Environmental Control in Tea Fields to Reduce Infection by *Pseudomonas syringae* pv. *theae*

T. Tomihama, T. Nonaka, Y. Nishi, and K. Arai

First, second, and third authors: Tea Research Division, Kagoshima Prefectural Institute for Agricultural Development, 3964 Nagasato, Chiran-cho, Minamikyushu-shi, Kagoshima, 897-0302, Japan; and fourth author: University of Kagoshima, 1-21-24, Korimoto, Kagoshima-shi, 890-0065, Japan.

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ABSTRACT

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Bacterial shoot blight (BSB) disease, caused by *Pseudomonas syringae* pv. *theae*, is a major bacterial disease of tea plants in Japan. BSB mainly occurs in the low-temperature season, and lesion formation by *P. syringae* pv. *theae* is enhanced by both low temperature and the presence of ice nucleation-active *Xanthomonas campestris* (INAX), which catalyzes ice formation at -2 to -4°C and is frequently co-isolated with *P. syringae* pv. *theae* from tea plants. Low temperature is thus the most important environmental factor influencing the incidence of BSB; however, the effects of low temperature on infection of the host by *P. syringae* pv. *theae* and

of environmental controls in fields on the occurrence of the disease are poorly understood. In this study, we show that ice formation on tea leaves by INAX enhanced *P. syringae* pv. *theae* invasion into leaf tissue. The natural incidence of BSB in the field was closely related to early autumn frost. Frost protection in late autumn, which prevented ice formation on tea plants, significantly decreased the incidence of BSB, and frost protection combined with bactericide application held the incidence under the economic threshold level. Our data indicate that environmental control in the field based on microbial interactions in the host offers a new strategy for plant disease control.

Additional keywords: disease triangle, frost-protective fan.

Tea (*Camellia sinensis*) is an important industrial crop worldwide, with up to 50,000 ha devoted to its cultivation in Japan (4). Since ancient times, green tea has been considered a health-promoting beverage, and in recent years, the possible cancer preventative activity of tea catechin has been studied extensively (36). The control of disease and pests is crucial for stable tea production. Bacterial shoot blight (BSB) disease, caused by *Pseudomonas syringae* pv. *theae*, is a major bacterial disease of tea in Japan. BSB causes both severe loss in yields of the first crop of the year and damage to the tea plant; however, BSB disease is difficult to manage due to permanent infestation by *P. syringae* pv. *theae* and lack of effective means to control the pathogen.

P. syringae pv. *theae* forms aggregates (30) similar to biofilms (18,21) on tea leaves, and epigallocatechin gallate (26), a major tea catechin, induces biofilm formation of *P. syringae* pv. *theae* on abiotic surfaces (32). In addition, compared to planktonic-grown cells, biofilm-grown *P. syringae* pv. *theae* cells on an abiotic surface or on tea leaves have remarkable resistance to the antibiotic kasugamycin (33). Although control of BSB mainly consists of the application of a mixture of copper and kasugamycin, eradication of aggregates of *P. syringae* pv. *theae* on tea leaf surfaces by bactericides is difficult, and efficiency of control by bactericides fluctuates, depending on both the incidence levels of BSB and the timing of application of the bactericides (33).

The disease triangle (1,12,19) is a basic concept of plant pathology, and this principle has been used to develop new ideas to predict and control disease (24). Recently, much attention has been focused on host-pathogen interactions in the disease triangle (3,11) and on the interactions between pathogens and antagonistic (8) or plant growth-promoting microorganisms (5). By contrast, environmental controls for disease management in the field have received very little attention.

