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Rapid communication

Long-term effect of niclosamide on inhibition of bacterial leaf blight in rice

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Abstract: Bacterial leaf blight is one of the major diseases in rice and affects yields. Thus, various methods have been applied to protect rice from this disease. Here, we show systemic translocation of the human drug niclosamide (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) in rice and its long-term effect on prevention of rice leaf blight. The development of *Xanthomonas oryzae* pv. *oryzae*-induced rice leaf blight was effectively inhibited in untreated systemic leaves as in niclosamide-treated leaves, although its effect gradually decreased in a time-dependent manner. Time-course examination after niclosamide treatment showed that the niclosamide level was highest after 3 h in non-treated distal leaves, suggesting fast systemic movement of niclosamide from the treated local site to untreated distal regions. Our data indicate that niclosamide controls rice leaf blight by its rapid systemic movement and that its effect is maintained for a long time.

Key words: Bacterial leaf blight, niclosamide, rice, systemic movement, *Xanthomonas oryzae* pv. *oryzae*

Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a phytopathogenic Gram-negative bacterium that causes blight disease symptoms. *Xoo* bacteria enter the plant through wounds or hydathodes and move through the xylem vessels. Rice leaf blight induced by *Xoo* bacteria is a serious disease that causes huge grain loss. Thus, many research groups have tried to protect rice from bacterial leaf blight. So far, three different methods have been applied to protect rice from bacterial leaf blight. The first method is biological control, which uses other bacterial strains such as *Rhizobacterium*, *Bacillus*, and *Pseudomonas* (Chithrashree *et al*. 2011; Gnanamanickam 2002). The second method is chemical control, which uses various chemicals including chlorine, antibiotics, benzothiadiazole, and probenazole (Chand *et al*. 1979; McManus *et al*. 2002; Iwai *et al*. 2007; Karthikeyan and Gnanamanickam 2011; Khan *et al*. 2012). The third method is genetic resistance, which uses genes that confer resistance to leaf blight (Sharma *et al*. 2000; Lia *et al*. 2006; Swamy *et al*. 2006; Shimono *et al*. 2007; Peng *et al*. 2012; Singh *et al*. 2012; Han *et al*. 2013; Nakayama *et al*. 2013; Cernadas *et al*. 2014; Goto *et al*. 2015). Several results substantially proved that these methods can be applied to improve rice yields by protecting rice from bacterial leaf blight. Recently, we also tested the effects of the human drug niclosamide [5-chloro-*N*-(2-chloro-4-nitrophenyl)--2-hydroxybenzamide] on rice leaf blight. Niclosamide, which was first identified as an oral antihelminthic drug and molluscicide, has various effects such as antiviral activity (Wu *et al*. 2004), anti-anthrax properties (Zhu *et al*. 2009), induction of autophagosomes (Balgi *et al*. 2009), inhibition of the Wnt/Frizzled pathway (Chen *et al*. 2009) and mechanistic target of rapamycin signaling (Fonseca *et al*. 2012), and uncoupling of mitochondrial oxidative phosphorylation (Weinbach *et al*. 1969). We found that niclosamide directly inhibits the growth of *Xoo* bacteria and also suppresses *Xoo*-induced leaf wilting by increasing the level of salicylate and inducing the expression of defense-related genes such as *OsPR1* and *OsWRKY45* (Kim *et al*. 2016). However, the long-term effect of niclosamide on prevention of rice leaf blight was not examined in the previous study. Therefore, we tried to investigate the systemic long-term effect of niclosamide on bacterial leaf blight in rice. Here, we show that niclosamide rapidly moves from local leaves to distal leaves and functions in the distal leaves for a long time, resulting in blockage of *Xoo*-caused leaf wilting.

