Characterization of disease suppression activity of Bacillus cyclic lipopeptide depending on the induced disease resistance in plant

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Characterization of disease suppression activity of *Bacillus* cyclic lipopeptide depending on the induced disease resistance in plant

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By
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DEDICATION

I dedicate this work to my family

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Summary

A number of *Bacillus* strains have been reported to be biological control agents against several kinds of plant diseases. Most of the beneficial strains produce cyclic lipopeptides (cLPs), and cLPs, well-known antimicrobial compounds, are considered to play key roles in the suppression of plant diseases. A few papers have characterized the induced disease resistance elicited by cLPs so far, however, it is still unclear about the specificity to induce the disease resistance among the combinations of cLP molecules and host plants, and the signaling pathways in the innate immune system in planta.

Surfactin and iturin A are *Bacillus* cLPs. Iturin A is composed of the heptapeptide NYNQPNS linked to a ß-amino fatty acid, and surfactin is composed of the heptapeptide ELLVDLL linked to a ß-hydroxy fatty acid. In the previous studies in my lab, both purified surfactin and iturin A show disease suppression against soil-borne diseases caused by *Fusarium oxysporum* on tatsoi and lettuce, respectively. However, excess amounts of cLP amendments in soil negate the disease suppression for both of surfactin and iturin A.

In my PhD thesis, I aimed to characterize the effect of surfactin and iturin A to suppress disease via induced disease resistance in various edible plants and *Arabidopsis thaliana*.

1. Comparative study of disease suppression on various edible host plants by *Bacillus* cyclic lipopeptides

To evaluate disease suppression activities of purified cLPs via induced disease resistance, I conducted a bioassay system by hydroponic culture of host plants. *Brassica oleracea* (cabbage), *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), *Glycine max* (soybean) and *Cucumis sativus* (cucumber) were used as host plants. Seedlings of host plants were treated with purified surfactin or iturin A on their roots by addition of cLP to hydroponic culture. Two-days after cLP-treatment, the bacterial

pathogen suspension with sterilized 10 mM $MgSO_4$ was inoculated on the abaxial side of leaves by infiltration method.

Most of the studied host plants were elicited by both cLPs treatment on roots to suppress diseases through induced disease resistance, except tomato and cucumber. On tomato, only surfactin showed significantly disease suppression at a range of 1 to 4 µM, whereas no disease suppressions were observed at a range of 0.25 to 32 µM of iturin A. On cucumber, although significantly disease suppressions were observed by iturin A treatments, surfactin treatments enhanced disease comparing with disease control. It was notable that the effective disease-suppressing concentrations varied by host and cLP, and 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.

These findings strongly suggested that cLPs elicit induced disease resistance on a variety of edible host plants.

2. Insights on suppression of bacterial leaf spot by *Bacillus* cyclic lipopeptides via induced resistance in *Arabidopsis thaliana*

Induced disease resistance in plants is characterized into two systems; systemic acquired resistance, SAR, is an immune system triggered by pathogen recognition, which uses salicylic acid (SA) as a signaling molecule, whereas induced systemic resistance, ISR, triggered by rhizobacteria recognition, which uses jasmonic acid (JA) and ethylene. These two systems are inducible and strictly regulate the expression of distinct defense genes in planta.

To identify the signaling system in induced disease resistance elicited by cLPs treatments, *A. thaliana* Col-0 and its mutants were used as host plants. As well as described above on edible host plants, I conducted a bioassay system by hydroponic culture.

On *A. thaliana* Col-0 wild type, significant disease suppression against the bacterial disease on leaves was observed following root-treatments of purified surfactin and iturin A, respectively. However, the ranges of concentration to show disease suppressions differed between cLPs. Surfactin conferred significant disease suppression at a range of 4 to 16 μM in hydroponic culture, whereas iturin A conferred suppression at a range of 0.5 to 2 μM. Moreover, the disease suppression was negated at 32 μM surfactin and 4 μM iturin A, respectively, whereas no abnormalities were observed at 32 μM surfactin and 4 μM iturin A without bacterial pathogen inoculations.

To evaluate the signaling pathways in *A. thaliana* conferring disease suppression by cLP treatments, a series of *A. thaliana* Col-0 mutants.

NPR1, nonexpressor of pathogenesis-related protein 1, has been identified as a mediator for the expression in both of SA signaling and JA signaling pathways in *A. thaliana*. In npr1 mutants, no disease suppression was observed using either surfactin- or iturin A-treatments, suggesting that the induced disease resistance elicited by cLPs was conferred through NPR1-dependent pathway.

ICS1, isochorismate synthase 1, is a biosynthetic enzyme for SA via isochorismate

pathway. Generated SA is an important plant hormone, and acts as a regulatory component during SA signaling of induced disease resistance in *Arabidopsis*. In an *Arabidopsis ics1* mutant, no disease suppression was observed following treatment with surfactin or iturin A, suggesting that SA biosynthesis through the ICS pathway is important in the induced disease resistance elicited by surfactin and iturin A.

JAR1, jasmonate resistant 1, is a jasmonate-amido synthetase in the biosynthesis of jasmonate-isoleucine conjugate which activates the JA signaling pathway in *Arabidopsis*. In *jar1* mutants, significant disease suppression was observed following treatments of 8 μM surfactin and 1 μM iturin A, similar to as was

observed in wild type Col-0, suggesting that the JA signaling pathway does not confer the disease suppression by induced disease resistance elicited by cLPs.

Those findings revealed that SA acid signaling pathway via isochorismate pathway was the major signaling pathway in the induced disease resistance elicited by both cLPs. Moreover, negations of disease suppression were observed by excess amount of surfactin or iturin A treatment, and the negation of disease suppression was not correlated to the antagonistic effect by induction of JA signaling pathway in host plants.

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Chapter 1

1. General background

1.1. *Bacillus subtilis*

Bacillus genus Cohn (1872) belongs to the family Bacillaceae, which comprises most of the spore-forming species. The representatives of this group are gram-positive bacteria spore forming, aerobic or facultative anaerobic, which are shaped into rods with variable sizes (0.5 X 1.2 µm) (Turnbull, 1996). During the sporulation process, ellipsoidal spores are produced with structural and chemical composition to resistance the environments conditions (Driks, 2004) making these bacteria one of the best microorganims for formulation of efficient biopeseticide products (Ongena and Jacques, 2008).

Bacillus subtilis strains are well-studied organisms of the genus Bacillus that has excellent physiological characteristic and highy adaptable metabolis, allowing the bacterium to survive in environments with constant changes (Su et al., 2020), in which facilitates their rational use (Cawoy et al., 2011). Therefore, *B. subtilis* is commonly used on the pharmaceutical, food, cosmetics, and chemical industries for the production of many products (Su et al., 2020). Futhermore, it has been considered an important PGPR (Plant Growth Promoting rhizobacteria) in agriculture due to their abilbity to act as phytopathogenic antagonists or playing a crucial role as plant growth promoters (Alina et al., 2015).

The interaction of *B. subtilis* increases the levels of nitrogen fixation, nutrient solubilization, phytonutrient synthesis and consequently promotes plant growth (Manjula&Podelie, 2005). According to these authors, the association of *B. subtilis* with plants provides the physiological increase of metabolites, optimizing the sensitivity of the radicle system to external conditions, which results in the facilitation of the perception and absorption of nutrients. In addition, members of this group have been considered one of the most efficient microrganisms in the control of plant diseases by acting as against many phytopathogens (Phae et al., 1990). Several species have been

reported acting as antagonistic to fungi and bacteria by suppressing their growth in both in vitro and in vivo enviroments (Phae et al., 1990; Asada&Shoda, 1996; Romero et al., 2006). Besides that, *B. subtilis* strains have showed efficient to protect plants by increasing host resistance to pathogens through induced systemic resistance (ISR) (Hashem et al., 2019). It has been reported effect to protect *Arabidopsis thaliana* against *Pseudomonas syringae* (Ryu et al., 2003), cucumber and tomato against *Colletotrichum lagenarium* and *Pythium aphanidermatum,* respectively (Ongena et al., 2005), melon against cucurbit powdery mildew (García-Gutiérrez et al., 2013), tobbaco leaves against Botritys cinerea (Cawoy et al., 2014), rice against *Rhizoctonia solani* (Chandler et al., 2015), maize (Gond et al., 2015), and grapevine leaves against *Plasmopara viticola* (Li et al., 2019). Figure (1) represents the signaling pathways triggered by rizhobateria in host plants. This characteristic can be attributed in large part, to the production of active ciclic lipopeptides (ASAKA; SHODA, 1996; Vater et al., 2002; BAIS et al., 2004; Romero et al., 2007).
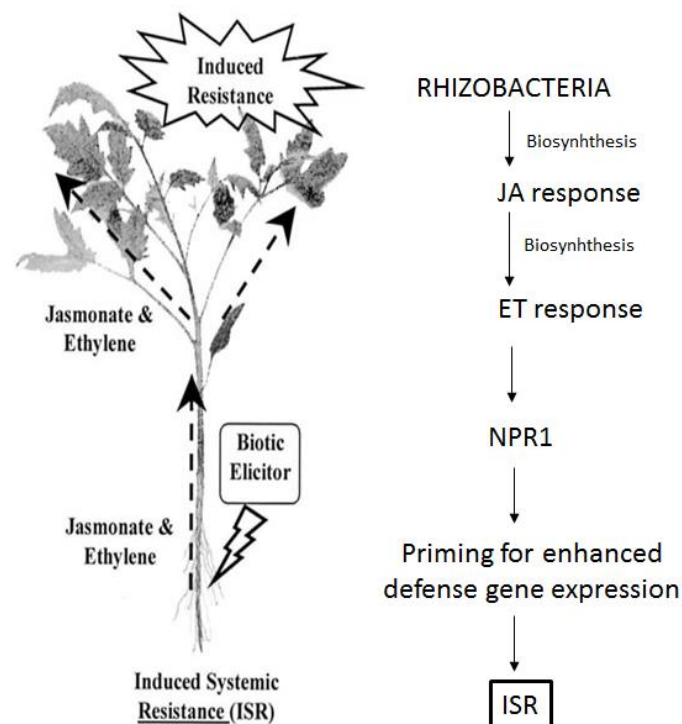
Fig 1. Schematic representation of the signaling pathways triggered rhizobacteria. The bacterium initiates the biosynthesis of jasmonic acid (JA) and ethylene (ET) that will

promote the activation of NPR1, a regulatory protein, responsible for the expression of defense gene featuring ISR pathway. (Adapted from: Vallad&Goodman, 2004).

