

Comparative Study of Disease Suppression on Various Host Plants by *Bacillus* Cyclic Lipopeptides

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Abstract

In biological controls using *Bacillus* spp., cyclic lipopeptides play a role as elicitors to induce disease resistance on various host plants. However, it is still unclear the specificity between cyclic lipopeptides and host plants to induce disease resistance. In this study, we aimed to clarify the specificity to induce disease resistance among cyclic lipopeptides on various host plants. Our data clearly showed both cyclic lipopeptides conferred disease suppression on most of host plants, but at different range of cyclic lipopeptide concentration. Our findings contribute to understanding the complex on the specificity of cyclic lipopeptide derived induced disease resistance.

Keywords

Bacillus, Cyclic Lipopeptide, Induced Disease Resistance, Surfactin, Iturin

1. Introduction

Cyclic lipopeptides (cLPs) are well known to play key roles in biological controls against several kinds of plant diseases by using *Bacillus* spp. as biological control agents [1]. These molecules are produced through the non-ribosomal synthesis of peptides, coordinated by a complex of multienzymes (NRPSs) which catalyze all necessary steps including the ordered selection and condensation of amino acid residues [2]. So far, a variety of cLPs derived from *Bacillus* spp. have been identified and are categorized into 3 families: surfactin family, iturin family and fengycin family, depending on the chemical structure and biosynthetic genes [2].

Surfactin and iturin A are well studied compounds among the *Bacillus* cLPs.

Surfactin A consists of a β -hydroxy fatty acid with a heptapeptide ELLVDLL, and iturin A consists of a β -amino fatty acid with a heptapeptide NYNQPNS. Both cLPs show antimicrobial activity [3] [4] [5].

cLPs also behave as elicitors to induce disease resistance on several kinds of plants. Rice [6], strawberry [7], and grapevine [8] have been reported to show disease suppression depending on the cLPs used to induce disease resistance. Although cLPs induce disease resistance on host plants with a broad host range, there is also some specificity among the combinations of cLP molecules and host plants. However, the factors that determine the ability of cLPs to induce disease in plants and how they work from different hosts are still unclear.

We previously reported that both purified surfactin and iturin A show disease suppression against soil-borne diseases caused by *Fusarium oxysporum* on tatsoi and lettuce, respectively [9] [10]. However, excess amounts of cLP amendments in soil negate the disease suppression for both of surfactin and iturin A.

In this study, we aimed to clarify the effect of surfactin and iturin A to suppress disease via induced disease resistance in various edible plants.

2. Materials and Methods

2.1. Plants and Microbes

Plants and microbes used in this study are listed in **Table 1**.

Table 1. Plants and microbes used in this study.

| Plants | | |
|---|--|--|
| Scientific name | Cultivar | Common name |
| <i>Brassica oleracea</i> | Ranten (Sakata Seed, Kanagawa, Japan) | Cabbage |
| <i>Solanum lycopersicum</i> | Frutica (Takii, Kyoto, Japan) | Tomato |
| <i>Oryza sativa</i> | Nipponbare (Nouken, Kyoto, Japan) | Rice |
| <i>Glycine max</i> | Okuharawase (Sakata Seed, Kanagawa, Japan) | Soybean |
| <i>Cucumis sativus</i> | Sagami Hanjiri (Noguchi Seeds, Saitama, Japan) | Cucumber |
| Microbes | | |
| Strain | Description | Bacterial suspension for inoculation (cells mL ⁻¹) |
| <i>Pseudomonas syringae</i> pv. <i>maculicola</i> MAFF 302783 | Causal agent of bacterial leaf spot of cabbage | 10 ⁵ |
| <i>P. syringae</i> pv. <i>tomato</i> MAFF 302665 | Causal agent of bacterial speck of tomato | 10 ³ |
| <i>P. syringae</i> pv. <i>oryzae</i> MAFF 301529 | Causal agent of bacterial halo blight of rice | 10 ⁹ |
| <i>P. syringae</i> pv. <i>glycinea</i> MAFF 301683 | Causal agent of bacterial blight of soybean | 10 ⁴ |
| <i>P. syringae</i> pv. <i>lachrymans</i> MAFF 301322 | Causal agent of marginal blight of cucumber | 10 ⁴ |
| <i>Bacillus subtilis</i> ATCC 21556 | Iturin A-producing bacteria | |

