Integrated disease management using environmental control in tea fields

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The occurrence of plant disease depends on interactions between the host plant, a pathogen, and the environment in a dynamic called “the disease triangle”[1-4]. Bacterial shoot blight (BSB) disease, caused by *Pseudomonas syringae* pv. *theae* (*Pst*), is a major bacterial disease of tea plants in Japan and substantially reduces tea productivity. BSB mainly occurs in the low-temperature season, and lesion formation by *Pst* is enhanced by both low temperature and the presence of ice nucleation-active *Xanthomonas campestris* (INAX), which catalyses ice formation at –2 to –4°C and is frequently co-isolated with *Pst* from tea plants[5]. Low temperature is thus the most important environmental factor to influence the incident; however, the effects of environmental controls in fields on the occurrence of the disease are poorly understood. Here we show that the natural incidence of BSB in the field is closely related to low temperatures in late autumn. Frost protection in late autumn, which protected tea plants against extremely low temperatures, significantly decreased the incidence of BSB, and frost protection combined with bactericide application held the incident 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 using integrated plant disease management based on the disease triangle concept.

Tea (*Camellia sinensis*) is an important industrial crop worldwide, with up to 50000 hectares devoted to its cultivation in Japan. 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[6]**.** The control of disease and pests is crucial for stable tea production. BSB disease (Fig.1a) caused by *Pst* 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 infection by the pathogen and lack of effective means to control the pathogen.

Pst forms aggregates[7] similar to biofilms[8,9] on tea leaves, and epigallocatechin gallate[10], a major tea catechin, induces biofilm formation of *Pst* on abiotic surfaces[11]**.** In addition, compared to planktonic-grown cells, biofilm-grown *Pst* cells on an abiotic surface or on tea leaves have remarkable resistance to the antibiotic kasugamycin[12]. Although control of BSB mainly consists of the application of a mixture of copper and kasugamycin, eradication of aggregates of *Pst* 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.

The optimum temperature range for disease development of *Pst* is approximately 10°C to 15°C (T.T. and N.T., unpublished data); BSB disease appears mainly from late autumn to spring and is not observed in the high-temperature season. Severe outbreaks of BSB (Fig. 1b) are associated with cold and frost damage[13]. In addition, INAX, which nucleates ice crystals at temperatures slightly below 0°C, is often isolated with *Pst* in lesions of BSB in tea fields (Fig. 1c), and enhances *Pst* lesion formation after exposure to –4°C[5]. Furthermore, seasonal population dynamics of *Pst* and INAX have been found to be synchronised in the low-temperature season in tea fields[5]. Therefore low temperature is the most important environmental factor for both damage of tea plants and enhanced virulence of *Pst* due to ice formation by INAX. Thus, we were interested in testing the effect of low temperature control in fields to reduce the incidence of BSB.

We began by describing a relationship between minimum daily air temperature

(T_{min}) and yearly incidence of BSB. BSB disease occurred early in the October 1986-March 1987 season and with higher severity than in the 1987-1988 season (Fig. 2a). The earlier onset of the lower T_{min} in October and November differed between the two seasons and there was a close relationship between the incidence of BSB in spring and the lower T_{min} in late autumn (Fig. 2a). No other obvious environmental factors, such as relative humidity, amount of precipitation, amount of insolation, or wind velocity, could explain the incidence of BSB we observed. Thus, we verified the relationship between the number of BSB-diseased leaves and the dynamics of T_{min} in October and November (low temperature index, see Methods Summary) in a tea field of our 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. 2b, r=-0.73, p=0.004). We observed the same seasonal occurrence of BSB in 25 tea fields around our Division during the period from 2004 to 2007. Low temperature, especially an early autumn frost, leads to damage to tea plants because they gradually acquire frost hardening in late autumn like fruit trees[14,15]. Our data indicate that low temperature in late autumn influenced the susceptibility of tea plants to BSB infection, and suggest that frost protection in late autumn would reduce the incidence of BSB.