The optimum temperature range for disease development of *P. syringae* pv. *theae* is approximately 10 to 15°C (T. Tomihama and Y. Nishi, unpublished data); BSB disease appears mainly from late autumn to spring and is not observed during the high temperatures of summer. Severe outbreaks of BSB are associated with cold and frost damage; however *P. syringae* pv. *theae* itself has no ice-nucleation activity (2,6). In addition, ice nucleation-active *Xanthomonas campestris* (INAX) (6), which nucleates ice crystals at temperatures slightly below 0°C (7), is often isolated with *P. syringae* pv. *theae* in lesions of BSB in tea fields; INAX enhances *P. syringae* pv. *theae* lesion formation after exposure of tea plants to -4°C (31). Furthermore, seasonal population dynamics of *P. syringae* pv. *theae* and INAX have been found to be occasionally synchronized in the low-temperature season in tea fields (31). Therefore low temperature is the most important environmental factor for both damage to tea plants and enhanced virulence of *P. syringae* pv. *theae* due to ice formation by INAX. Thus, we were interested in testing the effect of low temperature on the infection of the host by *P. syringae* pv. *theae* and of low temperature control in fields to reduce the incidence of BSB.

In this report, we first present evidence that ice formation on tea leaves by INAX accelerated the invasion of *P. syringae* pv. *theae* into tea leaf tissues probably through stomata. Second, we discovered a close relationship between yearly incidence of BSB and minimum air temperature ($T_{\min}$) in late autumn when frost occurs. Furthermore, we show that the integrated use of frost

Corresponding author: T. Tomihama
E-mail address: ttsuyoshi@kiad.pref.kagoshima.jp

*The e-Xtra logo stands for "electronic extra" and indicates that there are three additional figures provided online showing frost protection by fans and effect of low temperature on bacterial invasion and propagation. Figures 1 and 2 appear in color online.

protection in late autumn with bactericidal control effectively reduced the incidence of BSB.

MATERIALS AND METHODS

Strains, medium, and inoculation. Strain K9301 of *P. syringae* pv. *theae* (32), strain No. 20 of INAX (31), and King’s medium B were used for all inoculation studies. Quantitative analyses of *P. syringae* pv. *theae* and INAX were performed with selective media as described (31). *P. syringae* pv. *theae* and INAX suspensions used for inoculation were obtained from cultures grown overnight at 25°C in liquid King’s medium B. Bacteria were collected by centrifugation and resuspended in sterilized water to the appropriate concentration in each experiment. In field experiments, inoculum was sprayed on the tea canopy surface at 100 ml/m². Incidence of BSB was judged by counting the number of leaves with at least one dark brown lesion in each plot (or row), and the counts were transformed to the number of diseased leaves per square meter of tea canopy surface. Two-tailed analysis of variance (ANOVA), the Student’s *t* test, and regression analysis of disease incidence were performed with JMP 5.0 (SAS Institute, Tokyo, Japan).

Bacterial invasion of leaves under low temperature. Although mature tea leaves of first shoots in spring can be freeze-damaged at temperatures below -4°C, tea leaves that had acquired full frost hardening in winter can tolerate temperatures as low as -9°C (22). Mature tea leaves (cultivar Yabukita) in spring were sampled to test the effects of INAX-mediated ice formation on tea leaves on *P. syringae* pv. *theae* infectivity. Twenty microliters of 10⁸ colony forming units (CFU)/ml or 10⁶ CFU of *P. syringae* pv. *theae* per ml or a 1:1 mixture of *P. syringae* pv. *theae* and INAX was spotted on the adaxial or abaxial surface of tea leaves. Inoculated leaves were left at room temperature until the drops dried, and kept under high humidity (relative humidity (RH) > 95%) at 15°C (control) or exposed to -3°C for 2 h (low temperature treatment) in a growth cabinet (COSMOPIA, Hitachi Co., Tokyo, Japan). Immediately after low temperature treatment, the leaves were judged visually for water-soaked regions. To count bacteria that had entered the leaf tissue 12 h after low temperature, inoculated leaves were dipped in 100 mM H₂O₂ for 1 min for surface sterilization and washed with sterilized water. After H₂O₂ treatment, disks that contained inoculated leaf regions were detached with a cork borer (10 mm diameter), and the bacterial population in the leaf disks was determined. For bactericide treatment, inoculated leaves were dipped in a solution of copper oxychloride (Dutch-Bordeaux, Hokko Chemical Co., Tokyo, Japan) at a dilution ratio of 1/500 for 1 min and kept under high humidity (RH > 95%) at 15°C for 24 h. Then disks that contained inoculated leaf regions were detached, and the bacterial population in the leaf disks for each treatment was determined. For microscopic analysis, inoculated leaves were immersed in 0.4% crystal violet

for 1 min and washed with sterilized water. Bacteria on tea leaves stained by crystal violet were observed with a Color 3D Profile Microscope (VK-8500, Keyence Co., Tokyo, Japan) as described (30). Experiments were replicated at least two times with similar results.

