Title: Specific growth inhibitors of *Ralstonia solanacearum*, *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, and *Clavibacter michiganensis* subsp. *michiganensis*

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Abstract (233 words)

Plant pathogenic bacteria cause huge yield losses in crops globally. Therefore, finding effective bactericides to these pathogens is an immediate challenge. In this study, we sought compounds that specifically inhibit the growth of *Ralstonia solanacearum*. As a result, we identified one promising compound, 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline, which inhibited the growth of *R. solanacearum* (*Rs*1002) from a pilot library of 376 chemicals provided from RIKEN. We further obtained its structural analogues and assessed their ability to inhibit *Rs*1002 growth. Then we identified five compounds, named ralhibitins A to E, that specifically inhibit growth of *Rs*1002 at >5 μg/ml final concentration. The most effective compounds, ralhibitins A, C, and E completely inhibited the growth of *Rs*1002 at 1.25 μg/ml. In addition, ralhibitins A to E inhibited growth of *Xanthomonas oryzae* pv. *oryzae* but not the other bacteria tested at a final concentration of 10 μg/ml. Whereas, ralhibitin E, besides inhibiting *R. solanacearum* and *X. oryzae* pv. *oryzae*, completely inhibited the growth of *X. campestris* pv. *campestris* and the Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* at 10 μg/ml. Growth inhibition by these compounds was stable at pH 6–9 and after autoclaving. Because *Rs*1002 grew in the culture medium in which ralhibitins were incubated with the ralhibitin-insensitive bacteria, the unaffected bacteria may be able to inactivate the inhibitory effect of ralhibitins. These results suggest that ralhibitins might be potential lead compounds for the specific control of phytopathogenic bacteria.

Keywords: bactericide; ralhibitins; *Ralstonia solanacearum*; lead compounds

1. Introduction

Crop production is constrained by bacterial pathogens resulting to yield losses that threaten global food security (Sundin et al. 2016). *Ralstonia solanacearum* (*Rs*) is one of the most destructive plant pathogenic bacteria and has a wide host range infecting more than 200 plant species in over 50 plant families; it is a causal agent of damage to many economically important crops including tomato, potato, eggplant, tobacco, and banana. Its direct economic impact is difficult to calculate, but it was estimated that losses of potato alone were US$1 billion each year worldwide (Elphinstone 2005). Thus, *Rs* was listed as the second most important plant pathogenic bacteria based on scientific and economic studies (Genin 2010; Hayward 1991; Mansfield et al. 2012). *Rs* is a soil-borne pathogen that infects plants via wounds or root tips; it invades the xylem vessels and systematically spreads to the aerial parts of the plant through the vascular system.

Rs species have been recognized as a complex group of phenotypically diverse strains that can be subdivided into four phylotypes: phylotype I (Asia), phylotype II (America), phylotype III (Africa), and phylotype IV (Indonesia) (Prior and Fegan 2005). However, a recent report proposed taxonomic revision of the *Rs* species complex into three genospecies: *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* (Safni et al. 2014; Prior et al. 2016). Since the entire genome sequence of *Rs* GM1000 was determined, molecular- and genome-level studies have developed (Salanoubat et al. 2002). Comprehensive analysis of effector genes has revealed the entire repertoire of effector proteins of *Rs* 1000 (Mukaihara et al. 2010).

To control bacterial wilt diseases, cultural as well as physical, biological, and chemical methods have been reported (Aino et al. 2014; Yuliar et al. 2015). Use of resistant cultivars to control bacterial wilt is the most economical, environmentally friendly, and effective method. Grafting with resistant root stocks for tomato and eggplant is also an important strategy.