1.2. Cyclic Lipopeptides

Cyclic lipopeptides (cLPs) are molecules composed of a fatty acid chain linked to a short oligopeptide which is cyclized to a form a lactone or lactam ring either between two amino acids in the peptide chain or between an amino acid and an amino- or hydroxylgroup bearing fatty acid moiety. The variations in the number, type (basic, acidic, aromatic, aliphalitic, cyclic, OH/SH-containing, α- or β-type), and configuration (D, L) of the amino acids in the peptide portion, the length (C_6-C_{18}) and the composition of the fatty acid moiety (β-OH groups, iso-, anteiso-methyl branched forms) are responsible to characterize a variety of molecules (Schenider et al., 2014). These molecules may act as antibiotics, antiviral and antitumor agents, immunomodulators or toxins and specific enzyme inhibitors. In addition, molecules derived from *Bacillus* spp. are often cited as playing a key role in the suppression of various diseases in plants. The mode of action of most of these compounds is not absolutely clear, although it is likely that their surface and active membrane properties play an important role (Cameotra, Makkar, 2004).

Amoung of a variety of cLPs, iturin and surfactin are members of particular antibiotic class from *Bacillus subtilis* (Lang, 2002), formed by a lipoheptapeptides cycles, which contain a β-amino fatty acid and an β-hydroxy fatty acid respectively (Bonmatin et al. 2003; Ongena and Jacques 2008). The production of these molecules is accomplished 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 (Stein, 2005).

1.3. Surfactin family

Surfacins are cyclic acidic lipopeptides produced by a several strains *Bacillus subtilis* formed by a ß-amino fatty acid (generally a ß-hydroxydecanoic acid, such as 3-hydroxy-13-methyl tetradecanoic acid) linked to the lactone ring via an amide bridge in the glutamate residue and an ester bond on the D-leu residue (Kakinuma et al., 1969). The hydrophobic moiety is formed of a carbonic chain ranging from C13 to C15 and the principal hydrophilic moiety is composed of Glu-Leu-Leu-Val-Asp-Leu-Leu, arranged as a peptide ring (Figure 2) (ARIMA et al., 1968, Kakinuma et al., 1969, LANG, 2002). Hydrophobic amino acid residues are located at positions 2,3,4,6, and 7 while an aspartyl residue is at the position 5 (Bonmatin et al., 2003). The strains but also the nutrients of the culture medium are resposible for the determination of the ß-hydroxy fatty acids (Bonmatin, 2003) that can present conformation as iso, anteiso C13, iso, normal C14, and iso, anteiso C15 (Peypoux et al., 1999).

These molecules have the ability to reduce the surface tension of the water from 72 mN/m to 27 mN/m at a concentration lower than 10 µmol/L (Arima, Kakinuma, and Tamura, 1968) and present critical micellar concentration (CMC) in water of 25 mg/l and lowers the surface tension to 27 nN/m (Rosenberg&Ron, 1999). Moreover, surfactins are soluble in many kinds of organic solvents, according to the orientation of the residues (Mulligan, 2005), including methanol, ethanol, acetone, chloroform, acetic acid, but insolube in ester, and hexane (Arima et al., 1968). The presence of L-Glu and L-Asp residues confers to surfactins high affinity for divalent cation at alkaline pH and aqueous solution (Mulligan et al., 2005) with very powerful surfactant properties and amphiphilic properties (Bonmatin et al., 1994). Due to the amphiphilic nature, surfactins can modifie the integity of biological membrane by associating and anchoring into lipid layers (Ongena&Jacques, 2007). Surfactin may also form in the bilipid membrane micellar aggregates with lamellar organization or small closed vesicles, which solubilize the membrane, as acting a detergent in this structure (CARRILLO et al., 2003).

$$R(CH_2)_{6-11}\text{-}CH\text{-}CH_2\text{-}CO \longrightarrow L\text{-}Glu \longrightarrow L\text{-}Leu \longrightarrow D\text{-}Leu$$
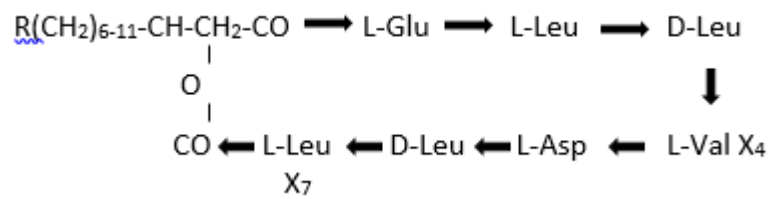
Fig 2. Primary structure of surfactin (adpted from Bonmatin et al., 2003)

The antimicrobial activity of this secondary metabolite are well know (Huang et al., 2008), but they also has been shown to present antiviral (Kracht et al., 1999), haemolytic (Dufour et al., 2005), antimycoplasma properties, and to induce cytotoxicity against many types of cancer by inhibiting cancer progression (Wu et al., 2017). In contrast, surfactin does not display a strong effect against fungi (Ongena et al., 2010). Besides that, surfactin as been reported as elicitor to induce resistance in several host plants. In tobacco and grapevine cells, surfactin induced early events related to defence such as alkalinization and oxidative burst (Jourdan et al., 2009; Farace et al., 2015). Experiments conducted on melon plants showed that surfactin was the major determinant to elicit defense response by activation of jasmonate-and salicylic acid pathways (Garcia et al., 2013). The root-treatment in tatsoi with surfactin suggested that the disease suppression observed on host plants was due to the induction of resistance promoted by this cLP (Yokota; Hayakawa, 2015).

The biological activities performed by surfactin can be explained by the chimeric structure of these molecules (VASS et al., 2001). It is believed that its antimicrobial activity is related to the strong interaction capacity that this lipopeptide possesses with its target membrane and its action on the stability of bilayers (CARRILO et al., 2003).

1.4. Iturin family

Iturin A, one of the most studied elements of the group, present a critical micellar concentration (CMC) of ca. 25 μM (Aranda et al., 2005). This molecule was the first element to be isolated from a *B. subtillis* strain in 1957 (figure 3), triggering studies that led to the identification of other members of iturin family (Bonmatin et al., 2003). Neutral or monoanionic lipopeptides that present the same pattern of chemical

constitution (Bonmatin et al., 2003) are classified into iturin A, C, D, and E, bacilomycins D, F, L, and mycosubtilin (Peypoux et al., 1978; Peypoux et al., 1978; Besson & Michel, 1986; Peypoux et al., 1980; Peypoux et al., 1985; Besson et al., 1977; Peypoux et al., 1976). The general structure of these molecules present seven aminoacids with the same LDDLLDL chiral and a commom number of residues: Asx, Glx, Pro, Ser, Thr, Tyr (Bonmatin et al., 2003) linked to one unique β-amino fatty acid chain. The length of the fatty acid may vary from one member to another: Iturin A and C and bacillomycin D and L, present a length of 14 to 15 carbons on fatty acid; while the fatty acid chain of bacillomycin F and mycosubtilin are composed by 16 to 17 carbons (Jacques, 2011).
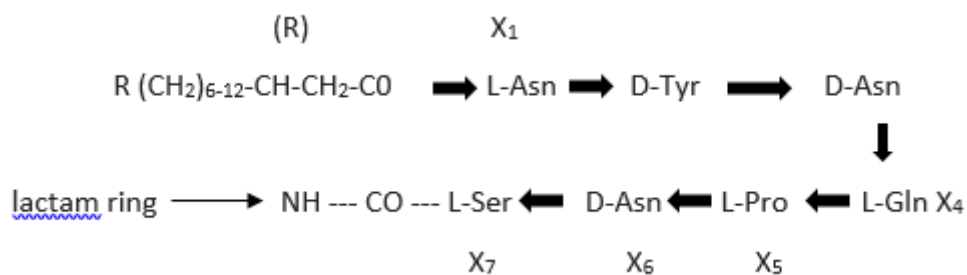
Fig. 3 Schematic Structure of Iturin A (adapted from Bonmatin et al., 2003).