Natural incidence of BSB and weather parameters. From 1981 to 1987 and from 2002 to 2007, we investigated the incidence of BSB in 8 to 25 rows of tea plants (area of each row was 90 m²) of our Tea Research Division (cultivar Yutakamidori, 38 years old in 2007). Incidences of BSB were recorded at 30-day intervals from 1 October to 1 March. Parameters of the physical environment were monitored with a weather recorder (NASCON-2000, Yokogawa Electric Co., Tokyo, Japan). The average minimum daily temperature (30-year average) was subtracted from the minimum daily temperature for 1 October to 30 November in a given year, and the means of this calculation were considered the low temperature indexes.

Frost protection by covering. A slightly sloped tea field (260 m², cultivar Yabukita, 40 years old in 2007), which had a gradient in the minimum temperature (*T*_{min}) along the slope, was divided into two blocks of differing *T*_{min} (Table 1) containing a 16-plot array (for various covering and inoculation treatments). Strain K9301 (10⁶ CFU/ml) was inoculated on 13 October before the frost-protective period (pre-frost protection inoculation treatment), or 1 December 2006 after the frost-protective period (post-frost protection inoculation treatment). The frost protection experiment consisted of tea plants (i) exposed to the naturally occurring low temperatures (non-frost protection), or (ii) tea plants protected from frost by covering with tunnel-shaped cheesecloth (Baron Screen, Koizumiseima, Kobe, Japan) that was manually applied on nights with predicted frost conditions (frost protection). Each tunnel covered one row of tea plants, and was applied 12 times during the experimental period from 13 October to 1 December (Table 1). Ice formation on tea leaves was judged every morning during the frost protection period. Incidences of BSB were investigated by counting the number of leaves with lesions in each plot on 1 December (48 days after the pre-frost protection inoculation treatment) and 20 January (50 days after the post-frost protection inoculation treatment). Frost hardening of tea plants before (15 October) and after frost protection (1 December) was monitored by collecting 10 tea buds from each plot, exposing them to artificial low temperature (-3°C, -6°C, or -9°C) for 2 h in a growth cabinet, and recording the severity of freezing damage to the buds. The percentages of freeze-damaged buds (buds showing necrosis) in each 2.8 m² plot was recorded; each treatment was replicated four times. Temperature on the tea canopy surface in the center of each block was monitored with a thermo-recorder RTR-53 (T&D Corp., Nagano, Japan).

Frost protection by fan and application of bactericides. A large tea field (900 m², cultivar Yabukita, 38 years old in 2007) was divided into two blocks (frost protection or non-frost

TABLE 1. Minimum temperature (*T*_{min}) during frost protection by covering

Block ^a	Treatment	Minimum temperature (°C) ^b											
		11/2 ^c	11/4	11/5	11/6	11/8	11/12	11/13	11/15	11/22	11/29	11/30	12/1
Low $T_{\min}$	Non-frost protection	4.9	−1.5	−2.4 ^d	3.3	2.0	3.3	−3.3 ^d	5.4	1.6	4.5	3.3	−3.8 ^d
	Frost protection	6.2	0.2	−0.1	5.4	4.5	6.2	−0.1	7.4	4.1	5.8	4.9	0.2
High $T_{\min}$	Non-frost protection	7.0	−0.6	−1.5	4.5	3.7	5.8	−1.5	6.6	2.8	5.4	4.9	−2.4 ^d
Difference between treatments in low $T_{\min}$ block		1.3	1.7	2.3	2.1	2.5	2.9	3.2	2.0	2.5	1.3	1.6	4.0
Difference between non-frost protection blocks		2.1	0.9	0.9	1.2	1.7	2.5	1.8	1.2	1.2	0.9	1.6	1.4