Recently, “high-grafting” was recognized as a more effective method to control bacterial wilt. High-grating was defined as grafting at a higher position (on the stem above the second or third leaf) than usual (at the epicotyls) (Nakaho et al. 2014). It is also important to use clean planting materials, to practice crop rotation and multi-cropping, and to amend the soil environment. As an example of a physical control method, sterilization by sunlight or hot water is used. On the other hand, many biological control agents (BCAs), such as *Bacillus thuringiensis*, have been reported for use as bacteriophages and avirulent strains of the pathogen (Takahashi et al. 2014; Buttimer et al. 2017). However, the only commercially available microbial pesticide is “Serunae-genki,” which consists of two types of *Pseudomonas fluorescens* (Aino et al. 2014; Yuliar et al. 2015). Even when BCAs are available, their efficacy is hampered by poor colonization and mass production challenges (Yuliar et al. 2015). There are two types of chemical control agents for bacterial wilt: the first type is soil fumigation materials such as chloropicrin and metam sodium, and the second type is a non-disinfectant chemical, validamycin A. Validamycin A is a major component of the validamycin complex produced by *Sterptomyces hygroscopicus* subsp. *limoneus*. Two functions of validamycin A are reported: it inhibits trehalase, which degrades trehalose in pathogens, and consequently limits nutrient acquisition (Asano et al. 1987), and it induces systemic acquired resistance (Ishikawa et al. 2005). Validamycin A is useful to protect eggplant from bacterial wilt, but it is not applicable to tomato plants due to its harmful effect. Beside validamycins, some biological compounds such as lansiumamide B (Li et al. 2014), flavonoids (Zhao et al. 2011), and L-histidine (Seo et al. 2016) are reported to suppress bacterial wilt. Thus, a multilateral approach to control bacterial wilt has been attempted. However, their protective effects against bacterial wilt are not perfect. In particular, specific compounds to attenuate the growth of *Rs* have not been identified.

In this study, we found that five structurally related novel halogen compounds, designated ralhibitins A to E, inhibit the growth of *Rs* at a final concentration of less than 5 μg/ml. No antibiotic compounds have been utilized for the control of bacterial wilt disease, and no chemical compounds that specifically inhibit the growth of *Rs* at such low concentrations have been reported so far. Furthermore, the growth inhibitory effect of ralhibitins is species-specific; ralhibitins also inhibited the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and ralhibitin E, containing an iodine-substituent, inhibited the growth of *Xanthomonas campestris* pv. *campestris* (*Xcca*) and *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Therefore, ralhibitins might be potential lead compounds as specific controlling agents of these four bacteria species.

2. Materials and methods

2.1. Bacterial strains

*Rs*1002 is a spontaneous nalixidic acid-resistant derivative of *Rs*1000 (Mukaihara et al. 2010). *Rs* OE1-1 (Mori et al. 2016) and *Burkholderia glumae* (*Bg*) Pg-10 (Maeda et al. 2007) were obtained from Prof. Hikichi, Kochi University. Three other *Rs* strains, 6601 (MAFF301070), 970825-11 (MAFF211479), and POPS 8409 (MAFF211271), and *Dickeya dadantii* (*Dd*) 92-31 (MAFF311041), *Acidovorax avenae* (*Aa*) NARCB200109, T13052 (MAFF106618), *Pantoea ananatis* (*Pa*) NARCB200120 AZ200124 (MAFF106629), *Pseudomonas protegens* (*Pp*) Cab57 (MAFF212077), *Xcca* XcA (MAFF301151), *Xcci* NS387 (MAFF311001), and four *Xoo* strains, T7174 (MAFF311018), H-9101 (MAFF210548), KXO 93-1 (MAFF210893), and T7174 (MAFF301226), were obtained from Genebank NARO Japan. *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000, *Chromobacterium violaceum* (*Cv*) Cv026 and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) EC1 were gifts of Prof. Collmer

(Cornell University, USA), Prof. Williams (Nottingham University, UK), and Prof. Tsuyumu (Shizuoka University, Japan), respectively. *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) 05M1-2 was obtained from the Agricultural Experiment Station, Okayama Prefecture. *Escherichia coli* (*Ec*) DH5α was obtained from Nippongene (Toyama, Japan). *Rhizobium radiobacter* (Ti) (*Rr*) GV3101 was a laboratory stock strain. Bacterial strains with respective growth media are listed in supplementary Table S1.