The biological activity of members of iturin group are represented first by strong antigungal activity against several species of yeast and fungi (Besson et al., 1978; Phae et al., 1990), but no antiviral properties and limited antibacterial action (Ongena&Jacques, 2007) compared to surfactin molecules. The dispersinos of iturin A in different concentrations, due to its amphiphilic character, tends to associate and form small vesicles, inducing the formation of pores in biological membrane (Grau et al., 2001). The interaction with the lipid part of the organism's membrane by forming pores and not membrane disruption or solubilization (Aranda et al., 2005) is probably the mechanism that allows iturins to kill the target (Bonmatin et al., 2003). Due to their characteristics such as surfactant, low toxicity, and low allergenic effect in humans and other animal, members of iturin family have been seen as a potential biopesticide (Phae et al., 1990).

As well as surfactin molecules, iturins have been reported as elicitor to induce disease resistance in several host plants. Inducing activity of iturin is not observed on tobacco cells and rice cells (Jourdan et al., 2009; Chandler et al., 2015). On the other hand, Iturin A treatment in chili pepper promoted high expression of defense-related genes *PR1*, *Peroxidase* and *PR10* that reflected in significant reduction of disease *Phytophthora capsici* (Park et al., 2015). Moreover, experiments conducted in grapevine showed that only iturin group was effect on induce defence gene expression against disease caused by *Botrytis cinerea* (Farace et al., 2015). Interestingly, the activity of iturin, as well as surfactin, also suggest that root-treatment induced disease resistance on tatsoi by suppressing disease against *Fusarium* yellows (Yokota; Hayakawa, 2015). . It is not clear how these cLPs act to induce mechanisms of defense. However, it seems that each family require a specific ability to ellecit different plant cells (Ongena&Jacques, 2008).

1.5. Plant immune system

Plants present an extremely defense mechanism, which acts in a dynamic and coordinated way to activate an effective imune response against the invader encountered (Jones & Dangl 2006). Common compounds of microorganisms such as bacterial flagellin or fungal chitin, in wich conserved microbial elicitors called pathogens associated molecular patterns (PAMPs or MAMPs) or endogenous molecules derived from damage caused by pathogen invasion called danger-associated molecular patterns (DAMPs) are recognized by receptor proteins called pattern recognition receptors (PRRs) (Boler&Felix, 2009; Dodds&Rathjen, 2010). This type of recognition represent the first line of defense called PAMP-triggered immunity (PTI) and can retains most of the invaders (Dodds&Rathjen, 2010). This recognition promotes cell wall alternations as deposition of callose and accumulation of defense-related proteins acting negatively on pathogen colonization (van Loon et al., 2006). However, pathogens present virulence factors that might suppress host immune response by inhibiting PTI (Jones&Dangl, 2006).

In turn, plants have a second defense mechanism that apparently remains inactive or latent, until the exposition of the host to induction agents in which resistance is mediated by recognition protein of specifc effector, resulting in effector-triggered immunity effector-induced immunity (ETI) (Jones&Dangl, 2006; Pieterse et al., 2012), characterized by a manifestation of gene to gene resistance (Flors, 1971). ETI is a faster and stronger version of PTI that often accompanies programmed cell death, in a process called hypersensitive response (HR), which occurs only at the site of infection not extending beyond the infected cell to prevent entry of additional pathogens into the host tissue (Jones&Dang, 2006; Pieterse et al., 2014). The local infection often elicit one or more long-distance signals that stimulate the defense process in parts of the plant that have not yet been damaged in a process called "systemic acquired resistance (SAR)" (Shah&Zeier, 2013). Similarly to SAR, root colonization by plant growth-promoting rhizobacteria (PGPR) also can induce a systemic resistance in a phenomenon termed "induced systemic resistance (ISR)" (Van Loon et al., 1998).

1.6. Systemic acquired resistance (SAR)

SAR was first demonstrated on *Nicotiana tabacum* var. Samsun NN host in which tobacco mosaic virus (TMV) induced not only a high level of resistance to TMV in the uninfected organs against subsequent infections, but also against to tobacco necrossis virus, to turnip mosaic virus, and to tobacco and tomato ringspot viruse (Ross, 1961). Since that, experiments have showed that the activation of SAR is effect against a broad and distinctive spectrum of pathogens, including bacteria, oomycetes, and fungi (Ryals et al., 1996). The expression of SAR in the systemic tissue depedens on the generation of a signal in the inoculated tissue transported systemically via the vasculate, generally the phloem (DEMPSEY&KLESSIG, 2012). The accumulation of salicylic acid (SA) and its derivative SA-glucoside (SAG) in the pathogen-free organs characterize the activation of SAR, which is normally accompanied by the expression of the SA-responsive PR (PATHOGENISIS-RELATEDED1) gene, a marker of SAR (Shah&Jyoti, 2013). The sequence of events associated with the establishment of SAR are show on figure 4.
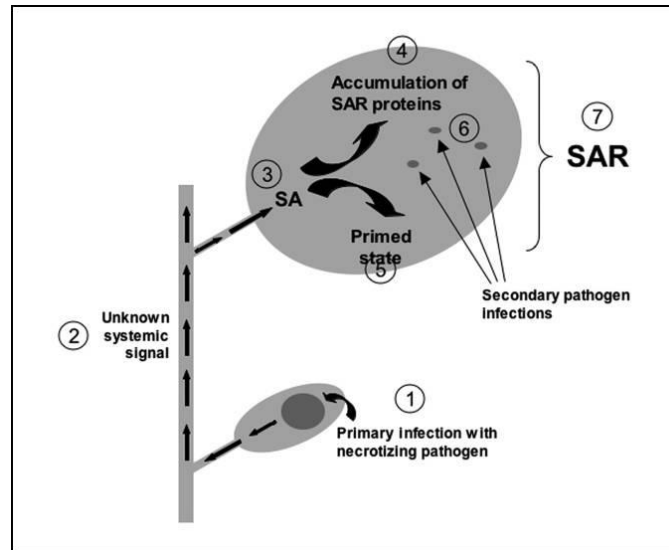
Fig 4. System of SAR activation. (1) The primary infection with a necrotizing pathogen promotes the distribution of unknown systemic signal throughout the plant (2), wich causes the accumulation of SA (3) and the activation of SAR genes (4). Adapted from Conrath, 2006.

The first evidence for this came from studies with transgenic Arabidopsis and tobacco plants unable to accumulate high levels of SA and donot acquire systemic resistance against necrotizing pathogens (Gaffney et al., 1993; Delaney et al., 1994). The synthesis of SA can be obtained via isochorimaste synthase (ICS) and phenylalanine ammonia-lyase (PAL), in wich ICS is the major pathway for SA accumulation in *A. thaliana* (Lefevere et al., 2020). The ICS branch is responsible for the conversion od isochorismate to SA by isochorismate pyruvate lyase (IPL) (Gao et al., 2015). The importance of accumulation and signaling of SA as a critical elicitor for the disease resistance conferred by SAR as been confirmed in tobacco and A. thaliana by genetic studies (Shad&Zeier, 2013).

The regulation of SA signaling is moderated by NPR1 (NON-EXPRESSOR OF PR GENES) gene (Durrant and Dong, 2004), in wich one of the target is PR1 genes, implicating a critical role of the protein secretory pathway for SAR (Shad&Zeier, 2013).

NPR1 can reside both in the nucleus and the cytosol, in wich the nuclear localization is the major required to the activation of *PR1* transcription (Kinkema et al., 2000). The importance of NPR1 to the activation of SAR via SA accumulation has been supported by two studies demonstration that the overexpression of NPR1 in transgenic plants did no lead to enhanced SA levels or expression of *PR* genes (Conrath, 2006).

1.7. Induced disease resistance (ISR)

Plants present a vast community of commensal and mutualistic microrganims called plant growth-promoting rhizobacteria (PGPR) that provide them essencial sources of growth promotion. However PGPR are well know for there service on growth promoting they also can enhance the plant immune system against a variety of pathogens by a plant-mediated immune response called rhizobacteria-induced systemic resistance (ISR) (Lugtenberg&Kamilova, 2009; Van Loon et al., 1998). ISR is characterized by the activation of latent defense mechanisms expressed not only at the site of induction but also systematically in plant tissue that are soatially separeted from the inducer (Pieterse et al., 2014). ISR was first described in carnation plants systematically protected against *Fusarium oxysporum* f. sp dianthi by *P. flouresncens* WCS417r (Van Peer et al., 1991), and in cucumber, when leaves showed resistance against *Colletotrichum orbiculare* induced by rhizobacterial strains (Wei et al., 1991).