To examine the effect of frost protection in late autumn on the incidence of BSB, we first used a tunnel-shaped cheesecloth covering over the tea plants to protect them on nights of predicted frost. In experimental fields of either high or low T_{min} as a result of the field slope, the frost protection raised the T_{min} by 1.23-4.14°C compared with that in non-protection plots in the low T_{min} block, and the lower extremes of temperature in the frost protection and non-protection plots in the low T_{min} block were –0.16°C and –3.85°C, respectively (see Supplementary Table 1).

In treatments where inoculation of *Pst* was performed before the frost-protective period (Fig. 3a; pre-protect inoculation), a significant difference in the number of BSB-diseased leaves between the blocks of low and high T_{min} was observed (by two-tailed analysis of variance (ANOVA): $F_{1,15}$=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 *Pst* was inoculated after the frost-protective period (Fig 3b; post-protect inoculation), although the difference in the number of diseased leaves between blocks was still significant ($F_{1,15}$=9.21; p=0.009), the degree of difference was smaller than for the pre-protect inoculation, and frost protection had no effect on the degree of BSB (p=0.508). Furthermore, frost protection had no effect on frost hardening of the tea plants (see Supplementary Table 2; Frost damage indicates the extent of frost hardening of tea plant). These data indicate that frost protection in late autumn had a substantial influence on the incidence of BSB during the frost protection period, but no effect on susceptibility and frost hardening of tea plant.

We have previously 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[16]. Frost-protective and thermo-regulative fans (see Supplementary Fig. 1) are widely used to protect the first crop of tea from frost injury in Japan (21734 hectares); however they are used for frost control of new shoots for only March and April. Thus we examined the effect of a management plan that integrated frost protection by fans in late autumn with regular application of bactericides.

During the experimental frost protection period of 76 days, the fans ran for 15 nights and raised the T_{min} by 0.88-5.33°C, and lower extremes of temperature in the frost protection and non-protection plots were –1.51°C and –6.31°C, respectively (see Supplementary Table 3). Frost protection by fan sufficiently reduced the incidence of BSB during the protective period compare to non-protection (Fig. 4a vs. 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[17] (the red line in Fig. 4b). However frost protection followed by two applications of bactericide kept the incidence under the economic threshold level (Fig. 4b). In contrast, the level of BSB in plots without frost protection was severe, and three applications of bactericide could not fully reduce the incident (Fig. 4a). The integrated disease management reduced the incidence of BSB to 3.5% of the incidence in control plots of the non-protection block, and the cost of running the fans per unit area was equal to a single application of bactericide. Such highly effective management of a plant bacterial disease with low cost has rarely been reported.

An occurrence of early autumn frost increases the susceptibility of plants to fungi attacks[15]. In addition, 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 *Pseudomonas syringae* pathovars[18]. Lindow and colleagues demonstrated that recombinant non-ice strains of *P. syringe* and *P. fluorescens* could be applied to sufficiently reduce the population of ice nucleation-active bacteria to decrease frost injury[19-21]. They have also examined integrated biological control of both fire blight and frost injury because protection of frost injury by biological agents can reduce the incidence of fire blight[22,23]. However, we found no report that showed that environmental frost protection could decrease such plant diseases.

The disease triangle[1-3] is a basic concept of plant pathology, and this concept has been used to develop new ideas to predict and control disease[4]. Severe occurrence of BSB is caused by interactions between susceptibility (frost hardening) of the tea plant, *Pst* (with INAX) and low temperature. Frost prevention appears to decrease BSB infection mainly by preventing damage as a result of ice formation in the tea leaves; however, other possibilities are that prevention of cycles of freezing and thawing reduces the infectivity of the pathogen or that water available to the pathogen is limited by ice formation. Furthermore, frost protection may have also prevented the enhancement of virulence of *Pst* by INAX-induced ice formation and chemical control by bactericide application most likely reduced the inoculum potential of the pathogen. In conclusion, low temperature enhances the extent of damage to the host by disease, and in contrast, frost protection and chemical control dramatically reduce the damage. Our study is a valuable example of the effect of the environment on host-pathogen interactions, allowing us to develop a new strategy for plant disease control in the field.