2.2. Chemical compounds and screening of growth inhibitors of *Ralstonia solanacearum Rs*1002

*Rs*1002 cultured overnight in BG medium was harvested by centrifugation and suspended in fresh BG medium adjusted to OD_{600} = 0.1. An aliquot of 150 µl of bacterial suspension was placed in each well of 96-well microtiter plates. To examine the effect on bacterial growth, 0.5 µl of each chemical (3 mg/ml in DMSO) was added to each well. As a control, the same amount of DMSO was also added. The microtiter plate was incubated for 24 h at 27°C in a DeepWell Maximizer (Taitec, Koshigaya, Japan), then absorbance at OD_{600} was measured using an iMARK Microplate Reader (BIO-RAD, Tokyo, Japan). To confirm the reliability of this assay system, we checked the growth inhibitory effects of the compounds in the authentic library (80 compounds) provided by RIKEN NPDepo (Natural Products Depository, https://www.jsbi.org/pdfs/journal1/GIW06/GIW06S03.pdf). Among the authentic library compounds, several antibiotics such as novobiocin, rifampicin, spiramycin I, neomycin B, streptomycin, tetracycline hydrochloride, and bicyclomycin significantly inhibited the growth of *Rs*1002 (data not shown), confirming that the assay system successfully function. Compound 1, 1-(4-bromophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline, and compound 2, 1-(4-chlorophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline, were purchased from Pharmeks

(Moscow, Russia), and their analogue compound, 1-(4-chlorophenyl)-2,3,4,9-tetrahydro-1H-β-carboline, was obtained from Alinda Chemical (Moscow, Russia).

2.3. Chemical synthesis of ralhibitins C, E, and F

To synthesize ralhibitin C, a mixture of tryptamine (1.8 g, 13 mmol) and 4-bromobenzaldehyde (1.9 g, 10 mmol) in acetic acid (50 ml) was reacted at 100°C for 20 h. The reaction mixture was added to H_2O (100 ml), and the suspension was extracted with ethyl acetate (AcOEt), washed with brine, and recrystallized. The precipitate was filtered, washed with AcOEt and hexane, and dried under vacuum to obtain a pale yellow powder (1.6 g). Ralhibitins E and F were synthesized by the same procedure using 4-iodobenzaldehyde and 4-fluorobenzaldehyde, respectively, instead of 4-bromobenzaldehyde. Purity of the compounds were analyzed by 1H NMR. Purity was ca. 97% by the calculation of number of protons derived from the compounds divided by the total number of proton in the products.

2.4. Dose-dependent inhibition by ralhibitins of growth of *Rs*, *Xoo*, *Xcca*, and *Cmm*

Dose-dependent inhibition of growth of *Rs*1002 and *Xoo* by ralhibitins A, B, C, D, and E was investigated. In the case of ralhibitin E, dose-dependent activity was also investigated using *Xcca* XcA and *Cm* 05M1-2. Ralhibitins were dissolved in DMSO to obtain 10 mg/ml stock concentrations, and a series of dilutions was made to determine the minimum inhibitory concentration (MIC) for each compound. *Rs*1002, *Xoo* strain T7174 (MAFF311018), *Xcca* XcA, and *Cmm* 05M1-2 were inoculated in 3 ml of BG, KB, Moka Rm, or LB medium, respectively, and incubated at 27°C overnight with shaking at 200 rpm to OD_{660} = 0.7. Ten μL of overnight culture of each bacterium was diluted with 3 ml of fresh medium, then 3 μL of the diluted bacterial suspension was further inoculated into 3 ml of respective fresh medium with

0.31, 0.63, 1.25, 2.5, 5, or 10 µg/ml of ralhibitins at final concentrations. After 24–48 h incubation at 27°C, absorbance at OD_{660} was measured using a MiniPhoto 518R (Taitec).