Rhizobacteria-mediated ISR is similar to pathogen-induced systemic acquired resistance (SAR) in wich both pathway induce resistance to pathogens in plant tissues uninfected (Van Loon et al., 1998). Similar to pathogen-induced SAR, ISR is mediated by the expression of NPR1 gene whereas instead SA it requires the accumulation of both JA and ethylene (Pieterse et al., 1998), in which methyl jasmonate (MeJa) and the ET precursor, ACC are effective in inducing resistance against phytopathogenic microflora (Thomma et al., 2001). Both JA and ET act activating defense responses, in that JA and derivatives induce the expression of defense-related genes, such as thionins and proteinase inhibitor, while ET activates several tyoes of pathogenesis-

related (PR) gene family (Choudhary et al., 2009). The similarities and difference from SAR and ISR activation are demonstraded on figure 5.
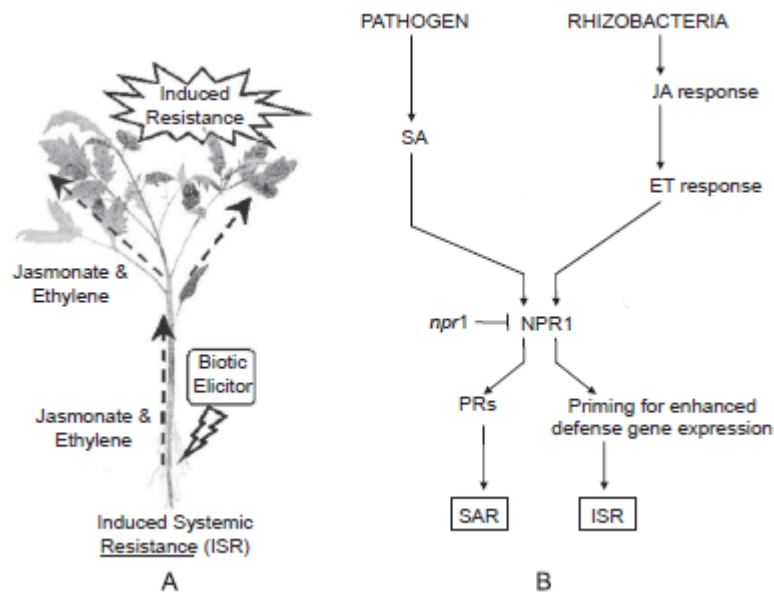
Fig 5. Representation of SAR and ISR. (A) Schematic figure of local and systematically activation of ISR in host plant. (B) Diagram of similarity and difference between SAR and ISR in *A. thaliana*. Boh pathways are mediated by the expression of NPR1 gene. Systemic acquired resistance, induced by pathogen, is dependent of SA accumulation and expression of PRs genes whereas Induced systemic resistance depends on the accumulation of JA and ET through the exposition to beneficit rizhobacteria to be activates. (Adapted from Vallad & Goodman, 2004).

The similarity between SAR and ISR and the importance of JA and ET signaling to the regulation of rhizobacteria-mediated ISR was well demonstraded by Pieterse et al., (1998) by using *A. thaliana* jasmonate response mutant *jar1*, the ethylene response mutant *etr1,* and the SAR regulatory mutant *npr1*. In this experiment, they demonstrated that *A. thaliana* showed resistance against leaf infection caused by *P. syringae* pv tomato promoted by the nonpathogenic, root-colonizing *Pseudomonas fluorescens* triggering induced systemic resistance (ISR) mediated by NPR1 via jasmonate and ethylene accumulation. In addition, methyl jasmonate–induced protection was blocked in *jar1*, *etr1*, and *npr1* plants, whereas 1-

aminocyclopropane-1-carboxylate–induced protection was affected in *etr1* and *npr1* plants but not in *jar1* plants.

The ability to develop ISR depends on specific recognition signal between the plant and the systemic resistance-inducing rhizobateria to promote the induction of resistance (Hakeem&Akhtar, 2016). The fault to elecit ISR seems to be doe to the absence of production of inducing components in the rhizosphere or an inability of the particular plant species to perceive such compounds (Van Loon, 2007).

1.8. The role of iturin and surfactin as inducers of resistance in plants

The studies involving the role of cLPs to protect plants against pathogen have showed that they present no only antimicorbial activities, that inhibit the pathogen growth, but also act as 'immuno-stimulators' by reinforcing host plant resistance potencial (Ongena &Jacques, 2008). The effect of iturin and surfactin to induce disease resistance have been characterized in several host plants. However, the efficiency or failure of these molecules to suppress disease displayed differents results depending on host plants.

Experiments performed in tomato and bean plants showed that surfactin from B. subtilis strain was the major cLP to induce ISR against disease caused by Botritys cinerea (Ongena et al., 2007). The role of surfactin as a resistance-inducing agent was also showed on rice (Chandler et al., 2015). Experiments carried out on leaf defied host plant against *Rhizoctonia solani* post surfatin and iturin treatment at final concentration of 35 µM. The results demonstrated that only surfactin was able to suppress disease by inducing ISR. Moreover, only surfactin induced- defense-related early events such extracelluar medium alkalinization and reactive oxygen, and stimulated the defense enzymes phenylalanine ammonilyase and lipoxygenase on tobacco cells (Jourdan et al., 2009).

By contrast, recently experiment in madarin fruit revelead that iturin A was the powerful cLP to stimulate the ISR system in response to stresses in postharvest mandarins (Tunsagool et al., 2019).

Treatment with both iturin and surfactin stimulated grapevine innate immune responser by activating SAR and ISR (Farace et al., 2015). These cLPs also elicited the gene expression of the pathogenisis-related proteins in strawberry triggering systemic resistance on leaves, resulting in the reduction of the severity of anthracnose disease caused by Colletotrichum gloeosporioides (Yamamoto et al., 2015).

It seems that the ability of these cLPs to elecit SAR and/or ISR depends on the host plant and the cLPs concentration used on the treatment. However, mode of action of these molecules by triggering resistance process in plants has not been fully elucidated. Although, it was suggested, that the surface and active membrane properties of each cyclic lipopeptide play an important role for their perception in plant cells (Jourdan et al., 2009). In fact, there is a strong suggestion that the recognition of surfactin by plant cells is mediated by interaction with lipids in the plasma membrane (Henry et al., 2011). The disturbance of the lipid layer is not enough to affect the viability of the cell, but triggers a cascade of molecular events that optimize plant defense (Jourdan et al., 2009).

1.9. *Arabidopsis thaliana*

A. thaliana is a plant belonging to the Brassicaceae family that is widely distributed in Europe, Asia and North America (Hoffmann, 2002). It was first described as *Pilosella siliquosa* by Johannes Thal, in the 16th century, and had its name changed several times until it became known today (Rédei, 1975). Due to its wide distribution, this species has a high number of ecotypes, in wich the Columbia and Landsberg ecotypes being outstanding for its use in laboratory tests (Meinke et al., 1998). The cycle of *A. thaliana* from germination followed by development, flowering, seed maturation and senescence corresponds to approximately 42 days. The plants develop in the form of rosette, whose size varies from two to 10 cm in diameter. In turn, the stems can reach 20 cm in height as the growing conditions (Meinke et al., 1998).

At the end of the year 2000, *A. thaliana* became the first plant organism and the third multicellular organism to possess the fully sequenced genome (The *Arabidopsis*

Genome Initiative, 2000). Subsequent to this event, the number of papers published using *A. thaliana* as a biological model increased significantly (Delatore; Silva, 2008).

One of the advantages of using *A. thaliana* in bioassays is its small size when compared to other plants. This feature makes it possible to grow in petri dishes obtaining a large number of plants at the same time and in small laboratory spaces (Meinke, 1998). This specie has a relatively small genome with five chromosomes, 20,000 genes and approximately 146 million base pairs, and about 85% of the genome consists of "coding" genes, ie DNA sequences that can be translated (Bevan; Walsh, 2006). In addition, most of the processes that occur in commercially important species also occur in *A. thaliana*. For example, from the comparison between *Arabidopsis* and rice proteomes, it was shown that 71% of the proteins predicted for rice showed similarity to *A. thaliana* proteins (BEVAN; WALSH, 2006). This similarity suggests that the cellular and biochemical functions of rice genes and possibly of other cultures can be established from experiments conducted in *Arabidopsis*. Thus, the determination of orthologous relationships between *A. thaliana* and other plants of great economic importance is a quick and easy way of transferring the information obtained in the model plant to commercial crops.

1.10. *Pseudomonas syringae*

The first isolation of *P. syringae* occurred in 1902 by van Hall from a diseased lilac (Syringa vulgaris). This specie is a gram negative bacteria, with an aerobic metabolism, and polar flagella that are taxonomically subdivided into pathogenic varieties (pathovars), based on their host isolation, that are subdivided into over 60 pathovars according to the pathogenic characters, nine genomespecies defined by DNA-DNA hybridization and 13 phylogenetic groups (Hwang et al., 2005). To interact with their host *P. syringae* strains uses a variety of virulence-associated systems that produce toxins, ice nucleation proteins, antimicrobial resistance, and secreted effectors (Hwang et al., 2005).

Pseudomonas syringae is one of the models for the scanning of plant-microbe interections in both natural and laboratory systems (Baltrus et al., 2017). The strains

of this specie are well know due to their ability to infect and cause a variety of symptoms on severeal host plants, including (but not limited to) apples, beets, beans, cabbage, cucumbers, oats, olives, peas, tobacco, tomato, rice, and *A. thaliana* (Horst, 1990; Jakob et al., 2002). The damage cause on host vary from leaf spots, and galls to death of tissues (Bender et al., 1999).

Antimicrobial such as copper (Cooksey, 1994) and streptomycin (Dye, 1953) have been used to control the infection caused by *P. syringae* strains in several crop plants (Hwang et al., 2005), however the appearance of resistant strains do exist (Cazorla et al., 2002). Unfurtanely, the extensive use of chemicals for controlling plant diseases cause risk to human health and influence the ecological balance leading to resistant strains of pathogens (Meena and Kanwar, 2015). One potential alternative for safer crop-management to control bacterial diseases is through beneficial microorganisms by using their componds (Fravel, 2005). Cyclic lipopeptides (cLPs) is one of that secondary metabolities that have gained attention due to their ability to control disease in different host plants by antagonistc activity against pathogens or by inducing resistance systems (Ongena & Jacques, 2008).

Chapter 2 – Comparative study of disease suppression on various host plant by Bacillus cyclic lipopeptides

2.1. 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 (Romero et al., 2007). 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 (Stein, 2005). 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 (Stein, 2005).