Methods Summary

Strain K9301 of *Pst* [11] and King's medium B were used for all inoculation studies. Qualitative analyses of *Pst* and INAX were performed with selective media PST-SM[5]. Inoculum of 24-h cultured strain K9301 was sprayed on the tea canopy surface at 100 ml/m^2. Incidence of BSB was judged by counting the number of leaves with at least one dark brown lesion in each plot and the counts were transformed to the number of diseased leaves per square meter of tea canopy surface. Two-tailed analysis of variance and regression analysis of incidences of disease were performed with JMP 5.0 (SAS Institute, Japan).

From 1981 to 1987 and from 2002 to 2007, we investigated the incidence of BSB in a tea field of our Tea Research Division (3000 m^2, cultivar Yutakamidori). Parameters of the physical environment were monitored automatically by weather recorder (NASCON2000, Yokogawa Electric Co., 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.

For frost protection, see Methods.

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End Notes

Supplementary Information is linked to the online version of the paper at .

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Author Contribution T.T. and N.T designed and conducted the experiments; T.T., N.T., N.Y. and A.K. analyzed the data and wrote the paper.

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Figure legends

Figure 1

Leaf symptom of BSB and co-isolation of *Pst* and INAX from infected tea leaves. **a**, Tea leaf infected with BSB. A dark brown lesion develops along the midrib of a tea leaf infected with *Pst*. **b**, Severe natural occurrence of BSB in a tea field. **c**, Colonies of *Pst* (white colonies, arrows) and INAX (small yellow colonies, dashed arrows) on selective PST-SM medium isolated from a BSB lesion.

Figure 2

Interaction between the natural occurrence of BSB and minimum daily temperature. **a**, Seasonal dynamics of BSB and daily minimum air temperature (T_{min}). Light gray and black bars represent the number of diseased leaves for the 1986-1987 season and 1987-1988 season, respectively. Broken and solid lines represent the T_{min} of each 10-day period for the 1986-1987 season and 1987-1988 season, respectively. Values of diseased leaves are the means ± s.e.m. (n=8). **b**, Relationship between the natural occurrence of BSB and low temperature indexes (see Methods Summary) for 1981-1987 and 2002-2007. 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 to 25).

Figure 3

Effect of frost protection by covering tea plants in late autumn on the incidence of BSB. Incidence of BSB in tea plants inoculated of *Pst* before the frost-protective period (**a**) and after the frost-protective period (**b**) are shown. In each panel, black and light gray bars represent the number of diseased leaves in frost protection and non-protection plots, respectively. Values are the means ± s.e.m. (n=4).

Figure 4

Effect of integrated disease management on the incidence of BSB. Incidence of BSB infection in tea leaves of plants in non-frost protection (**a**) and frost protection (**b**) fields are shown. In each panel, squares represent the untreated control, triangles represent copper oxychloride and antibiotic kasugamycin treatment. The red line indicates the economic threshold level of BSB (50 diseased leaves/m^2 at the beginning of March), at which control measures should be taken to prevent the incident from reaching the economic injury level (10% loss of yields) [14]. 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 ± s.e.m. (n=3).