2.5 Thermostability assay

The thermostability of ralhibitins was determined after exposure to different temperatures. Briefly, 10 mg/ml ralhibitins in DMSO were incubated at different temperatures (4°C, 37°C, or 50°C) for 24 h or were autoclaved at 121°C for 20 min. Then growth inhibition activity was measured as described above.

2.6 pH assay

The growth inhibition by ralhibitins at different pHs in BG medium was investigated. First, the pH of the BG medium was adjusted to 6.0, 7.0, or 9.0 by the addition of HCl or KOH. Overnight cultured *Rs*1002 was inoculated into fresh BG medium at different pHs, then each ralhibitin was added at a final concentration of 10 µg/ml. After 24 h incubation at 27°C, absorbance at OD_{660} was measured as described above.

2.7 Growth inhibition activity of ralhibitins cultured with unaffected bacteria

Bacterial pathogens *Bg* Pg-10, *Pcc* EC1, and *Rr* GV3101, were grown overnight at 27°C in 3 ml BG medium with 10 µg/ml ralhibitions at a final concentration. *Rs*1002 was cultured with each spent culture filtrate overnight at 27°C, then absorbance at OD_{660} was measured using a MiniPhoto 518R (Taitec).

2.8 Statistical analysis

All results are expressed as mean±standard deviation (SD). Statistical evaluations were performed using Graph-pad Prism version 7 (GraphPad Software, Inc., San Diego, CA, USA). The data were subjected to analysis of variance (ANOVA), and significant differences between means were calculated by Tukey's t test. Differences at $P \leq 0.05$ were considered significant.

3. Results

3.1. Identification of growth inhibitors of *R. solanacearum*

To obtain effective compounds that specifically inhibit the growth of *Rs*1002, we first screened a pilot library that consists of 376 chemical compounds provided by RIKEN. Fortunately, we found one promising compound, as shown in Fig. 1 (number 1, NPD252), that completely inhibited the growth of *Rs*1002 in BG medium at a final concentration of 10 μg/ml. To examine the relationship between chemical structure and growth inhibitory activity, we further analyzed the inhibitory effect of the eight structurally similar molecules of compound number 1. As shown in Fig. 1, only numbers 1, 2, and 7 showed a strong inhibitory effect on the growth of *Rs*1002. Other similar compounds, numbers 3, 4, 5, 6, 8, and 9, did not effectively suppress the growth of *Rs*1002. Therefore, the common structural motif in numbers 1, 2, and 7, namely 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline, seems to be essential for their growth inhibition activity on *Rs*1002, but is probably not sufficient for the activity. To confirm this idea we commercially obtained the compound 1, 2, and their analogue compound, 1-(4-chlorophenyl)-2,3,4,9-tetrahydro-1H-β-carboline. Furthermore, we chemically synthesized compound 7, 1-(4-bromophenyl)-2,3,4,9-tetrahydro-1H-β-carboline and their analogues, 1-(4-iodophenyl)-2,3,4,9-tetrahydro-1H-β-carboline, and 1-(4-fluorophenyl)-2,3,4,9-tetrahydro-1H-β-carboline. Hereafter, we call these compounds, numbers 1, 2, and 7, ralhibitins A, B, and C, and three newly obtained compounds ralhibitins D, E, and F, respectively, as shown in Fig. 2A, B. The

dose-dependent inhibitory effect of ralhibitins was investigated, and we found that ralhibitins A, C, and E were the most effective: completely inhibiting the growth of *Rs*1002 at >1.25 µg/ml, and that ralhibitin B and D inhibited the growth of *Rs*1002 at >2.5 µg/ml and >5 µg/ml, respectively, but ralhibitin F did not show a significant inhibitory effect on *Rs*1002 (Fig. 2C). Ralhibitins A to E also completely inhibited the growth of four other strains of *Rs* including phylotype I (Rs1002, RsOE-1, 301070 and 211479) and phylotype IV (211271) at 10 µg/ml (Fig. 3).