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 (Peypoux et al., 1978; Arima et al., 1968); Kakinuma et al., 1969).

cLPs also behave as elicitors to induce disease resistance on several kinds of plants. Rice (Chandler et al., 2015), strawberry (Yamamoto et al., 2015), and grapevine (Farace et al., 2015) 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 abilitiy of cLPs to induce disease in plants and how they work from different host are still unclear.

We previous reported that both purified surfactin and iturin A show disease suppression against soil-borne diseases caused by *Fusarium oxysporum* on tatsoi and lettuce, respectively (Yokota and Hayakawa, 2015; Fujita and Yokota, 2019). 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.

2.2. Materials and methods

2.2.1. 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
Brassica oleracea	Ranten (Sakata Seed, Kanagawa, Japan)	Cabbage
Solanum lycopersicum	Frutica (Takii, Kyoto, Japan)	Tomato
Oryza sativa	Nipponbare (Nouken, Kyoto, Japan)	Rice
Glycine max	Okuharawase (Sakata Seed, Kanagawa, Japan)	Soybean
Cucumis sativus	Sagami Hanjiro (Noguchi Seeds, Saitama, Japan)	Cucumber

Microbes

Strain	Description	Bacterial suspension for inoculation (cells mL^{-1})
Pseudomonas syringae pv. *maculicola* MAFF 302783	Causal agent of bacterial leaf spot of cabbage	10^5

P. syringae pv. *tomato* MAFF 302665	Causal agent of bacterial speck of tomato	10^3
P. syringae pv. *oryzae* MAFF 301529	Causal agent of bacterial halo blight of rice	10^9
P. syringae pv. *glycinea* MAFF 301683	Causal agent of bacterial blight of soybean	10^4
P. syringae pv. *lachrymans* MAFF 301322	Causal agent of marginal blight of cucumber	10^4
Bacillus subtilis ATCC 21556	Iturin A-producing bacteria	

2.2.2. 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.

2.2.3. 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 (Hoagland and Arnon, 1950) unless otherwise described. Population of bacterial suspensions for inoculation on host plants are listed in Table 1.

2.2.4. Rice

Rice plants were cultivated with Kasugai solution (Kasugai, 1939). 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) (Kuwata, 1985).

2.2.5. Other plants

Bacterial pathogen (Table 1) was inoculated on King's B agar plate at 25 °C for 2 days. Bacterial colonies were obtained and suspended in sterilized 10 mM $MgSO_4$ 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.

2.2.6. Statistical analysis

All statistical analysis were 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 were performed by Student's T-test.

2.3. Results and discussion

2.3.1. 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* (Yokota and Hayakawa, 2015). 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 (Ongena and Jacques, 2008). By contrast, members of iturin family present limited antibacterial and no antiviral activity but they have a strong *in vitro* antifungal action (Phae et al., 1990). Beyond that, these molecules have been reported as inducers of defense response in host plant by stimulation of the induced systemic resistance phenomenon (Ongena et al., 2007; Yamamoto et al., 2015).

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 6 (a) and (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 (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.

2.3.2. 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 6 (c) and (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 (Solanaceae) as tomato, surfactin treatment of tobacco cells elicits induced disease resistance related events, whereas no responses were observed following iturin A treatments (Jourdan et al., 2009). Our data suggests that a specificity between surfactin and Solanaceae plants may exist for eliciting induced disease resistance.

Similar to cabbage, high surfactin treatments over 8 µM negated disease suppression activities whereas no disease symptoms were observed with up to 32 µM of surfactin treatment without pathogen inoculation.

2.3.3. 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 6 (e) and (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 (Chandler et al., 2015). 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.

2.3.4. Soybean

On soybean, both purified surfactin and iturin A treatments showed significant disease suppression against bacterial blight of soybean (Figure 6 (g) and (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.

2.3.5. 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* (Ongena et al., 2005).

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 6 (i) and (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.
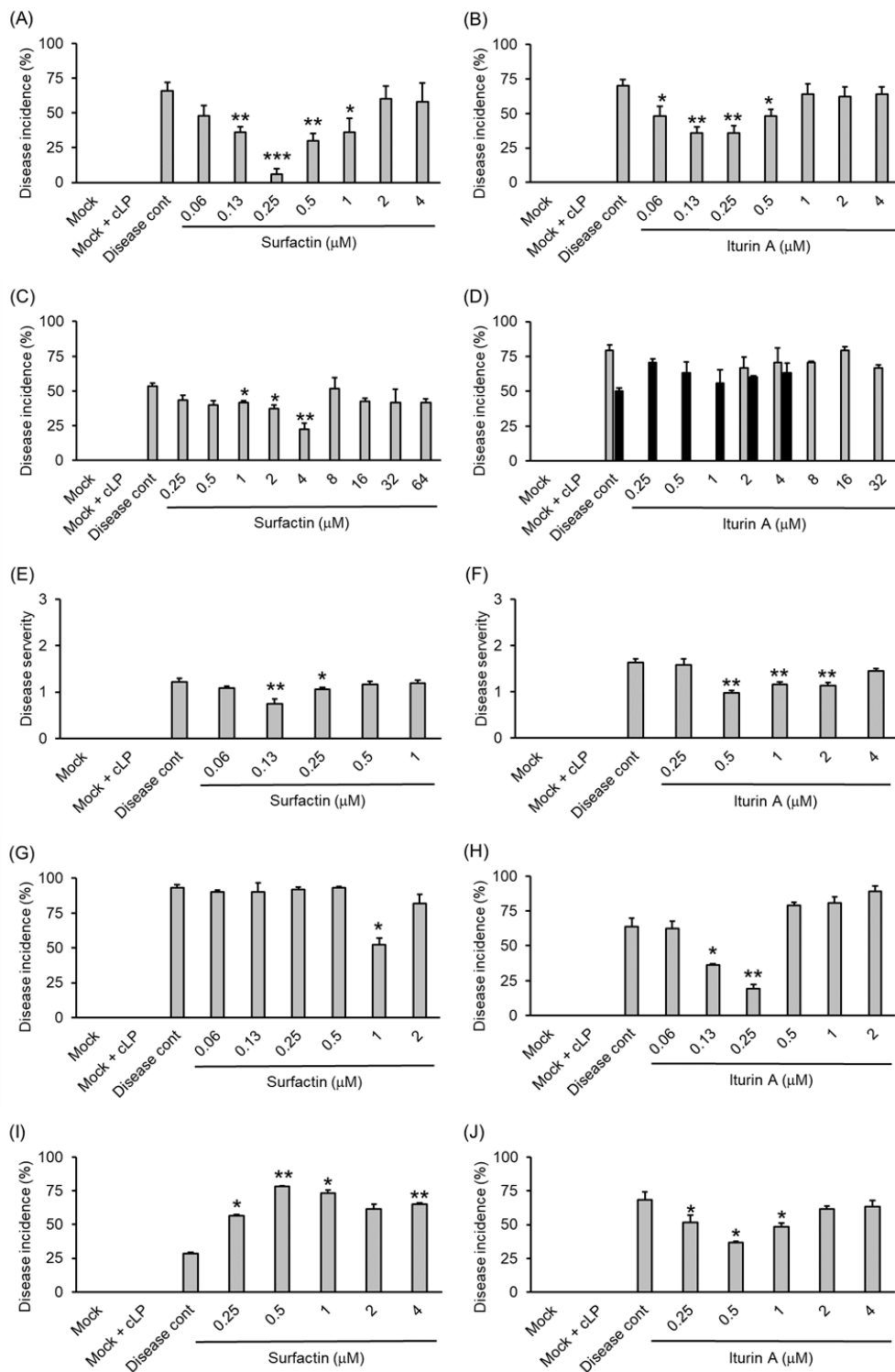
Fig 6. Disease suppression following root treatment of surfactin or iturin A against bacterial leaf disease on various kinds of host plants. (A) surfactin-treatment on cabbage (n=5), (B) iturin A-treatment on cabbage (n=5), (C) surfactin-treatment on tomato (n=3), (D) iturin A-treatment on tomato (n=3), (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 +/- 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.

Chapter 3 – Insights on suppression of bacterial leaf spot by *Bacillus* cyclic lipopeptides via induced resistance in *Arabidopsis thaliana*

3.1. Introduction

Cyclic lipopeptides (cLPs) derived from *Bacillus* spp. have been reported as antimicrobial compounds with a broad host range (Bais et al. 2004; Phae et al. 1990), as well as major factors that confer biological control activities against several kinds of plant diseases (Bais et al. 2004; Romero et al. 2007). So far, a variety of cLPs derived from *Bacillus* spp. have been identified and categorized into 3 families: the surfactin family, the iturin family and the fengycin family, depending on the chemical structure and biosynthetic genes (Stein 2005). Since *Bacillus* derived cLPs show antimicrobial activities, this antimicrobial activity has been the assumed mechanism of biological control of plant diseases when using *Bacillus* strains. However, cLPs have been demonstrated to induce disease resistance in host plants with a broad range (Ongena et al. 2007).

Plants have innate immune systems, known as induced disease resistance, for defense against pathogens. Induced disease resistance in plants is characterized into two systems; systemic acquired resistance, SAR, is an immune system triggered by pathogen recognition, which uses salicylic acid (SA) as a signaling molecule (Ryals et al. 1994), whereas induced systemic resistance, ISR, triggered by rhizobacteria recognition, uses jasmonic acid (JA) and ethylene (Pieterse et al. 2014). These two systems are inducible and strictly regulate the expression of the defense genes independently in planta (Pieterse et al., 2014).