^a A slightly sloped tea field, which had a gradient in the *T*_{min} along the slope, was divided into two blocks of differing *T*_{min} (low *T*_{min} and high *T*_{min} block).
^b Minimum temperatures on frost protective nights are shown. Minimum temperatures were monitored with a thermo-recorder. Minimum temperatures in the frost-protected high *T*_{min} block were not monitored.
^c The numbers are dates.
^d Ice formation observed on tea leaves.

protection) of six plots each (for various bactericide treatments). Thermo-regulation fans (NK-607YD, Matsushita Electric Co., Tokyo, Japan) were used from 16 October to 31 December 2007; the fans were set to activate at 4°C on the tea canopy surface from 17 October to 13 November, 2°C from 14 November to 30 November, 1°C for 1 December to 14 December, and 0°C from 15 December to 30 December, and the stop temperature to inactivate the fans was set to the start temperature plus 2°C. Ice formation on tea leaves was judged every morning during the frost protection period. Inoculation (10^5 CFU/ml of strain K9301) was performed on 16 October 2007 before the frost protection period, and the fans ran for 15 nights during the experimental period. We applied copper oxychloride with the antibiotic kasugamycin (Kasumin-Bordeaux, Hokko Chemical, Tokyo, Japan) at a dilution ratio of 1/500 and application dose of 4 ml/m² at the first appearance of BSB (1 November 2007 in the non-frost protection block, 4 January 2008 in the frost protection block) and only copper oxychloride (Dutch-Bordeaux, Hokko Chemical Co., Japan) after the initial incident once a month thereafter. Incidences of BSB were recorded at 15-day intervals from 1 November 2007 to 1 March 2008. Yields of the first crop of tea were measured on 2 May 2008 by plucking new shoots in each plot and a non-inoculated control plot using a riding-type tea plucking machine (Valiant, Matsumoto Kiko Co., Kagoshima, Japan), and the amounts were scaled to the yields per m² of tea canopy surface. The area of each plot was 7.2 m², and each treatment was replicated three times. Temperature on the tea canopy surface in the center of each block was monitored with a thermo-recorder RTR-53.

RESULTS

Ice formation on tea leaves by INAX enhances *P. syringae* pv. *theae* invasion into tea leaf tissues. Artificially exposing tea leaves to -3°C, a temperature at which INAX is able to nucleate ice crystals, caused water-soaked regions to appear when a high concentration (10^8 CFU/ml) of a mixture of *P. syringae* pv. *theae* and INAX was dropped on the abaxial (lower) leaf surface, but not when a high concentration of only *P. syringae* pv. *theae* (Fig. 1A) or a low concentration (10^6 CFU/ml) of *P. syringae* pv. *theae* and INAX (data not shown) was applied. When the high concentration mixture of *P. syringae* pv. *theae* and INAX was applied on the abaxial surface of tea leaves, populations of both *P. syringae* pv. *theae* and INAX in surface-sterilized leaves were significantly higher in -3°C-exposed water-soaked leaves compared with non-treated leaves (Fig. 1B, Student's *t* test, $P = 0.002$ for *P. syringae* pv. *theae*, $P < 0.001$ for INAX). When only *P. syringae* pv. *theae* was applied, there was no significant difference in the population of *P. syringae* pv. *theae* on surface-sterilized leaves between temperature treatments (Fig. 1B). Similarly, significantly higher populations of *P. syringae* pv. *theae* and INAX survived on -3°C-treated, water-soaked leaves after bactericide treatment compared to leaves exposed to 15°C (Fig. 1C, Student's *t* test, $P < 0.001$ for both *P. syringae* pv. *theae* and INAX). The surviving population of bacteria in -3°C-exposed water-soaked leaves after surface sterilization by hydrogen peroxide was significantly higher on the abaxial surface than adaxial (upper) surface (Fig. 2A, by two-tailed ANOVA, $F_{1,8} = 88.04$, $P < 0.001$). Many bacterial cells after staining with crystal violet were observed within and around stomata of -3°C treated, water-soaked tea leaves caused by co-inoculation of *P. syringae* pv. *theae* with INAX (Fig. 2B). In contrast, bacterial cells in non-water-soaked leaves by single inoculation of *P. syringae* pv. *theae* were mainly observed in the cell junction of the epidermal layer of leaves (Fig. 2C). No crystal violet-stained cells were observed in noninoculated leaves (data not shown).