2.2. Cyclic Lipopeptides

Surfactin sodium salt was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Iturin A was purified from solid-state cultures of *B. subtilis* ATCC21556 by organic solvent extraction followed by column chromatography, as previously described [9]. Briefly, solid-state culture of *B. subtilis* ATCC 21556 was extracted by ethyl acetate (EtOAc): 1-butanol (BuOH) = 7:3 (v/v), and then, organic phase was dried up by a rotary evaporator. The resulting residue was dissolved in EtOAc: methanol (MeOH) = 90:10 (v/v) and applied to a silica gel column (SI60µm SIZE:60., Shoko Science, Kanagawa, Japan). Iturin A was eluted by a linear gradient of EtOAc - MeOH (90:10 to 50:50 within 30 min). The elution containing iturin A were combined and dried up by a rotary evaporator. The resulting residue was dissolved in 50% MeOH, and applied to an ODS column (Octa Decyl Silyl) resin (Wakogel 50C18, Wako Pure Chemical Industries, Osaka, Japan). Iturin A was eluted by a linear gradient of MeOH - DDW (50:50 to 75:25 within 60 min, followed by 75:25 for 30 min). Purified Iturin A was >98% pure measured at 210 nm by HPLC.

2.3. Disease Suppression Assay

Disease suppression assay was conducted in an air-controlled greenhouse at 24°C. Host plants were propagated by a hydroponic culture with Hoagland solution [11] unless otherwise described. Population of bacterial suspensions for inoculation on host plants is listed in **Table 1**.

2.3.1. Rice

Rice plants were cultivated with Kasugai solution [12]. At the seven-leaf stage, purified surfactin or iturin A was added to hydroponic solution for root-treatment on host plants.

Pseudomonas syringae pv. *oryzae* MAFF 301529, the causal agent of bacterial halo blight on rice, was grown on LB agar plate at 25°C for 2 days. The bacterial colony was obtained and suspended in sterilized 0.1% w/v Tween 20 solution. Five microliters of bacterial suspension was inoculated on true leaves by puncturing method. After inoculation of pathogen, rice plantlets were covered by plastic bags for 24 h in the greenhouse to maintain high humidity. Disease severity was rated using a 0 to 3 rating scale (0, no disease symptom; 1, pale green or pale yellowish symptoms; 2, yellow or yellowish-brown symptoms; 3, conspicuous halo symptom) [13].

2.3.2. Other Plants

Bacterial pathogens (**Table 1**) were grown on King's B agar plate at 25°C for 2 days. Bacterial colonies were obtained and suspended in sterilized 10 mM MgSO₄ solution. The bacterial suspension was inoculated by infiltration on the abaxial side of leaves. After inoculation of pathogen, plantlets were covered by plastic bags for 24 h in the green house to keep high humidity. Disease incidence was expressed in terms of the percentage of disease symptoms.

2.3.3. Statistical Analysis

All statistical analysis was achieved using R v.3.5.1 (<https://www.r-project.org/>). Disease severity on rice was performed by Wilcoxon's U-test. Disease incidence on other host plants was performed by Student's T-test.

3. Results and Discussion

3.1. Cabbage

We previously reported on disease suppression by soil amendments of purified surfactin or iturin A against Fusarium yellows of tatsoi, *Brassica rapa* var. *rosularis* [9]. We found that both surfactin and iturin A significantly suppress disease caused by this soil-borne pathogen. However, the disease suppression by surfactin and iturin A can be negated by applying excess amounts of either cLP in soil.

Surfactin is a strong haemolytic, antibacterial and antiviral molecule [14]. By contrast, members of iturin family present limited antibacterial and no antiviral activity but they have a strong *in vitro* antifungal action [15]. Beyond that, these molecules have been reported as inducers of defense response in host plant by stimulation of the induced systemic resistance phenomenon [7] [16].

Significant disease suppression against bacterial cabbage leaf spot was observed after root-treatment of host plants with both surfactin and iturin A at a range of 0.125 to 1 μM (Figure 1(a) and Figure 1(b)). Moreover, disease suppression was negated after application of more than 2 μM of root-treatments for both of surfactin and iturin A, respectively, whereas no disease symptoms were observed with 4 μM of surfactin or iturin A root-treatments without pathogen-inoculation (Figure 1(a) and Figure 1(b)).

This data more clearly demonstrates that the disease suppression is due to the effects of induced disease resistance by surfactin and iturin A, instead to the antimicrobial activities of surfactin and iturin A directly against soil-borne pathogens. Furthermore, it is notable that the loss of disease suppressive activities of surfactin and iturin A at high concentrations occurred via lack of induced disease resistance, with no visible plant disease symptoms observed unless a pathogen is applied.