A few papers have characterized the induced disease resistance elicited by cLPs so far, however, it is still unclear about the specificity to induce the disease resistance among the combinations of cLP molecules and host plants, and the signaling pathways in the innate immune system in planta. For example, Farace et al. (2015) reported on conferred disease suppression in grapes by purified cLPs against a fungal leaf disease, *Botrytis cinerea*, and gene expression analysis on the defense genes in grapes. With respect to their findings on grapes, the induced disease resistance response, as

measured by gene expression analysis, varied among the treatments of surfactin, plipastatin, and mycosubtilin, which is a member of iturin family. Moreover, the relationship between disease suppression and activation of signaling pathways have not been confirmed in some cLPs.

We demonstrated disease suppression by soil amendments of both purified surfactin and iturin A against soil-borne diseases caused by *Fusarium oxysporum* on tatsoi and lettuce, respectively (Yokota and Hayakawa 2015; Fujita and Yokota 2019). They also reported that an excess amount of cLP amendment in soil negates the disease suppression for both cLPs.

Besides that, we also investigated the disease suppression elicited by purified surfactin- and iturin A-treatments against bacterial leaf diseases on various kinds of edible plants (Altrão et al., 2022). 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. However, it is still unclear why in excess concentration of cLPs disease suppression was negated.

In this study, we aimed to evaluate the conferring of disease suppression by treatments of purified surfactin and iturin A on *Arabidopsis thaliana* host plants and to clarify the signaling pathway(s) connected to the induced disease resistance elicited by both cLPs.

3.2 Materials and methods

3.2.1 Plants and bacteria

A. thaliana (L.) Heynh. Columbia (Col-0) ecotype was used as wild type. The *npr1* (Cao H. et al. 1997), *ics1* (Alonso et al. 2003), and *jar1* (Susa and Staswick 2008) *Arabidopsis* mutants were purchased from The Arabidopsis Information Resource

(TAIR). *Pseudomonas syringae* pv. *maculicola* MAFF 302783, a causal agent of bacterial leaf spot of Brassica species, was used as the bacterial pathogen on *A. thaliana* (Igarashi et al. 2010). *B. subtilis* ATCC 21556 was used as iturin A-producing bacteria for purification of iturin A (Yokota and Hayakawa 2015).

3.2.2. cLPs

Iturin A was purified from solid state cultures of *B. subtilis* ATCC 21556 by organic solvents extraction followed by silica and ODS column chromatography according to Yokota and Hayakawa (2015). Surfactin was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

3.2.3. Antibacterial activity assays

Growth inhibition of the bacterial pathogen by the purified surfactin or iturin A was evaluated by measuring OD_{600} of the cultures after 48 h in Luria-Bertani broth (1 % [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl) amended with concentrations (0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50 μM) of surfactin or iturin at 25 °C.

3.2.3. Disease suppression assay in *A. thaliana* wild type

Conferring of disease suppression by cLPs were evaluated against bacterial leaf disease on *A. thaliana* by root-treatments of purified surfactin or iturin A. Host plants were propagated by hydroponic culture with Hoagland solution (Hoagland and Arnon, 1950) at 24 °C, 10 h light, light condition 100 μmol m^{-2} s^{-1}. At the 6-true leaf stage of host plants, purified surfactin or iturin A were added to hydroponic solution for cLP-treatment on roots.

Two-days post cLP-treatment, the bacterial pathogen suspension (10^5 cells ml^{-1}) with sterilized 10 mM $MgSO_4$ was inoculated on the abaxial side of leaves by infiltration method. Disease symptoms were evaluated for 6 days post pathogen

inoculation. Disease incidence was calculated by (number of disease symptoms / number of bacterial pathogen inoculation) × 100.

3.2.4. Measurement of bacteria in leaf

Growth of bacteria in leaves was measured at 4 days post inoculation. Detached leaves were weighed, and then homogenized in 10 ml of sterilized water by a homogenizer (Physcotron NS-50, Microtech, Chiba, Japan). The suspension was plated using appropriate dilutions on King's B agar plates and incubated at 25 °C for 3 days.

3.2.5. Disease suppression by purified cLPs on A. thaliana mutants

The mutants of *A. thaliana* npr1 (transcriptional regulator NPR1 mutant Col-0), ics1 (salicylic acid synthase ICS1 mutant: Col-0), jar1 (jasmonic acid synthase JAR1 mutant: Col-0) was used to investigate whether disease suppression effect of cLP treatment is due to the induction of the same pathway. Hydroponic cultivation was carried out as the same parameters as described above.

Seedlings were divided in six experiments plots: Disease control (cLP untreated plot), iturin A treatment group at 1 and 4μM (final concentration in hydroponic solution), and surfactin treatment group (Surfactin Sodium Solt: Wako Pure Chemicals) at 8 and 16μM.

Two days post cLP treatment, *P. syringae* pv. *maculicola* MAFF 302783 was inoculated at 10^5 cells/ml. Pathogen inoculation was performed on five individuals per test plot in six leaves per individual. Disease development were analyzed for 5 days post pathogen inoculation. Disease incidence rate was calculated by (number of cases/ number of inoculated pathogens) × 100.

3.2.6. Statistical analysis

Statistical analyses were performed with one way ANOVA test.

3.3. Results

3.3.1. Antibacterial activity of Iturin A and Surfactin

P. syringae pv. maculicola MAFF 302783 growth was not inhibited at any concentrations of surfactin and iturin A tested (Figure 7a and b).

(a)
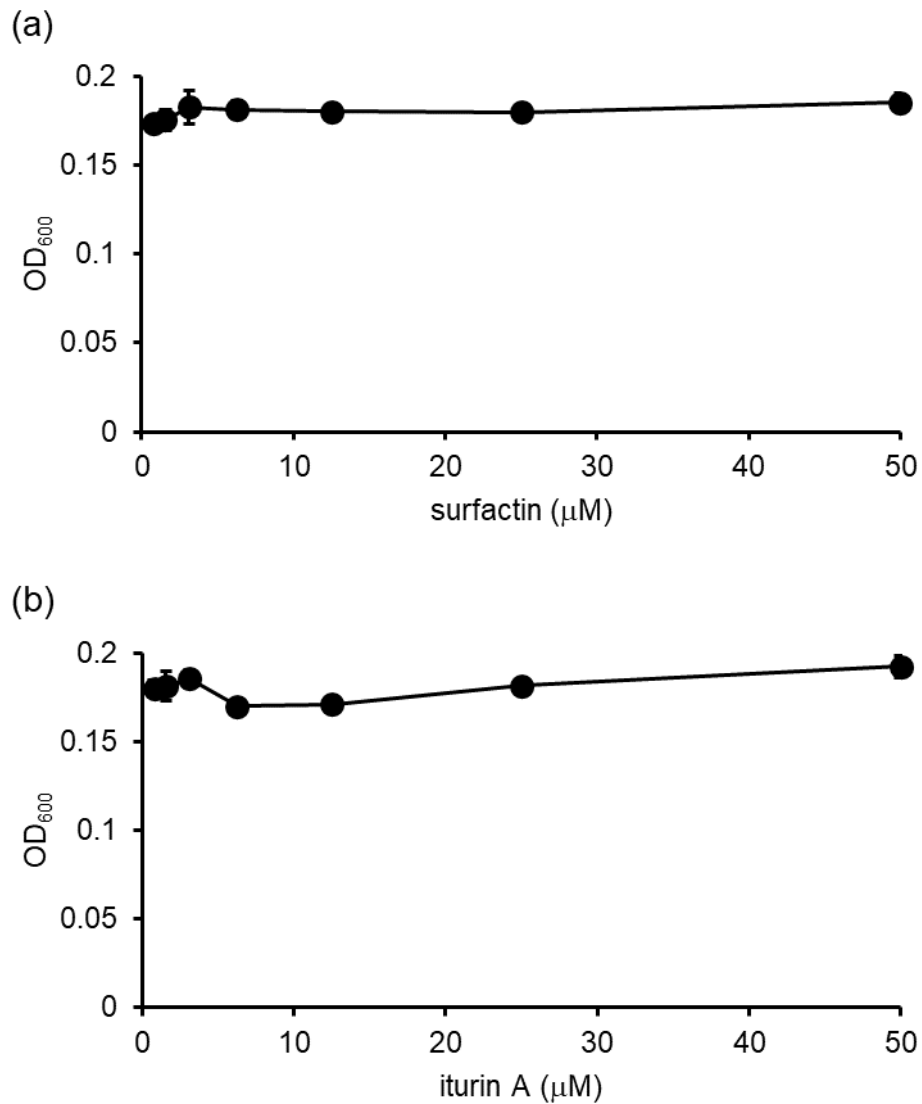

(b)

Fig. 7 Mean (±SE) OD_{600} to estimate population of *Pseudomonas syringae* pv. *maculicola* MAFF 302783 after 48 h in LB broth amended with different concentrations of (a) surfactin or (b) iturin A. (n = 3 cultures/concentration).