The natural incidence of BSB in the field correlates with early autumn frost. BSB occurred earlier in the October 1986 to

Fig. 1. Effect of low temperature on bacterial invasion in tea leaves. **A**, Formation of a water-soaked region that extended beyond the point of inoculation on a tea leaf by *Pseudomonas syringae* pv. *theae* (*Pst*) with (right) or without (left) co-inoculation of *Xanthomonas campestris* (INAX). The bacterial population in control (kept at 15°C) and low temperature-treated (-3°C for 2 h) tea leaves following inoculation. Colony forming units (CFU) were counted after leaf surface sterilization by **B**, hydrogen peroxide or **C**, copper bactericide treatment. In each panel, white, black, and shaded bars represent the population of *P. syringae* pv. *theae*, of *P. syringae* pv. *theae* in leaves co-inoculated with *P. syringae* pv. *theae* and INAX, and of INAX in leaves co-inoculated with both bacteria, respectively. Values are the means $\pm$ standard error ($n = 4$).

Fig. 2. The stomata are considered to be the main avenue for bacterial entry after freezing. **A**, Effect of choice of leaf surface for inoculation of -3°C -exposed tea leaves on the size of the survival population of bacteria after sterilization with hydrogen peroxide. In each panel, white, black, and shaded bars represent the population of *Pseudomonas syringae* pv. *theae* (*Pst*), of *P. syringae* pv. *theae* in leaves co-inoculated with *P. syringae* pv. *theae* and *Xanthomonas campestris* (INAX), and of INAX in leaves co-inoculated with both bacteria, respectively. Values are the means $\pm$ standard error ($n = 4$). Microscopic analysis of bacteria on the abaxial surface of -3°C -exposed leaves inoculated with **B**, *P. syringae* pv. *theae* or **C**, *P. syringae* pv. *theae* and INAX. After -3°C exposure, crystal, violet-stained bacteria on the leaf surface were observed microscopically. Scale bar = 20 μm .

March 1987 season (Fig. 3A) and with higher severity than in the 1987 to 1988 season (Fig. 3A). For the 1986 to 1987 season, there was a close relationship between a lower minimum temperature ($T_{\min}$) in late autumn and the incidence of BSB in spring (Fig. 3A). No other obvious environmental factors, such as RH, amount of precipitation, amount of insolation, or wind velocity could explain the observed incidence of BSB. Thus, the relationship between the number of BSB-diseased leaves and $T_{\min}$ dynamics in October and November was examined closer (low temperature index, see Material and Methods) in a tea field at the Tea Research Division for the seasons from 1981 to 1987 and 2002 to 2007. There was an apparent negative relationship between the incidence of BSB in March and a low temperature index (Fig. 3B, $r = -0.73$, $P = 0.004$). The same seasonal occurrence of BSB was observed in 25 tea fields around our division from 2004 to 2007 (data not shown).

Frost protection in late autumn significantly decreases the incidence of BSB. To examine the effect of frost protection in late autumn on the incidence of BSB, a tunnel-shaped cheesecloth covering was applied over the tea plants to protect them on nights of predicted frost. In experimental fields of either high or low $T_{\min}$, frost protection raised the $T_{\min}$ of the tea canopy by 1.3 to 4.0°C compared with that in nonprotected plots in the low $T_{\min}$ block, and the lowest minimum temperature recorded in the frost-protected and non-frost-protected plots in the low $T_{\min}$ block were -0.1 and -3.8°C , respectively (Table 1). In addition, no ice formation was observed on tea leaves during nights of frost protection in frost-protected plots in the low $T_{\min}$ block (Table 1).