3.2. Tomato

On tomato, significant disease suppression against bacterial speck was observed by root-treatment of surfactin at a range of 1 to 4 μM (Figure 1(c) and Figure 1(d)). On the other hand, no significant disease suppression was observed at a range of 0.25 to 32 μM of iturin A treatments.

In tobacco (*Nicotiana tabacum*), which belongs to the same family (Solanaaceae) as tomato, surfactin treatment of tobacco cells elicits induced disease resistance related events, whereas no responses were observed following iturin A treatments [17]. Our data suggests that a specificity between surfactin and Solanaaceae plants may exist for eliciting induced disease resistance.

Similar to cabbage, high surfactin treatments over 8 μM negated disease

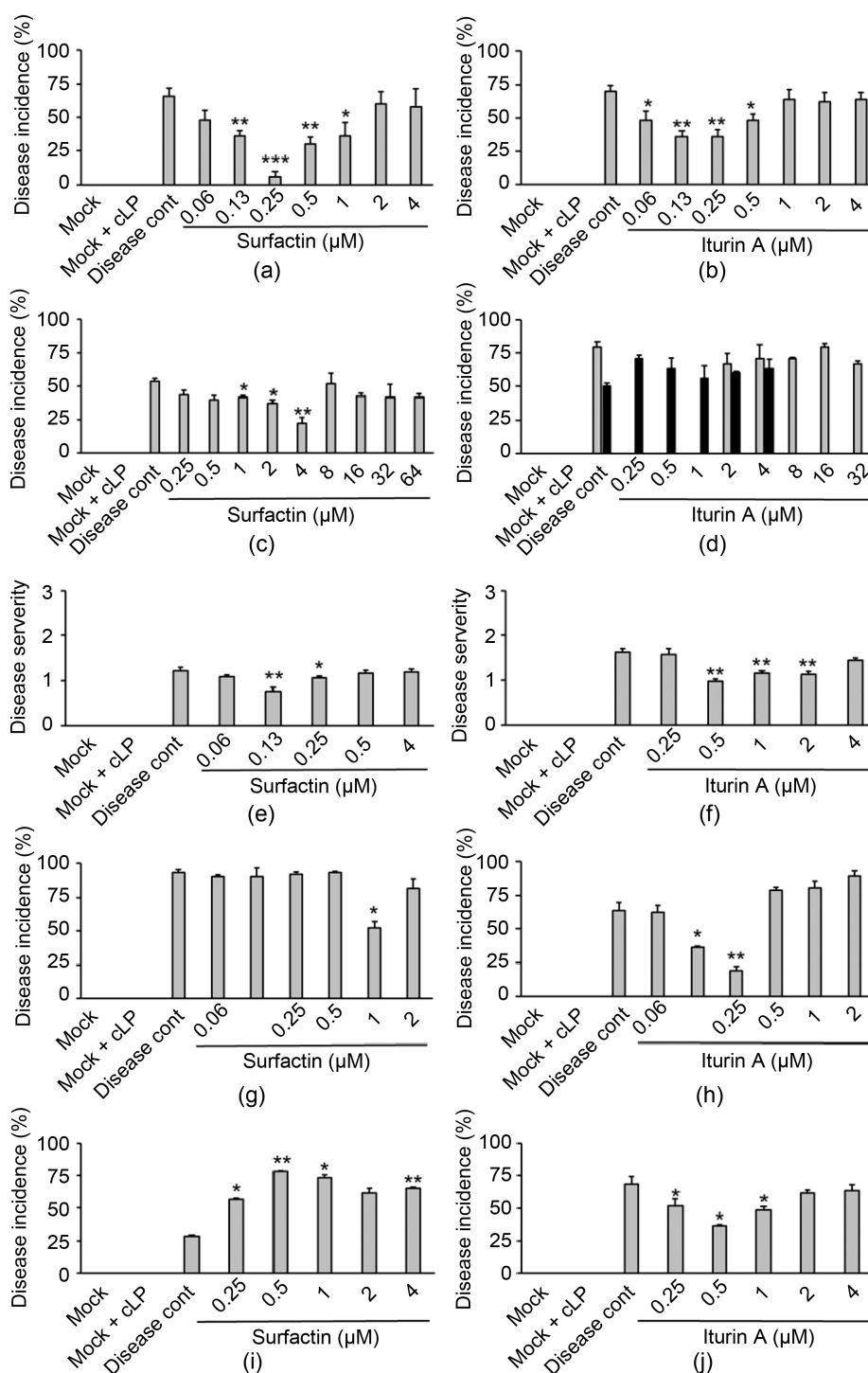


Figure 1. Disease suppression following root treatment of surfactin or iturin A against bacterial leaf disease on various kinds of host plants. (a) surfactin-treatment on cabbage ($n = 5$), (b) iturin A-treatment on cabbage ($n = 5$), (c) surfactin-treatment on tomato ($n = 3$), (d) iturin A-treatment on tomato ($n = 3$), (e) surfactin-treatment on rice ($n = 3$), (f) iturin A-treatment on rice ($n = 3$), (g) surfactin-treatment on soybean ($n = 3$), (h) iturin A-treatment on soybean ($n = 3$), (i) surfactin-treatment on cucumber ($n = 3$), (j) iturin A-treatment on cucumber ($n = 3$). Mean \pm SE of disease incidence or disease severity are represented. Asterisks indicate significant difference from disease control: *, $P < 0.05$; **, $P < 0.01$. The concentrations of cLP in Mock + cLP were set as the highest concentration for each experiment. Two independent experiments testing a range of concentrations of iturin A-treatments are represented by gray and black bars in panel D.

suppression activities whereas no disease symptoms were observed with up to 32 μM of surfactin treatment without pathogen inoculation.

3.3. Rice

On rice, both surfactin and iturin A showed disease suppression activity against bacterial leaf blight of rice after root treatments. However, the range of concentrations that elicited disease suppression differed between surfactin and iturin A (**Figure 1(e)** and **Figure 1(f)**). The range of concentration that showed significant disease suppression by surfactin was 0.06 to 0.5 μM in hydroponic culture. This disease suppression range was lower than that observed for iturin A treatments, which was 0.5 to 2 μM in hydroponic culture. Moreover, similar to the above plants, 1 μM of surfactin or 4 μM of iturin A treatments negated the observed disease suppression, whereas no disease symptoms were observed at 1 μM of surfactin- and 4 μM of iturin A-treatments, respectively, in pathogen-free plants.