3.3.1. Disease suppression by purified cLPs on *A. thaliana* wild type

Significant disease suppression against the bacterial disease on leaves was observed following root-treatments of purified surfactin and iturin A, respectively (Fig. 8a and b). However, the ranges of concentration to show disease suppressions differed among cLPs. Surfactin conferred significant disease suppression at a range of 4 to 16 μM in hydroponic culture, whereas iturin A conferred suppression at a range of 0.5 to 2 μM. Moreover, the disease suppression was negated at 32 μM surfactin and 4 μM iturin A, respectively, whereas no disease symptoms were observed at 32 μM surfactin and 4 μM iturin A without bacterial pathogen inoculations, respectively.
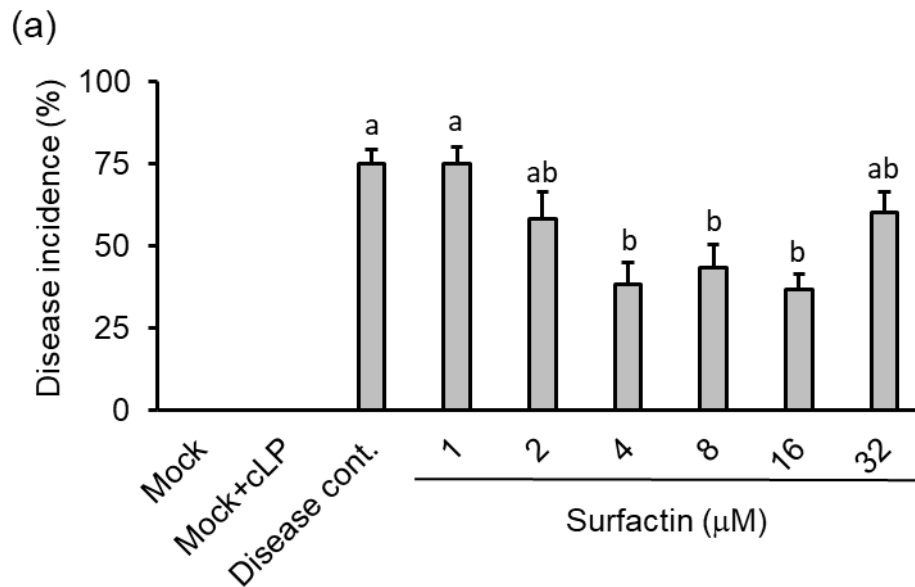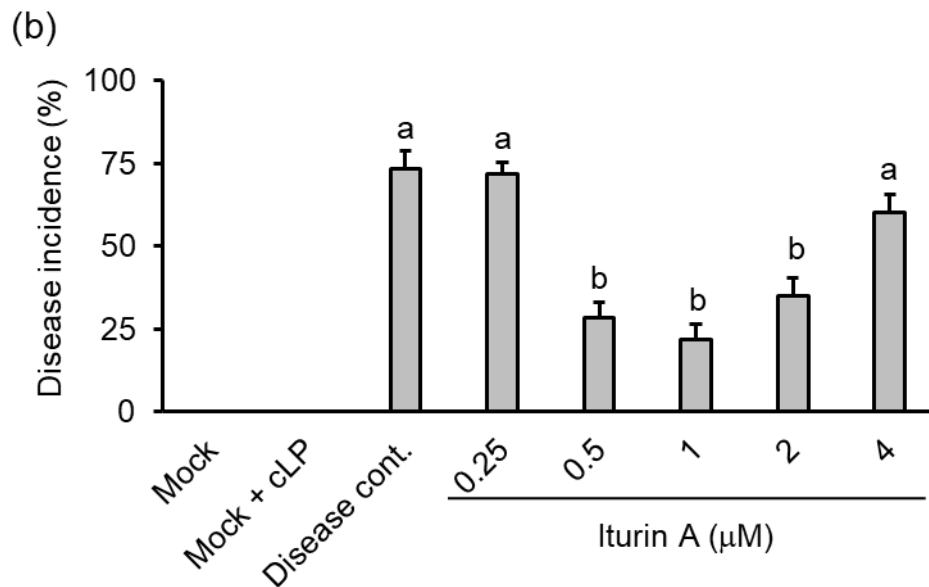
Fig. 8 Disease suppression by purified cLPs on *Arabidopsis thaliana* wild type. **a** Root-treatments of surfactin. **b** Root-treatments of iturin A. Means + SE (n = 10). cLP concentrations in Mock + cLP were the highest concentrations for each cLPs. Different letters correspond to significant differences in the values (p<0,01) in one-way ANOVA followed by Tukey's test.

3.3.2. Bacterial pathogen growth in leaves

The bacterial populations in leaves corresponded to the conferred disease suppression and were significantly lower at the 0.5, 1, and 2 μM iturin treatments compared to the disease control. Moreover, in plants treated with excess amounts of iturin (more than 4 μM), the repression of bacterial populations in leaves was negated, which agrees with the disease suppression assays (Fig. 9a). Although no significant differences on the bacterial populations were observed among the surfactin treatment, the bacterial population by 8 μM surfactin was more than 10 times lower compared with disease control (Fig. 9b).
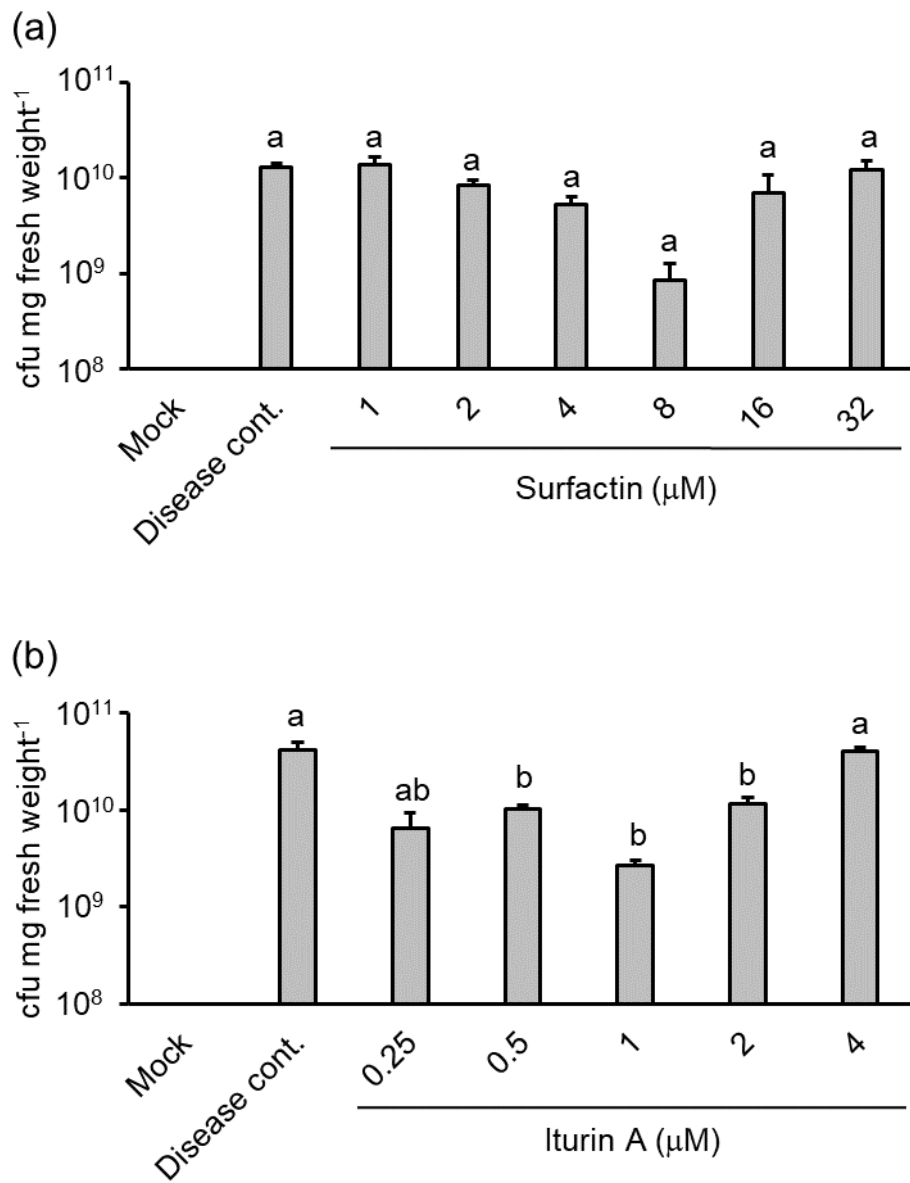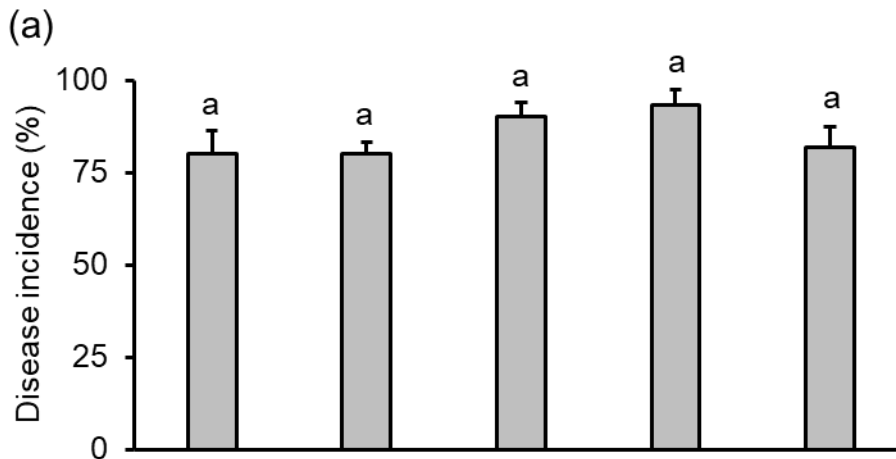
Fig. 9 Bacterial pathogen growth in leaves. (A) Root-treatments of surfactin. (B) Root-treatments of iturin A. Means + SE (n = 5). Different letters correspond to significant differences in the values (p<0,01) in one-way ANOVA followed by Tukey's test.