3.2. Species-specific inhibitory activity of ralhibitins on bacterial growth

We also investigated the effect of ralhibitins on growth of different bacteria at 10 µg/ml. The effects on bacterial growth of *Aa* T13052, *Bg* Pg-10, *Cv* Cv026, *Cmm* 05M1-2, *Dd* 92-31, *Ec* DH5α, *Pa* NARCB200120 AZ200124, *Pcc* EC1, *Pp* Cab57, *Pto* DC3000, *Rr* GV3101 (pMP90), *Xcca* XcA, *Xcci* NS387, and *Xoo* (strains, T7174, 210548, 301226, and 210893) were investigated (Fig. 3). Five ralhibitins A, B, C, D, and E completely inhibited the growth of all strains of *Rs* and *Xoo* tested. Ralhibitin E also completely inhibited the growth of *Xcca* and the Gram-positive bacteria *Cmm. Cmm* was also partially inhibited by ralhibitins A, B, and C. However, ralhibitin F partially inhibited the growth of *Rs* 211479 and *Xoo* (Fig. 3). No ralhibitin inhibited the growth of non-plant pathogens including the biocontrol bacterium *P. protegens* (*Pp*) Cab57.

Dose-dependent activities of ralhibitins on the growth of *Xoo*, *Xcca*, and *Cmm* were investigated (Fig. 4). Ralhibitins C and D effectively inhibited the growth of *Xoo* at 2.5 µg/ml, whereas ralhibitins A and E inhibited it at 5 µg/ml and B at 10 µg/ml. Furthermore, ralhibitin E partially inhibited the growth of *Xcca* and *Cmm* at 5 µg/ml and completely inhibited it at 10 µg/ml.

3.3. Thermo- and pH stability of ralhibitins

Exposure of ralhibitins to different temperatures, including autoclave treatment, did not affect their activity against *Rs*1002 (Fig. 5a). This shows that the antibacterial activity of ralhibitins is highly thermostable. *Rs*1002 can grow in BG medium in a pH range of 6 to 9. At pH 7 and 9, all ralhibitins completely inhibited growth of *Rs*1002 at 10 µg/ml; however, at pH 6, growth inhibition by the ralhibitins was reduced except for ralhibitin E (Fig. 5b).

3.4. Inactivation of ralhibitins with insensitive bacteria

The mechanism for specific growth inhibition of *Rs*1002 is not clear yet. Most bacteria tested are insensitive to ralhibitins or they can inactivate or detoxify them. We examined whether ralhibitins cultured with different bacteria (*Bg*, *Pcc*, and *Rr*) still retained the capacity to inhibit growth of *Rs*1002. We found that *Rs*1002 was able to grow to the control level in all culture media of *Bg*, *Pcc*, and *Rr* that contained ralhibitins (Fig. 6).

4. Discussion

Plant pathogenic bacterial diseases are still difficult to control due to a lack of effective bactericides. Therefore, the growth inhibitors of *Rs*, one of the most important and destructive plant bacterial pathogen were investigated in this study. As a result of screening, we found that the ralhibitins initially identified as growth inhibitors of *Rs* also inhibited the growth of *Xoo*. *Xoo* is a causal agent of bacterial blight disease of rice leading to high economic losses, and was listed as the top 4th of important plant pathogenic bacterium (Mansfield et al. 2012). Although bismerthiazole and thiadizole copper have been used in controlling the pathogens

such as *Rs* and *Xoo*, their efficacy is limited by phytotoxicity, low efficiency and the development of antibacterial resistance (Yang and Bao 2017). Ralhibitins that we found in this study were more effective to inhibit the growth of these bacteria at low concentrations. Ralhibitins A, C, and E were the most effective against *Rs* at 1.25 µg/ml (Fig. 2), whereas ralhibitin D was the most effective compound against *Xoo* at 5 µg/ml (Fig. 4). Recently, 2-mercapto-5-substituted-1,3,4-oxadiazole/thiadiazole derivatives were reported to have antibacterial activity against *Xoo* and *Rs,* and the half-maximal effective concentration values were 14.69 and 15.14 µg/ml against *Xoo* and *Rs*, respectively (Li et al. 2015). Thus, ralhibitins have a much higher activity than the compounds previously reported.