In treatments where inoculation of *P. syringae* pv. *theae* was performed before the frost-protective period (Fig. 4A; pre-frost protective inoculation), a significant difference in the number of BSB-diseased leaves between the blocks of low and high $T_{\min}$ was observed ($F_{1,15} = 8.31$; $P = 0.012$); within each $T_{\min}$ block, frost protection dramatically decreased the incidence ($F_{1,15} = 10.96$; $P = 0.005$). When *P. syringae* pv. *theae* was inoculated after the frost-protective period (Fig. 4B; post-frost protection inoculation), the difference in the number of diseased leaves between blocks was still significant ($F_{1,15} = 9.21$; $P = 0.009$), but the degree of difference was smaller than for the pre-protection inoculation, and frost protection had no effect on the degree of BSB infection ($P = 0.508$). Furthermore, although we observed a significant difference in frost damage of tea buds between buds taken before (15 October) and those taken after (1 December) the frost protection period in the non-frost-protected plot of the low $T_{\min}$ block ($P < 0.001$), frost protection had no effect on freezing damage of buds collected on 1 December compared to non-frost-protected buds ($P = 0.513$) (Table 2; freezing damage under artificial freezing conditions indicates the extent of frost hardening of the tea plants).

Frost protection combined with bactericide application holds the incidence of BSB under the economic threshold level. During the experimental frost protection period of 76 days from 16 October to 31 December 2007, the fans ran for 15 nights and raised the $T_{\min}$ by 0.9 to 5.2°C , and the lowest minimum temperatures in the frost-protected and non-frost-protected plots were -1.5 and -6.3°C , respectively (Table 3). In addition, ice formation on tea leaves was observed on only 1 day (6 December) in the frost-protected plots compared with 8 days in the non-frost-protected plots (Table 3). Frost protection by fans sufficiently reduced the incidence of BSB during the frost protective period compared to non-frost-protected plants (Fig. 5A versus B, $F_{1,9} = 12.15$; $P = 0.006$). After the frost protection period, the numbers of diseased leaves gradually increased, and frost protection alone failed to maintain the incidence of BSB under the economic threshold level (10% loss of yields (28); the dotted line in Fig. 5B). However, frost protection followed by two applications of bactericide kept the incidence below the economic threshold level

(Fig. 5B). In contrast, the level of BSB in plots without frost protection was severe, and three applications of bactericide could not fully control the disease (Fig. 5A). The integrated disease management program reduced the incidence of BSB to 3.5% of the incidence in control plots of the non-frost-protected block. In yields from the first crop of tea in the frost protection plot block that received bactericide treatments did not differ significantly (Student's t test, $P = 0.810$) from the yields in noninoculated plots within the nonfrost protection block (Fig. 5C). Thus, the integrated disease management sufficiently reduced loss in yields of the first crop of tea to prevent economic loss.

Fig. 3. Correlation between the natural occurrence of bacterial shoot blight (BSB) and minimum daily temperature. **A**, Seasonal dynamics of BSB and minimum daily air temperature (T_{min}). White and black bars represent the number of diseased leaves for the 1986 to 1987 season and the 1987 to 1988 season, respectively. Broken and solid lines represent the T_{min} for each 10-day period for the 1986 to 1987 season and the 1987 to 1988 season, respectively. Broken and solid arrows represent the first frost event for the 1986 to 1987 season and the 1987 to 1988 season, respectively. Values represent the mean number of diseased leaves in 90 m² plot ($n = 8$). **B**, Relationship between the natural occurrence of BSB and low temperature indexes (see Materials and Methods) for 1981 to 1987 and 2002 to 2007. The solid line shows the linear regression and dotted lines show the 95% confidence levels fitted to the data (diamonds represent each year, $n = 13$). Values are the means ($n = 8$ for 1981 to 1987, $n = 25$ for 2002 to 2007).