Materials and Methods

Plant materials and chemicals

Nipponbare rice (*Oryza sativa* L.) was used in this study. To prepare rice plants, seeds were directly sown in ster-

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ilized rice field soils as described in a previous study (Zainudin *et al*. 2008) and seedlings were transplanted in pots. The plants were grown before *Xoo* inoculation or niclosamide treatment in a growth chamber set on a 16 : 8 h (L : D) with a 28°C/26°C temperature cycle. A stock solution of niclosamide (Sigma-Aldrich, USA) was prepared by dissolving the chemical in dimethyl sulfoxide at a concentration of 50 μg · ml^{-1}.

Treatment with niclosamide and *Xoo* bacteria

Soil-grown rice plants were treated with niclosamide or *Xoo* bacteria as described in a previous study (Kim *et al*. 2016). For treatment with *Xoo* bacteria only, 80-day-old rice was treated with the *Xoo* strain PXO99. PXO99 cells were prepared as follows. Briefly, the cells were grown at 28°C to an optical density of 1.0 at 600 nm in peptone-sucrose broth containing 15 μg · ml^{-1} cephalexin (Sigma-Aldrich, USA). Finally, PXO99 cells (more than 10^9 cell-forming units per ml) resuspended in distilled water were used for inoculation. For examination of the long-term and systemic effect of niclosamide, half of the plant was covered by polythene bags and then 8 μg · ml^{-1} niclosamide was sprayed on the uncovered leaves. After incubation for 0, 8, 16, 32, and 64 h, each sample was inoculated with *Xoo* bacteria. After 16 days, the leaves were photographed and the lesion length was measured using ImageJ Software. Bacterial growth was also examined as described in a previous study (Kim *et al*. 2016). Briefly, the leaves were ground with a mortar and pestle and then each sample was suspended in sterile water. The samples were plated onto peptone-sucrose agar containing 15 μg · ml^{-1} cephalexin. The bacterial population was scored by counting the number of colonies every 4 days after inoculation. The lesion lengths and bacterial populations were expressed as the mean value ± the standard deviation. All data are expressed as the mean values from 15 leaves per treatment. The experiment was repeated five times under the same conditions.

Measurement of the amount of niclosamide

Half the leaves of 80-day-old rice were covered by polythene bags, and the remaining systemic leaves were sprayed with 8 μg · ml^{-1} niclosamide. After treatment, local and distal leaves were harvested after incubation for 0, 3, 6, 9, 12, 24, 48, and 96 h. Niclosamide was extracted from 0.5 g of each sample using absolute methanol, and the niclosamide concentration was determined by high-performance liquid chromatography (HPLC) separation and fluorescence detection. Niclosamide levels were expressed as the mean value ± the standard deviation. The experiment was repeated five times under the same conditions.