The ralhibitins used in this study had either a bromine (bromo), chlorine (chloro), and iodine (iodo) substituent in the para position of the benzene ring that is required to inhibit bacterial growth (Figs. 2, 3, and 4). If this position had a hydroxyl or methyl substituent, growth inhibition was lost (Fig. 1, compound numbers 8 and 9). The importance of para position of these halogens is also demonstrated by loss of activity of the chlorine (chloro) substituent in the ortho position (Fig. 1, compound number 5). This is in agreement with a study that reported increasing the potency of a triadimenol fungicide by incorporation of a chlorine substituent in the para position (Jeschke 2010). Du et al. (2018) also reported improved antibacterial activity with a chlorine atom at the para position of the benzene ring of quinazolin-4-one derivatives but poor activity when the chloro was at the meta/ortho positions, and they attributed this phenomenon to steric hindrance. Our work therefore identified a halogen group at the para position of the benzene ring to be essential for biological activity of ralhibitins.

Ralhibitin F, which has a fluoro substituent, had only low inhibitory effects on the growth of *Rs* and *Xoo* (Fig. 3). This result is similar to the study in which bromine- and chlorine-substituted derivatives of 4-hydroxycoumarin showed higher antibacterial activity against

Bacillus subtilis and *Staphyloccocus aureus* (Zavrsnik et al. 2007), and fluorine-substituted flavonoids showed low antibacterial properties against *S. aureus* and *E. coli* as compared to other halogen substitutions (Bahrin et al. 2016).

In this study, bromine-, chlorine-, and iodine-substituted derivatives from 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline showed high antimicrobial activity against *Rs* and *Xoo*. Thus, the moiety of 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline seems to be also essential for the antimicrobial activity. Recently, it was reported that 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline derivatives has anti-leishmanial activity (Ashok et al. 2016). In addition to the ralhibitins A to E, we expect that 1-(4-iodophenyl)-6-methoxy-2,3,4,9-tetrahydro-1H-β-carboline will also show high antimicrobial activity because a methoxy substituent in ralhibitins A and B did not affect the antimicrobial activity of ralhibitins C and D. Thus, bromo and iodo ralhibitins may show the highest antibacterial activity. Cushnie and Lamb (2005) reported that the antimicrobial potency of flavonoids can be enhanced by halogenation of the B ring, and the efficacies of 3-chloro and 4-bromo derivatives of 3-methyleneflavanone were four times greater against *Enterococcus faecalis* and two times greater against *S. aureas* than the original compound. Similarly, another derivative from the halogenation of the B ring (2', 4'-dichloro) had a 2–4-fold increase in potency against *E. faecalis* and a 4–8-fold improvement in activity against *S. aureas* as compared to the original compound (Cushnie and Lamb 2005). High antifungal activity has also been linked to the presence of a chlorine or bromine moiety attached to the C_2 of the phenyl ring in 2-bromo-2-chloro-2-(4-chlorophenyl sulfonyl)-1-phenylethanone (Bondaryk et al. 2015).