DISCUSSION

Plant-pathogenic bacteria must enter the leaf tissue to initiate pathogenesis, and this entry is a critical first step in the establishment of infection (20,34). Stomata, which are natural openings on the surfaces of leaves that conduct gas exchange necessary for photosynthesis and control water loss, are passive portals of entry for plant-pathogenic bacteria (34). Our data show that *P. syringae* pv. *theae* inoculated on abaxial leaf surfaces could survive efficiently within water-soaked leaf regions caused by ice formation by INAX, even after surface sterilization of the leaves (Fig. 1B and C). There are many more stomata on the abaxial surface than on the adaxial surface of tea leaves (according to our microscopic observations), and many bacterial cells were observed within and around stomata in water-soaked regions caused by ice formation (Fig. 2C). It has been reported that bacteria in the intercellular spaces of leaf tissue can survive after surface sterilization by hydrogen peroxide (10). We have also reported that the population of *P. syringae* pv. *theae* in wounded tissue of tea leaves cannot be reduced by copper bactericide treatment (27). In addition, ice on leaves can enter plants through stomata and hydathodes (23). Sule

Fig. 4. Effect of frost protection by covering tea plants in late autumn on the incidence of bacterial shoot blight (BSB). Incidence of BSB in tea plants inoculated with *Pseudomonas syringae* pv. *theae* **A**, before the frost-protective period and **B**, after the frost-protective period is shown. In each panel, black and white bars represent the number of diseased leaves of tea plants in frost-protected and non-frost-protected plots, respectively. Values are the means $\pm$ standard error ($n = 4$).

and Seemuller showed a relationship between ice formation and infection of sour cherry leaves by ice nucleation-active *P. syringae* pv. *syringae* (25). They assumed that bacteria were passively drawn into the plant and stomata were the main avenues for bacterial entry after freezing. Others have also noted that the ingress and spread of *P. syringae* is promoted by frost-related water-soaking of tissues (35). Thus, our data clearly show that ice formation, initially on the tea leaf surface, accelerates invasion of *P. syringae* pv. *theae* into tea leaves possibly through stomata. This indicates that prevention of ice formation on tea leaf surfaces may be sufficient to inhibit *P. syringae* pv. *theae* infection.

The natural incidence of BSB in the field was closely related to early autumn frost (Fig. 2A and B). The occurrence of frost that caused ice formation on and within leaves enhanced the infection of *P. syringae* pv. *theae* into tea plants. Thus, we predicted that the natural incidence of BSB would be earlier and more severe in the following spring after the early autumn frost had occurred; however, another possibility is that the early autumn frost led to freezing damage (dehydration) of the tea plants, and the population of *P. syringae* pv. *theae* in the intercellular spaces of freezing-damaged leaf tissues increased more rapidly than in non-damaged leaf tissues. The enhanced development of disease lesions of *P. syringae* pv. *theae* by INAX-mediated ice formation was more severe in the frost-sensitive cultivar Yutakamidori than the frost-tolerant cultivar Yabukita (*unpublished data*). We are currently studying the interactions between frost hardening of several tea cultivars and bacterial growth in the intercellular spaces under low temperature conditions.

We have reported that an application of copper bactericide with the antibiotic kasugamycin at the initial appearance of BSB lesions and regular application of copper bactericide once a month after the initial appearance can control the outbreak of BSB (29). Frost-protective and thermo-regulative fans are widely used to protect the first crop of tea from frost injury in Japan (on 21,734 ha); however they are used for frost control of new shoots for only March and April. The effect of a BSB management plan that integrated frost protection by thermo-regulated fans in late autumn with regular application of bactericides was examined.

Frost protection by either covering or operation of thermo-regulative fans in late autumn had a substantial influence on the

incidence of BSB during the frost protection period (Fig. 4A and 5) but little effect on later susceptibility and frost hardening of the tea plants (Fig. 4B, Table 2). Our data indicate that environmental control in the field through the use of frost-protective fans, which are already in use in many tea fields in Japan, to prevent ice formation on tea leaves can dramatically reduce both the incidence of BSB disease of tea plants and yield loss of the first crop of tea (Fig. 5). In addition, the cost of running the fans per unit area is equal to a single application of bactericide. Such highly effective management of a plant bacterial disease with low cost has rarely been reported.

It has been recognized that heavy rains are associated with the transition of *P. syringae* from epiphytic growth to endophytic parasitism, resulting in *P. syringae*-related disease outbreaks (10). In contrast, an association between ice formation and enhanced disease development has been reported for bacterial plant diseases caused by relatively “weak” pathogens, particularly those belonging to *P. syringae* pathovars (9). Lindow and colleagues demonstrated that recombinant non-ice forming strains of *P. syringae* and *P. fluorescens* could be applied to sufficiently reduce the population of ice nucleation-active bacteria to decrease frost injury (13,14,17). They have also examined integrated biological control of both fire blight and frost injury because protection from frost injury by biological agents can reduce the incidence of fire blight (15,16). However, we found no report that showed that environmental frost protection could decrease plant diseases.

