-Aviter *et al*., 2007; Walker *et al*., 2009; Banno *et al*., 2009; Qi *et al*., 2012; Duan *et al*., 2012; Liang *et al*., 2015; Torres-Calzada *et al*., 2015; Piccirillo *et al*., 2018 Rosenzwing *et al*., 2017).

The addition of SHAM in QoI fungicide sensitivity (*in vitro*) should be considered to add if SHAM shows low toxicity to fungi and increases the fungicide sensitivity.

Nowadays, the resistance to azoxystrobin or other QoI fungicides has not report yet for the case of *P. infestans*. The sensitivity of this fungicide group is necessary to be continually determined. Since the Fungicide Resistant Action Committee (FRAC) considered the QoI fungicides have high risk of resistant development and the sensitivity of *P. infestans* to this fungicide group is desired. Thus, to identify the suitable test condition for the sensitivity of *P. infestans* to QoI fungicides, this study aimed to determine the effect of SHAM on mycelial growth of *P. infestans* that SHAM should be amended in media to determine azoxystrobin sensitivity *in vitro* and to determine the baseline sensitivity of azoxystrobin in *P. infestans* population obtained from various fields in Northern Thailand.

Materials and methods

Isolate collection

Thirty-six isolates of *P. infestans* were isolated from symptomatic leaves of potato plants which were randomly sampled from four locations in Thailand including Mae Sai (Chiang Rai) and Mae Wang, Phrao and Chiang Dao (Chiang Mai) (Table 2). The distance between each field exceeded 10 km. Infected leaf was rinsed with sterile water and cut the fresh lesion size of about 3 x 3 cm^2, and placed on petri dish lid overplayed a slice of peeled-potato tuber with 5-8 mm thin. After the sample lid, it was placed and incubated on the water agar (WA) petri dish for 7 days at 18 ℃ in darkness, a hyphal tip grown on WA was cut under compound microscope and transferred to corn agar (CA; sweet corn 200 g, agar 15 g, boiled and adjusted with distilled water to obtain final volume of 1 liters). The culture was incubated at 18 ℃ in darkness for 14 days and used as a pure culture. For long-term storage, the pathogen was cultured on CA slant and subcultured every 4-6 months for further use.

SHAM toxicity test

Salicylhydroxamic acid (SHAM; 99% a.i.; Sigma-Aldrich, Singapore) was dissolved in methanol to obtain 10 mg/ml stock solution and kept at 4 ℃ in dark until use. SHAM was serially diluted with methanol to obtain final concentration of 5, 10, 20, 50 and 100 µg/ml in CA. The CA amended with only methanol at concentration of 0.5% (v/v) was used as the control. Active

mycelial plugs were cut from the margin of 14 days-old colonies of representative six isolates (PIKNL1_6, PITDL1_6, PIKWL1_2, PIMSL1_4, PIMSL2_12 and PIMSL3_18) and placed on the center of each tested plates. Four replications were performed for each isolate in this test. Inoculated plates were incubated at 18 ℃ for 14 days in the dark. The colony diameter of each plates was measured vertically twice with a ruler and determined their percentage inhibition of mycelial growth (PIMG). The mean (±SD) of PIMG of each isolate at each SHAM concentration was calculated using variance analysis (ANOVA) by Statistix 8.0 software. The dose of SHAM with less effect to mycelium growth (< 30 µg/ml) was selected to be used in azoxystrobin sensitivity assay.

Sensitivity to azoxystrobin with or without of SHAM

Azoxystrobin (25% w/v SC, Syngenta, Thailand) was dissolved with sterile water to obtain 10 mg/ml stock solution and kept at 4 ℃ in dark until use. Azoxystrobin was serially diluted to obtain final concentrations of 0.01, 0.1, 1, and 10 µg/ml in CA with or without addition of SHAM at a concentration of 5 µg/ml or 10 µg/ml as expected alternative oxidative inhibitor. CA amended with only SHAM was used as control. The mycelial plugs cut from the margin of 14 days-old colonies of six isolates were placed at the center of each plate with four replications. Inoculated plates were incubated at 18 ℃ for 14 days in the dark. The colony diameter of each isolate was measured and determined the PIMG. Median effective concentration (EC_{50}) was calculated using dose-response curve procedure in GraphPad Prism® (version 6.01; San Diego, CA, USA). Data of mean EC_{50} to azoxystrobin in present or absent of SHAM for each isolate were analyzed using variance analysis (ANOVA) by Statistix 8.0 software.