Ralhibitin E, which possesses iodine, had an inhibitory effect against not only *Rs* and *Xoo*, but also *Xcca* and *Cmm*. *Xcca* is a causal agent of black rot *Brassica* species, and was also listed as the top 5th of important plant pathogenic bacterium, whereas *Cmm* is a Gram-positive

bacterial pathogen which causes tomato bacterial canker, and was ranked as next most important plant pathogenic bacterium after the top 10 (Mansfield et al. 2012). The MIC of ralhibitin E against *Xcca* and *Cmm* was 10 μg/ml (Fig. 4). Iodine was reported to have desirable biocidal properties manifesting as rapid virucidal, fungicidal, sporicidal, and bactericidal effects (Zubko and Zubko 2013). This may explain the broad activity range of ralhibitin E against both Gram-negative and Gram-positive bacteria in this study. It is also reported that iodine had a higher activity against *S. aureas* and *Pseudomonas aeruginosa*, and that the bacterial biofilm was completely eliminated within 24 h after treatment (Thorn et al. 2009). Some bactericides such as bronopol, oxolinic acid, oxytetracycline, streptomycin, and 8-hydroxy-quinoline were investigated against *Cmm*, and their MIC were determined to be between 4–8 μg /ml (de Leôn et al. 2008). Despite this progress, there are currently no chemicals to comprehensively control the disease (de Leôn et al. 2008). Therefore, ralhibitin E may be another candidate for this bactericide. There is no effective method to control *Xcca*; however, control strategies like the use of zinc sulphate, sodium hypochlorite, antibiotics, and seed treatments have been used (Vicente and Holub. 2013). Thus, ralhibitin E is also expected to be a potential bactericide of black rot disease in brassicae. The ralhibitins did not inhibit the growth of non-pathogens like the biocontrol bacterium *P. protegens*. This is especially important in integrated disease management with cultural, bio-control, and chemical methods.

It has been reported that physical factors like pH and temperature affect antimicrobial activity in vitro (Hada and Sharma 2017). Similarly, storage conditions of antimicrobials can decrease their activity (White et al. 1991). Some antibiotics like amoxicillin are affected by temperature and pH. The amoxicillin storage temperature range is (2–8°C) and inappropriate condition renders them ineffective due to degradation (Naidoo et al. 2006). In our study, ralhibitins were found to be stable over a wide range of temperature and pH. These properties

are important in the storage and distribution of ralhibitins especially in the developing countries with a hot climate and lacking refrigeration facilities. Because ralhibitins are highly stable, they have much advantage for application.

Resistance to antibiotics has been linked to a number of causes, such as the failure to penetrate the outer membrane of Gram-negative bacteria, modification of their targets, efflux of the compound from the cell or destruction of the antibiotic by enzymes (Wright 2005). Our study revealed that *Rs* grew in the spent culture medium of ralhibitin-insensitive bacteria such as *Bg*, *Pcc*, and *Rr* with ralhibitin (Fig. 6), implying that these bacteria could inactivate the inhibitory effect or detoxify ralhibitins.

Introduction of halogen groups to the active ingredients of crop protection compounds has made them environmentally friendly, improved their efficacy, increased their economic viability, and improved membrane permeability (Jeschke 2010). Modification of lead compounds in their structure and functional groups can improve their physio-chemical characteristics, target interaction, and overall biological potency (Jeschke 2010). For over 35 years, halogen-bearing compounds have been of significant value in agriculture, mainly due to their high electronegativity, high target molecular interaction, enhanced biological potency, and thermal and metabolic stability (Jeschke 2017). Statistics between 1998–2008 showed that the majority (78.5%) of new agrochemicals had halogen substituents (Jeschke 2010). In the pharmaceutical industry, about 50% of the top marketed drugs have halogen substituents (Coates and Shing 2016). These reports confirm the prominent role played by halogen substitutions in the antimicrobial potency of compounds and suggest the great potential of halogen-containing antibiotics for disease control.