3.3.3. Disease suppression by purified cLPs on *A. thaliana* mutants

To clarify the signaling pathways of induced disease resistance triggered by cLP-treatments, we conducted disease suppression assays with a series of *A. thaliana* mutants as host plants. In NPR1 and ICSI mutants disease suppression was not observed independent to the cLPs concentration applied (Fig. 10a and b).

JAR1, jasmonate resistant 1, is a jasmonate-amido synthetase in the biosynthesis of jasmonate-isoleucine conjugate which activates the JA signaling pathway in *Arabidopsis*. In *jar1* mutants, significant disease suppression was observed following treatments of 8 μM surfactin and 1 μM iturin A (Fig. 10c), similar to as was observed in wild type Col-0 (Fig.8a and b). Our results strongly suggest that the JA signaling pathway does not confer the disease suppression by induced disease resistance elicited by cLPs.

(a)
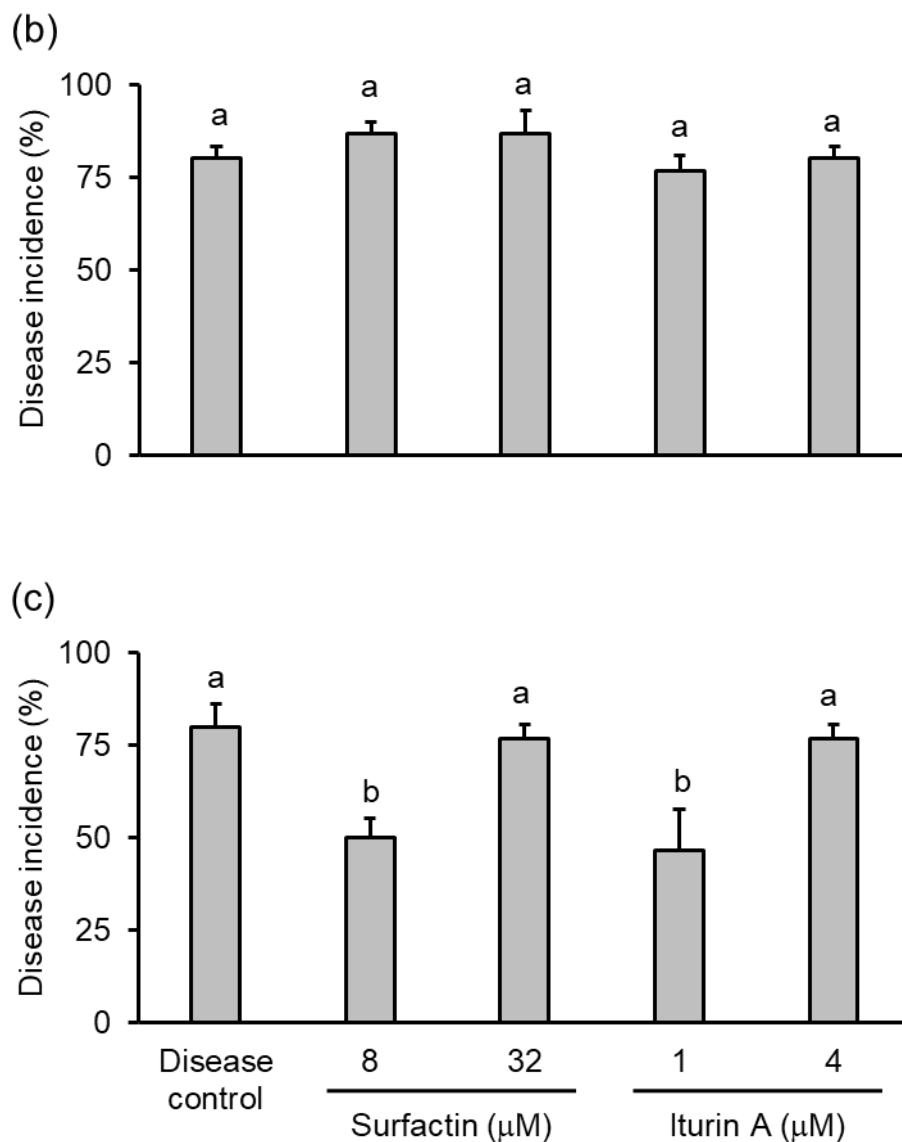
Fig. 10 Disease suppression by purified cLPs on *Arabidopsis thaliana* mutants.
(A) *npr1* mutant. (B) *ics* mutant. (C) *jar1* mutant. Means + SE (n = 5). cLP
concentrations in Mock + cLP were the highest concentrations for each cLPs. Different
letters correspond to significant differences in the values (p<0,01) in one-way ANOVA
followed by Tukey's test.

3.4. Discussion

We demonstrated the existence of a specific concentration range of these cLPs
(from 0.5 to 1 µM of iturin and from 4 to 16 µM of surfactin) to suppress disease in *A.*

thaliana under root-treatment. We also confirmed the disease suppression by root treatments of cLPs on various edible plants (unpublished data). Among the edible plants, the concentrations of cLP treatments to confer disease suppressions varied among surfactin and iturin A, respectively, similar to as was observed in *A. thaliana* in this study. Moreover, the negation of disease suppression by applying an excess amount of cLP treatments was observed on the edible plants similar to *A. thaliana* in this study. Therefore, it appears that the negation of disease suppression under an excess amount of cLP treatments might be a general characteristic of the induced disease resistance elicited by cLPs on a wide range of plants.

Igarashi et al. (2010) evaluated induced disease resistance using an assay system with *P. syringae* pv. *maculicola* MAFF 302783 as the causal bacterial pathogen in the infected leaf tissue of *A. thaliana* Col-0, similar to this study. They clearly showed that the bacterial populations in the pathogen infected leaves of host plants were repressed under induced disease resistance elicited by a commercial product consisting of glutamate fermentation by-product. We therefore deduced the number of bacterial population in leaves by cLP treatments were depending on the induced direase resistance on host plants.

NPR1, nonexpressor of pathogenesis-related protein 1, has been identified as a mediator for the expression in both of salicylic acid (SA) signaling and jasmonic acid (JA) signaling pathways in *A. thaliana* (e. g. Pieterse et al.2014). In *npr1* mutants, no disease suppression was observed using either surfactin- or iturin A-treatments (Fig. 3A), suggesting that the induced disease resistance elicited by cLPs were conferred through NPR1-dependent induced disease resistance pathways.

ICS1, isochorismate synthase1, is a biosynthetic enzyme for SA via the ICS pathway in *Arabidopsis* (Wildermuth et al. 2001). Generated SA is an important plant hormone, and acts as a regulatory component during SA signaling of induced disease resistance in *Arabidopsis*. In an *Arabidopsis ics1* mutant, no disease suppression was observed following treatment with surfactin or iturin A (Fig. 3B), suggesting that SA biosynthesis through the ICS pathway is important in the induced disease resistance elicited by surfactin and iturin A, respectively.

On the other hand, the JA signaling pathway also has a well-established role in induced disease resistance in *Arabidopsis*. Although the molecular mechanisms of the JA signaling pathway are still complex especially against bacterial diseases, Subramanian et al. (2011) reported that JA signaling elicited by a treatment of extracts of *Ascophyllum nodosum* is important to significantly confer disease suppression against a bacterial disease caused by *P. syringae* pv. tomato DC3000 on SA signaling deficient *Arabidopsis* mutants.

Moreover, we observed the negation of disease suppression by the treatments of 32 mM surfactin and 4 mM iturin A on *jar1* mutants, respectively, similar to wild type Col-0 (Fig. 1). Since it is well known that both the SA and JA signaling pathways are antagonistic to each other in *Arabidopsis* (Backer et al. 2019), the negation of disease suppression via the SA signaling pathway by an excess amount of cLP treatment should correlate to an antagonistic action on the JA signaling pathway. However, our results on *jar1* mutants clearly showed that an antagonistic effect between SA and JA signaling pathways did not result in a negation of disease suppression by excess amount of cLP treatments.

4. Conclusion

In my PhD thesis, I characterized disease suppression activities of *Bacillus* cLPs depended on induced disease resistance. The cLPs, surfactin and iturin A, showed disease suppressive activities against bacterial leaf diseases by eliciting of induced disease resistance on a variety of edible host plants. On the other hand, the specificity to induce the disease resistance among the combinations of cLP molecules and host plants were observed on a particular host plants, tomato and cucumber. Moreover, under the excess amount of cLP treatments, the disease suppression were negated for both of cLPs whereas no abnormalities were observed without bacterial pathogen inoculations.

On *A. thaliana*, the signaling pathway in induced disease resistance elicited by *Bacillus* cLPs was SA acid signaling pathway via isochorismate pathway. Moreover, negations of disease suppression were observed by excess amount of surfactin or iturin A treatment, and the negation of disease suppression was not correlated to the antagonistic effect by induction of JA signaling pathway in host plants.

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