In vitro sensitivity to azoxystrobin

Azoxystrobin was dissolved and serially diluted with sterilized water to obtain final concentrations of 0, 0.01, 0.1, 1, and 10 µg/ml in CA. SHAM 5 µg/ml was added and used as alternative oxidative inhibitor. The active mycelial plug was cut from the margin of 14 days-old colony and placed at the center of each plate. Four replications for each concentration were performed. The plates were incubated at 18 ℃ for 14 days in the dark. The diameter of each colony was measured and determined their PIMG and EC_{50}.

Results

SHAM toxicity test

Six representative isolates were randomly selected from different location and tested the toxicity of SHAM on mycelial growth. SHAM at concentration of 5 µg/ml exhibited little effect to mycelial growth of all isolate with mean of PIMG of 5.33%. SHAM at concentration of 10 µg/ml showed inhibitory effect to the isolates with mean of PIMG of 12.06%, and not significantly different with the concentration of SHAM at 5 µg/ml (Figure 1). The increase of the concentration of SHAM correlated with an increase of the mycelial growth inhibition. At the concentration of 100 µg/ml, the average PIMG of all isolates was 96.29%. Thus, SHAM at concentration of 5 and 10 µg/ml were used for testing the *in vitro* effect of azoxystrobin with SHAM.

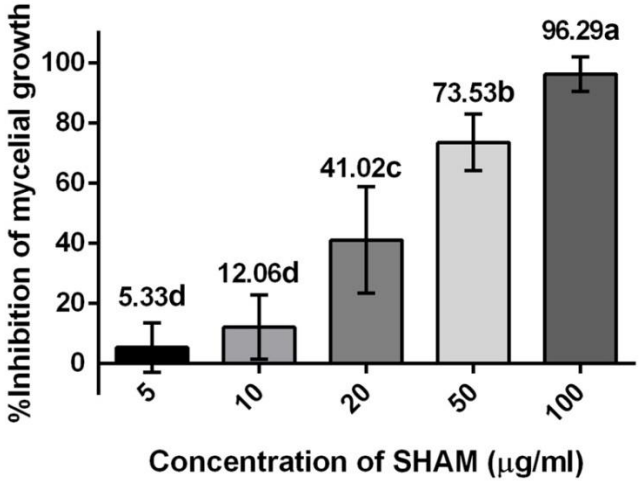

Figure 1. Toxicity of salicylhydroxamic acid (SHAM) on mycelial growth of *Phytophthora infestans* causing late blight disease of potato. The number on the bar was the mean of six isolates followed by the different letter indicated significantly different based on the analysis of least significant difference (LSD) test at *P* value = 0.05

Sensitivity to azoxystrobin with or without of SHAM

To prove the effect of SHAM addition to the sensitivity of azoxystrobin *in vitro*, EC_{50} of six representative isolates to azoxystrobin with or without of SHAM were examined. We found the EC_{50} of azoxystrobin without SHAM that

was rangeg from 0.0329-0.1518 µg/ml with mean of 0.0873 ±0.0459 µg/ml (mean ± SD) (Table 1). The EC_{50} of azoxystrobin with SHAM at 5 or 10 µg/ml were ranged from 0.0088-0.0993 µg/ml (mean ± SD = 0.0390 ±0.0329 µg/ml) and 0.0087-0.0736 µg/ml (mean ± SD = 0.0254 ±0.0243 µg/ml), which dramatically decreased from not amended SHAM for 55.34 and 70.90%, respectively. Moreover, the azoxystrobin sensitivity in presented SHAM at 5 or 10 µg/ml were not significantly different. These results suggested that alternative oxidase could be activated in *P. infestans* when faced with azoxystrobin, quinone outside inhibitor (QoI), and addition of SHAM at concentration of 5 µg/ml in the artificial medium was efficient way to inhibit the interfering by the action of alternative respiration.