Our study revealed that ralhibitins with a halogen group at the para position of the benzene ring in 1-phenyl-2,3,4,9-tetrahydro-1H-β-carboline were highly potent against plant

pathogenic bacteria. The results showed that the ralhibitins (A-E) had greater antibacterial activity against *Rs* and *Xoo* than previously reported thiadizole derivatives or other compounds as described above. Additionaly, Ralhibitin E could inhibit the growth of *Xcca* and *Cmm* showing specific activity against plant phytopathogenic bacteria. Thus, ralhibitins are highly active and stable, therefore they can be potential lead compounds for the specific control of *Rs*, *Xoo*, *Xcca* and *Cmm*. Further studies will focus on physiochemical factors that affect ralhibitin's activity like adhesion, spread, retention and the presence of suitable carriers to improve performance of ralhibitin formulations in the natural condition. Investigation of other factors like the right dosage, and timing of application will further enhance future economic feasibility of ralhibitins.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

Fig. 1. Inhibitory effect of chemicals on growth of *Rs*1002. (a) Structure of chemical compounds. Number 1 was screened from the pilot library, and numbers 2–9 are its analogues. (b) Activities of each compound to inhibit the growth of *Rs*1002. Number and name of compounds used in NPDepo are number 1 (NPD252), 2 (NPD5838), 3 (NPD5839), 4 (NPD8268), 5 (NPD8269), 6 (NPD10580), 7 (NPD8265), 8 (NPD8264) and 9 (NPD5835). Overnight-cultured *Rs*1002 in 3 ml of BG medium was centrifuged, then bacterial density was adjusted to OD_{660}= 0.1 with fresh BG medium. One hundred and fifty µl of *Rs*1002 suspension was added to each well of a 96-well microtitre plate. As a control treatment 0.5 µl of DMSO was added, and 0.5 µl of each compound (3 mg/ml, DMSO) was added to obtain a final concentration of 10 µg/ml. After incubation for 24 h at 27°C in microtitre plates, the absorbance at OD_{600} was measured. The relative absorbance (OD_{600}) of the DMSO control was set to '1'. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations, and asterisks indicate that results are statistically significant compared to the DMSO control (*P<0.001). The final concentration of kanamycin (Km) was 50 µg/ml.

Fig. 2. Dose-dependent inhibition of *Rs*1002 by ralhibitins. (a) Supplier and chemical structural formula of each ralhibitin. (b) Structures of six ralhibitins. (c) Activity of each ralhibitin to inhibit the growth of *Rs*1002. *Rs*1002 was incubated in 3 ml of BG medium without or with one ralhibitin for 24 h at 27°C in a glass tube, then the absorbance at OD_{660} was measured. The relative absorbance (OD_{660}) of the DMSO control was set to '1'. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at *P<0.05 were considered significant.

Fig. 3. The growth inhibition effect of ralhibitins on various bacterial strains. Various bacterial strains listed in supplementary Table S1 were incubated in 3 ml of medium with or without ralhibitins at 10 µg/ml for 24–48 h at 27°C in a glass tube, then the absorbance at OD_{660} was measured. The relative absorbance (OD_{660}) of the DMSO control was set to '1'. Results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

Fig. 4. Dose-dependent activity of ralhibitins to inhibit the growth of *Xoo* T7174 (a), *Xcca* (b), and *Cmm* (c). The relative absorbance (OD_{660}) of the DMSO control was set to '1'. Results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

Fig. 5. Stability of ralhibitins. (a) Effect of temperature on inhibition of the growth of *Rs*1002 by ralhibitins. (b) Activity of ralhibitins on inhibition of the growth of *Rs*1002 at different pH of BG medium. Ralhibitins were applied at a final concentration of 10 µg/ml. After incubation for 24 h at 27°C in a glass tube, the absorbance at OD_{660} was measured. The results shown are means of three independent experiments with three replicates. Error bars represent standard deviations. Mean differences at $*P<0.05$ were considered significant.

Fig. 6. Inhibitory effect of pre-culture with ralhibitins. Three bacteria (*Pcc*, *Bg*, and *Rr*) were incubated with 10 µg/ml ralhibitins overnight. Then the filtrate of each culture supernatant was used for the medium of *Rs*1002. Controls were precultured medium without ralhibitins.