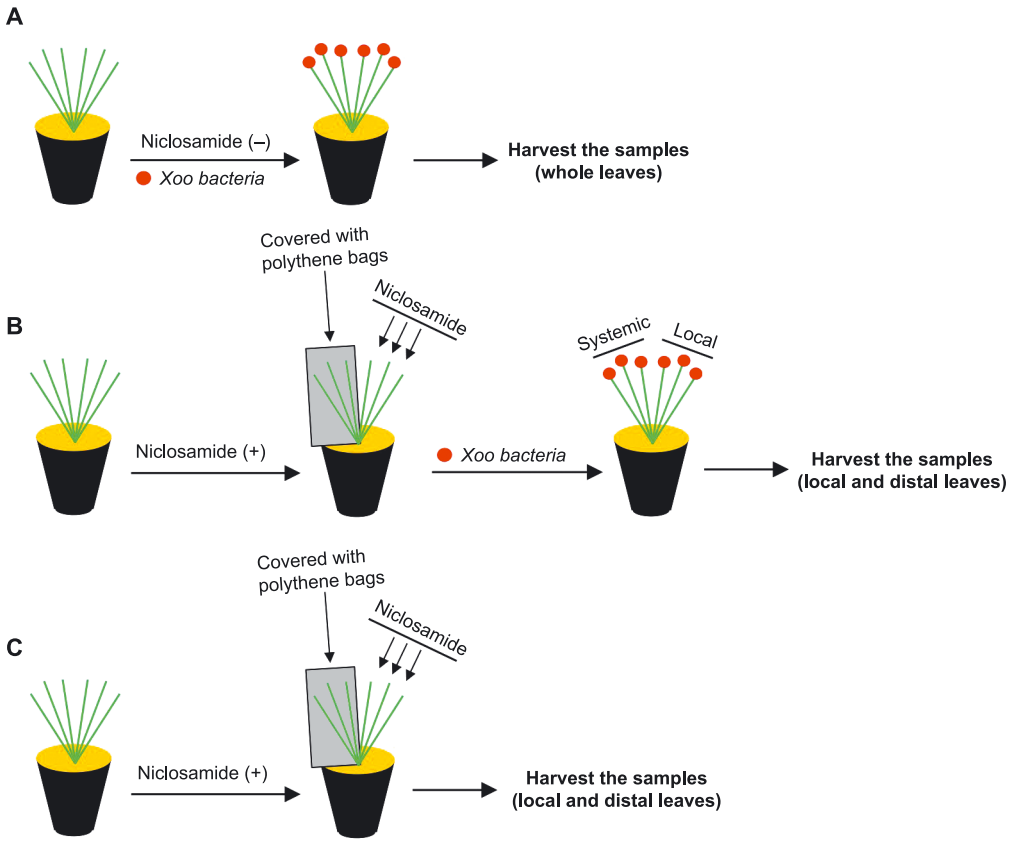

Fig. 1. Schematic representation of the treatment of rice with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) bacteria and niclosamide: A – Mock treatment. Leaves of 80-day-old rice were treated with *Xoo* bacteria, and then samples were harvested; B – Leaves of 80-day-old rice were treated locally and systemically with 8 μg · ml^{-1} niclosamide and then inoculated with *Xoo* bacteria PXO99. Both local and distal leaves were harvested; C – Leaves of 80-day-old rice were treated with 8 μg · ml^{-1} niclosamide, and then both local and distal leaves were harvested

Results and Discussion

In recent years, plant-derived metabolites have been demonstrated to have curative effects. Thus, a large number of studies have been performed to isolate effective chemicals and metabolites. It was also reported that several chemicals including antibiotics can effectively prevent plant diseases caused by bacteria and fungi (Chand *et al*. 1979; McManus *et al*. 2002; Iwai *et al*. 2007; Karthikeyan and Gnanamanickam 2011; Khan *et al*. 2012; Hoshi *et al*. 2015). Therefore, we inferred that human drugs can also have curative effects on plant diseases. In a previous study, we found that the human drug niclosamide indeed has an inhibitory effect on rice leaf blight caused by *Xoo* bacteria (Kim *et al*. 2016).

We wanted to determine how long the inhibitory effect of niclosamide on rice leaf blight persists and how fast niclosamide can translocate from the local inoculation site to systemic distal regions. To this end, we first treated rice plants with niclosamide and incubated them for the indicated amounts of time. After incubation, the plants were inoculated with the PXO99 strain and then further incubated for 16 days (Fig. 1A and B; Fig. 2). Lesion development was completely inhibited up to 8 h in untreated systemic leaves as in niclosamide-treated local leaves (Fig. 2A and B). The inhibitory effect of niclosamide on lesion development was detected even after 64 h in untreated systemic leaves, although its effect gradually decreased (Fig. 2A and B), indicating that niclosamide can systemically move a long distance and function in untreated distal regions for more than 64 h.