Table 1. Median effective concentration (EC_{50}) of *Phytophthora infestans* to azoxystrobin amended with or without of salicylhydroxamic acid (SHAM)

Isolate	Median effective concentration (EC_{50})		
	Azoxystrobin	Azoxystrobin + SHAM 5 µg/ml	Azoxystrobin + SHAM 10 µg/ml
PiKWL1_2	0.0490	0.0170	0.0163
PiKNL1_6	0.0781	0.0442	0.0736
PiTDL1_6	0.1518	0.0993	0.0259
PiMSL1_4	0.1299	0.0433	0.0120
PiMSL2_12	0.0819	0.0212	0.0159
PiMSL3_18	0.0329	0.0088	0.0087
Mean ±SD[1/]	0.0873 ±0.0459[a]	0.0390 ±0.0329[ab]	0.0254 ±0.0243[b]

[1/] Means EC_{50} ± SD of six representative isolates follow by the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant different test

Baseline sensitivity to azoxystrobin

The sensitivity of 36 isolates of *P. infestans* to azoxystrobin which obtained from four locations in Thailand was determined the inhibition of mycelial growth. The EC_{50} values of 36 isolates were ranged from 0.0005 to 0.4415 µg/mL and the mean EC_{50} value was 0.0531 ± 0.0896 (mean ± SD) µg/mL (Table 2). There was no significant difference found in their means of EC_{50} value among four locations. The frequency distribution of EC_{50} values was displayed as unimodal curve albeit with a long right-hand tail (Figure 2). These data are noted as the baseline sensitivity of *P. infestans* to azoxystrobin in northern Thailand and for monitoring azoxystrobin resistance in the country.

Table 2. Azoxystrobin sensitivity of *Phytophthora infestans* isolates obtained from various potato production fields in Chiang Mai and Chiang Rai, Thailand

Location	Number of isolates	Range of EC_{50} value (μg/mL)[1/]	Mean EC_{50} ± SD (μg/mL)[2/]
Mae Wang, Chiang Mai	7	0.0044-0.0170	0.0089 ± 0.0051[a]
Phrao, Chiang Mai	8	0.0148-0.0980	0.0518 ± 0.0265[a]
Chiang Dao, Chiang Mai	4	0.0024-0.1189	0.0466 ± 0.0511[a]
Mae Sai, Chiang Rai	17	0.0005-0.4415	0.0736 ± 0.1242[a]
Total	36	0.0005-0.4415	0.0531 ± 0.0896

[1/] Median effective concentration (EC_{50}) values were calculated based on fungicide dose response curve using GraphPad Prism® software

[2/] Averages of EC_{50} in each column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) at *p*-value of 0.05

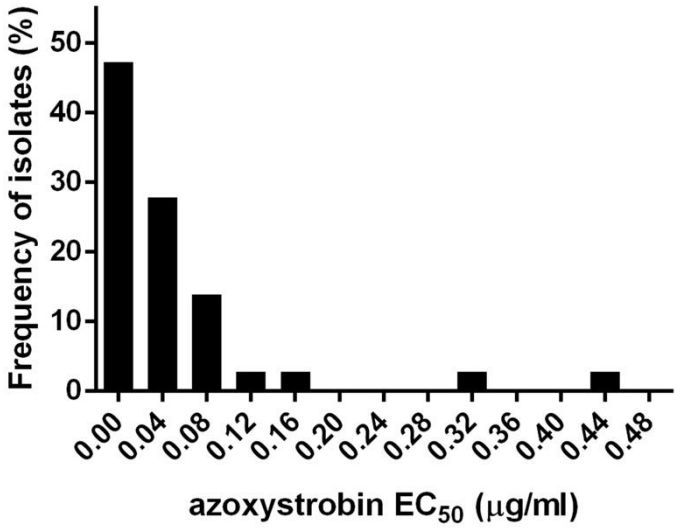

Figure 2. Frequency distribution of azoxystrobin sensitivity of 36 isolates of *Phytophthora infestans* collected from different locations in Thailand. EC_{50} values were grouped in class intervals of 0.04 μg/ml. Values on X-axis indicated the midpoint of the interval