On disease suppression through treatment of purified surfactin or mycosubtilin, a member of iturin family, against rice sheath blight on rice, and followed the rice plant response through gene expression analysis for induced disease resistance related genes [6]. With respect to their findings, both surfactin and mycosubtilin elicited expression of induced disease resistance related genes on rice. However, in that study, only surfactin showed disease suppressive activity at the 35 μM treatment used against the fungal disease on rice, whereas no disease suppression was observed following mycosubtilin treatment at the same concentration. Based on our findings, we propose that mycosubtilin might also show disease suppression against rice fungal disease at a different range of mycosubtilin concentration.

3.4. Soybean

On soybean, both purified surfactin and iturin A treatments showed significant disease suppression against bacterial blight of soybean (**Figure 1(g)** and **Figure 1(h)**). However, the concentration of surfactin that showed significant disease suppression was 1 μM in hydroponic culture, which was higher than the significantly effective range of iturin A treatment (0.13 and 0.25 μM in hydroponic culture). Moreover, 2 μM of surfactin or 0.5 μM of iturin A treatments negated the disease suppression, whereas no disease symptoms were observed by both surfactin- and iturin A-treatments at 2 μM in pathogen-free soybean plants.

3.5. Cucumber

The treatment of cucumber seeds with a mixture of 1 μM of semi-purified of iturin, surfactin and fengycin, respectively, showed no disease suppression against cucurbit anthracnose on cucumber leaf caused by *Colletotrichum lagenarium* [18].

In this study, iturin A treatments of cucumber roots showed significant disease suppression against cucurbit angular leaf spot at a range of 0.25 to 1.0 μM in

hydroponic culture, whereas the negation of disease suppression was observed at 2 μM and higher of iturin A in hydroponic culture (**Figure 1(i)** and **Figure 1(j)**), similar to the other plants above. On the other hand, surprisingly, significant disease enhancement was observed following surfactin treatments at a range of 0.25 to 4 μM at 3 days post inoculation. With respect to the previous report [15], we deduce that by including surfactin in the mixture of cLPs, it might have acted antagonistically against the disease suppression by iturin A.

4. Conclusions

In this present study, we investigated that disease suppression elicited by purified surfactin- and iturin A-treatments against bacterial leaf diseases on various kinds of edible plants. The induce resistance promoted by cLPs required different concentration range of iturin A or surfactin depending on host plant. Based on our observations, most of the studied host plants were elicited by cLP treatment on roots to suppress diseases through induced disease resistance, and the effective disease-suppressing concentrations varied by host and cLP.

The negation of disease suppressive activity that was observed at excess concentrations of either surfactin or iturin A for all host plants was confirmed to be through loss of disease suppression. It is still unclear how host plants recognize cLPs. Further study should be done by analyzing gene expression of induced disease resistance related genes at a variety of cLP concentrations.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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