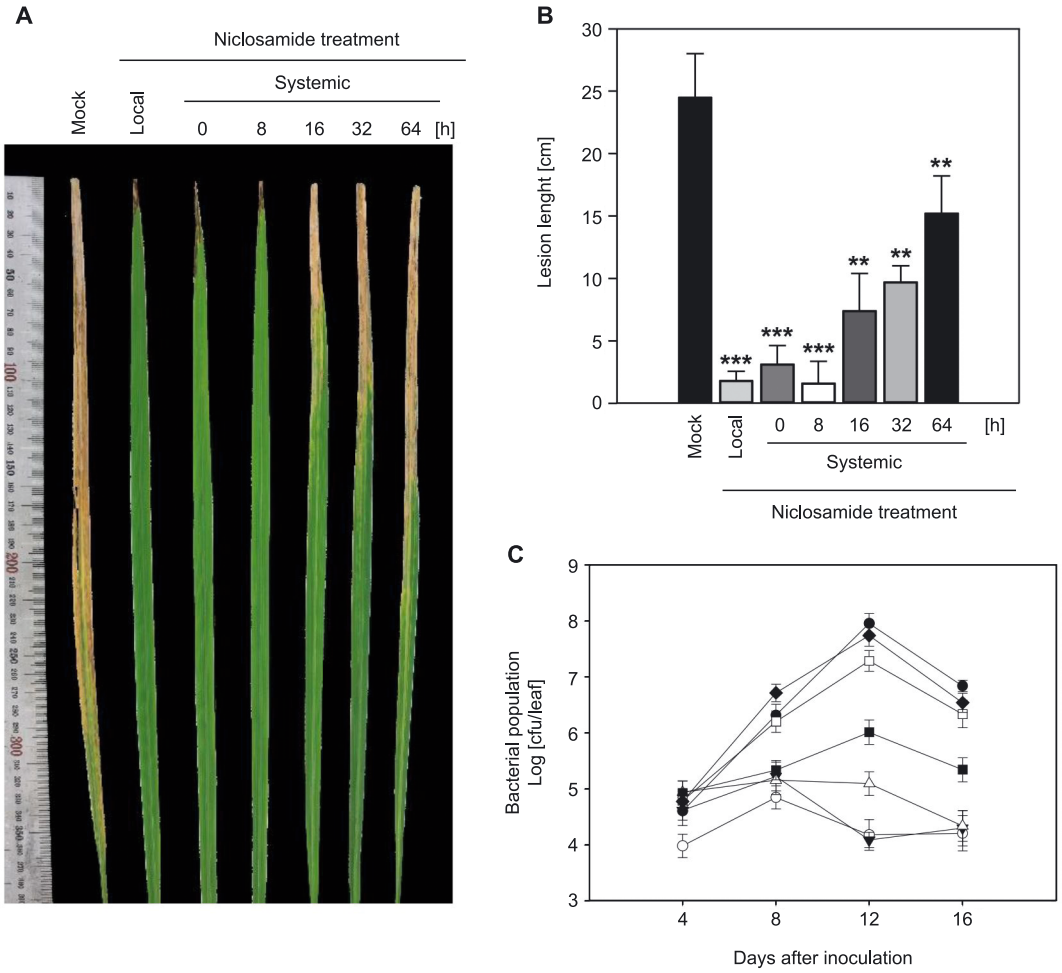

Fig. 2. The effect of niclosamide on the disease responses of rice to the representative *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99: A – Leaves of 80-day-old rice were locally or systemically treated with 8 μg · ml^{-1} niclosamide for the indicated amount of time and then inoculated with the bacterial suspension using the leaf clipping method. The samples were further incubated for 16 days and then photographed; B – Lesion development was examined by measuring lesion length using the leaves of PXO99-treated plants (Mock), locally niclosamide-treated plus PXO99-treated plants (Local), and systemically niclosamide-treated plus PXO99-treated plants (Systemic). The standard deviations of the means are indicated by vertical bars. Asterisks indicate significant differences from the vector control using the p-value calculated using the t-test (**p < 0.001, ***p < 0.0001); C – Leaves of PXO99-treated plants and locally and systemically niclosamide-treated plus PXO99-treated plants were sampled at the indicated time points, and the bacterial population was estimated by counting the number of bacteria grown in peptone-sucrose medium containing cephalexin. The standard deviations of the means are indicated by vertical bars; ● – *Xoo* only; ○ – *Xoo* plus local niclosamide treatment; ▼ – *Xoo* plus systemic niclosamide treatment for 0 h; Δ – *Xoo* plus systemic niclosamide treatment for 8 h; ■ – *Xoo* plus systemic niclosamide treatment for 16 h; □ – *Xoo* plus systemic niclosamide treatment for 32 h; ◆ – *Xoo* plus systemic niclosamide treatment for 64 h