Discussion

QoI fungicides has molecular mechanism in blocking electron flow of mitochondrial respiration chain by interfere the electron transfer at outer quinol oxidation site (Qo) of cytochrome bc1 complex (complex III) (Becker *et al*.,

1981; Bartlett *et al*., 2002; Gisi *et al*., 2002; Sierotzki, 2015). In determination of QoI sensitivity, SHAM usually be added into artificial media for suppress the alternative respiration due to AOX activity (Wood and Hollomon, 2003; Malandrakis *et al*., 2006; Markoglou *et al*., 2006; Walker *et al*., 2009; Duan *et al*., 2012). Addition of SHAM in the artificial medium is efficient way to examine the true value of the effect of QoI fungicides by inhibition of the interfering caused with the action of alternative respiration. However, SHAM has been used in different fungi with different dose. SHAM was usually used at 100 µg/ml in determining the QoI fungicide sensitivity for *Colletotrichum graminicola* (Avila-adame *et al*., 2003), *C. gloeosporioides* (Piccirillo *et al*., 2018), *C. truncatum* (Torres-Calzada *et al*., 2015), *Phyricularia grisea* (Vincelli and Dixon, 2002) *Alternaria* spp. (Rosenzwing *et al*., 2017), *A. solani* (Pasche *et al*., 2004), *Phytophthora cactorum* (Rebollar-Aviter *et al*., 2007), while at 50 µg/ml for *Botrytis cinerea* (Banno *et al*., 2009), and at 10 µg/ml for *Phytophthora capsici* (Qi *et al*., 2012), respectively. Furthermore, SHAM should not be added in *Sclerotinia sclerotiorum* (Liang *et al*., 2015). According to previous reports, SHAM also has effects to suppress the essential enzymes of fungi such as esterase, peroxidase, laccase, and putative melanogenesis enzyme (Hsiao and Bornman, 1993; Faure *et al*., 1995; Tsukamoto *et al*., 1999; Baek *et al*., 2008). Thus, the necessity of SHAM addition for QoI sensitivity assay is still under an argument, and the use of SHAM with a particular concentration was evaluated in this study for examination of azoxystrobin sensitivity in *P. infestans* for pathogen growth. We found that SHAM at concentration less than 10 µg/ml had no significant effect to the pathogen and increased the azoxystrobin sensitivity of 55.34% with 5 µg/ml and 70.90% with 10 µg/ml treatment, respectively. Based on these results, we concluded that SHAM at 5 µg/ml should be added in azoxystrobin sensitivity assay in *P. infestans* for suppress the action of AOX.

Evaluation of thirty-six isolates of *P. infestans* to azoxystrobin combined with SHAM 5 µg/ml showed high sensitivity to azoxystrobin (EC_{50} < 1 µg/ml). According to Rekanović *et al*. (2012), the EC_{50} of *P. infestans* obtained from Serbia to azoxystrobin (without of SHAM combination) was ranged from 0.019-0.074 µg/ml. Earnshaw and Shattock (2012) and Saville *et al*. (2015) also reported that *P. infestans* was sensitive to azoxystrobin. Our finding suggested that azoxystrobin still show high potential to control this pathogen from four locations in this study. Since, the QoI fungicides is considered to have high risk of resistant development (FRAC, 2020), and several fungal pathogens have been reported to be resistance to QoI fungicides such as *Botrytis cinerea* (Banno *et al*., 2009), *Pyricularia grisae* (Vincelli and Dixon, 2001), *Phyllosticta* sp. (Stammler *et al*., 2013), *Pestalotiopsis longiseta* (Yamada and

Sonoda, 2012). Moreover, oomycetes pathogens also have been reported to resistance to this fungicide group such as *Plasmopara viticola* (Toffolatti *et al*., 2011), *P. capsici* (Qi *et al*., 2012; Ma *et al*., 2018).

Thus, the risk management of fungicide resistance using a rotation or mix of different fungicides with different mode of actions, integrated pest management, and the resistant risk assessment by continue determination of the sensitivity to the fungicides are needed to protect the risk of fungicide resistant development in future.

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