Severe occurrence of BSB disease is caused by interactions between the tea plant, *P. syringae* pv. *theae* (with INAX), and low temperature (ice formation). Frost prevention appears to decrease BSB mainly by preventing infection by *P. syringae* pv. *theae*. Furthermore, frost protection also may prevent the enhancement of virulence of *P. syringae* pv. *theae* upon INAX-induced ice formation, and chemical control by bactericide application most likely reduced the inoculum potential of the pathogen. In conclusion, low temperature enhanced the extent of damage to the host by *P. syringae* pv. *theae*; by contrast, frost protection and chemical control dramatically reduced the damage. This study is a valuable example of the effect of the environment on host-pathogen interactions, allowing the development of new strategies for plant disease control in the field by modifying environmental conditions.

TABLE 2. Freezing damage of tea buds after exposure to artificial low temperature

Sampling time	Block ^a	Treatment	Frost damage of tea buds (%) ^b		
			−3°C	−6°C	−9°C
Before the frost protection	Low T_{min}	Non-frost protection	37.5	100.0	100.0
After the frost protection	Low T_{min}	Non-frost protection	0.0	6.3	15.0
		Frost protection	0.0	6.3	23.8
	High T_{min}	Non-frost protection	0.0	12.5	21.3
		Frost protection	0.0	7.4	26.3

^a A slightly sloped tea field, which had a gradient in the minimum temperature (T_{min}) along the slope, was divided into two blocks of differing T_{min} (low T_{min} and high T_{min} block).

^b Before (15 October) and after (1 December) the frost protection period, 10 buds from each plot were collected and exposed to −3°C, −6°C, or −9°C for 2 h. Freezing damage is presented as the means ($n = 4$) percentage of 10 buds exhibiting damage. Lack of freezing damage indicates the extent of frost hardening of the tea plant.

TABLE 3. Minimum temperature during frost protection by thermo-regulative fans

Block	Minimum temperature (°C) ^a														
	11/3 ^b	11/12	11/13	11/19	11/20	11/22	11/23	11/25	12/2	12/6	12/9	12/10	12/17	12/20	12/21
Non-frost protection	2.4	2.0	−1.5 ^c	−4.3 ^c	0.7	−0.1	−2.4 ^c	−0.1	−2.4 ^c	−6.3 ^c	0.2	−3.5 ^c	−1.9	−2.9 ^c	−3.8 ^c
Frost protection	5.4	5.8	3.7	−0.1	2.4	2.4	2.8	2.8	1.6	−1.5 ^c	1.1	0.7	0.2	0.2	0.7
Difference between blocks	3.0	3.8	5.2	4.2	1.7	2.5	5.2	2.9	4.0	4.8	0.9	4.2	2.1	3.1	4.5

^a Minimum temperatures on frost protective nights are shown. Minimum temperatures were monitored by a thermo-recorder.

^b The numbers are dates.

^c Ice formation observed on tea leaves.

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Fig. 5. Effect of integrated disease management on the incidence of bacterial shoot blight (BSB) and the tea yields from the first harvest. Incidence of BSB in tea leaves of plants in **A**, non-frost-protected and **B**, frost-protected fields is shown. In each panel, squares represent the untreated control, diamonds represent copper oxychloride and kasugamycin treatment. The dotted line indicates the economic threshold level of BSB (50 diseased leaves/m² at the beginning of March), when control measures are recommended to keep yield losses from exceeding 10% (28). Arrows indicate the application of a mixture of copper oxychloride and kasugamycin, and broken arrows indicate the application of copper oxychloride alone. Incidence values are the means $\pm$ standard error (SE) ($n = 3$). **C**, Effect of integrated disease management on tea yields from the first harvest. Values are the means $\pm$ SE ($n = 3$).

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