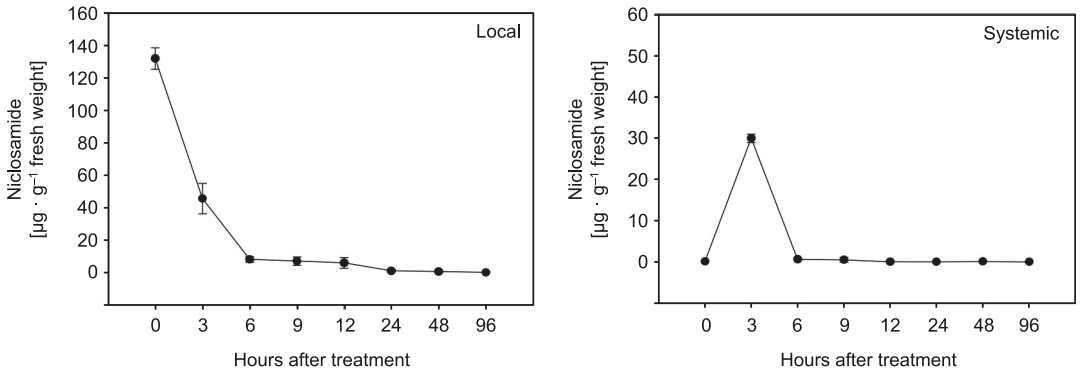

Fig. 3. Examination of the amount of niclosamide in local and systemic leaves. Eighty-day-old rice leaves were treated with 8 µg · ml^{-1} niclosamide, and then niclosamide-treated local leaves and non-treated systemic leaves were harvested at the indicated time points. The samples were ground with liquid nitrogen, and niclosamide was extracted with methanol. Niclosamide concentrations were measured by highperformance liquid chromatography (HPLC). The standard deviations of the means are indicated by vertical bars

Next, bacterial growth was examined at 4, 8, 12, and 16 days after PXO99 inoculation using niclosamide-treated samples. As expected, bacterial growth was completely inhibited even at 16 days in untreated distal leaves incubated for 8 h after niclosamide treatment (Fig. 2C). Upon incubation for longer than 8 h, the inhibitory effect of niclosamide on bacterial growth gradually decreased in untreated systemic leaves in proportion to the increase in the incubation time after niclosamide treatment (Fig. 2C). A previous study showed that niclosamide can directly inhibit bacterial growth and lesion development or indirectly inhibit bacterial growth and lesion development via a salicylic acid signaling pathway (Kim *et al*. 2016). Therefore, this result also indicates that, as in the inhibitory effect on lesion development, niclosamide can systemically move a long distance and directly or indirectly inhibit bacterial growth in untreated distal regions for more than 64 h.

Finally, we examined the speed of niclosamide movement from local leaves to systemic leaves and also the amount of niclosamide remaining in local and systemic leaves. Rice plants were treated with niclosamide for 96 h as described in the Materials and Methods and Figure 1C. Niclosamide-treated leaves and untreated systemic leaves were harvested at the indicated time points, and niclosamide concentrations were determined by HPLC. Niclosamide levels rapidly increased up to 3 h in systemic leaves according to the decrease in the amount of niclosamide in local leaves (Fig. 3). After 3 h, its level gradually decreased up to 6 h and was then maintained for a long time at a low basal level (Fig. 3). This indicates that niclosamide can rapidly move from niclosamide-treated local leaves to untreated distal leaves and then exert its inhibitory function in the prevention of bacterial leaf blight in rice. This also suggests that niclosamide can block the spread of rice leaf blight for a long time because it persisted for more than 4 days, although its level was low.

Our data prove that the human drug niclosamide can prevent the spread of *Xoo*-induced leaf blight by rapid movement from the local site to distal regions in rice, suggesting that other human drugs may also have curative effects on various plant diseases.

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