Methods

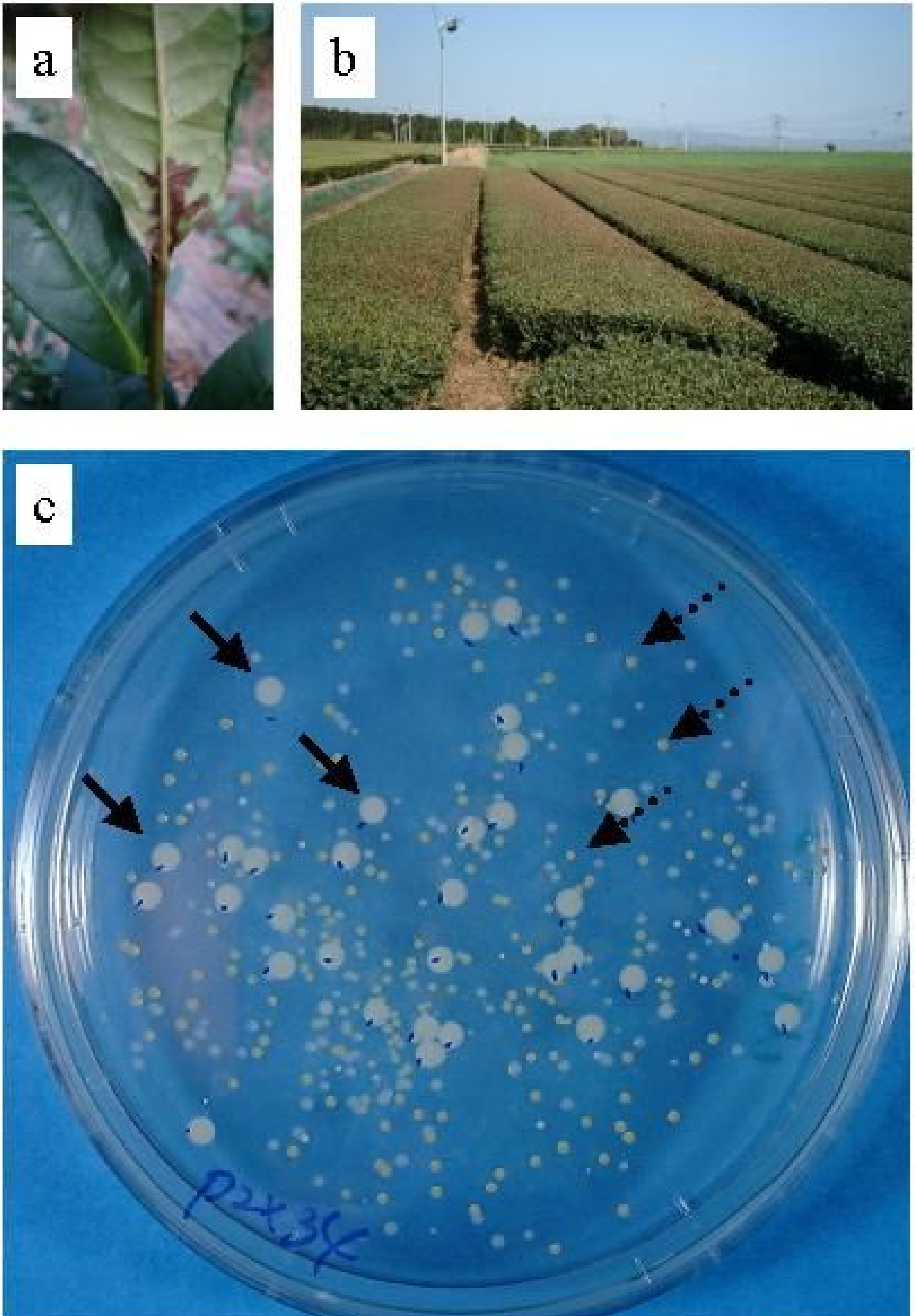
Frost protection by covering: A slightly sloped tea field (260 m^2, cultivar Yabukita), which had a gradient in the T_{min} along the slope, was divided into two blocks of differing T_{min} (see Supplementary Table 1) containing a sixteen-plot array (for the various covering and inoculation treatments). 10^6 cfu/ml of strain K9301 was inoculated on 13 October before frost-protective period (pre-protect inoculation treatment), or 1 December 2006 after frost-protective period (post-protect inoculation treatment). The frost protection experiment consisted of two treatments. Tea plants were (i) exposed to the naturally occurring low temperatures (non-protection), or (ii) protected from frost by covering with tunnel-shaped cheesecloth (Baron Screen®, Koizumiseima, Japan) that was manually applied on nights with predicted frost conditions from 13 October to 1 December (frost protection). Each tunnel covered one row of tea plants. The cheesecloth tunnel coverings were applied 12 times during the experimental period. Incidences of BSB were investigated at 1 December (48 days after the pre-protect inoculation treatment) or 20 January (50 days after the post-protect inoculation treatment). Frost hardening of tea plants after frost protection was monitored by collecting tea buds from plots, exposing them to –3°C, –6°C and –9°C for 2 hours, and recording the degree of frost damage of the buds. Percentages of frost damaged buds showing necrosis per plots was evaluated visually. The area of each plot was 2.8 m^2 and each treatment was replicated four times. Temperature on the tea canopy surface in the center of each block was monitored automatically with the thermo recorder RTR-53 (T&D Corp., Japan).

Frost protection by fan and application of bactericides: A large tea field (900 m^2, cultivar Yabukita) was divided into two blocks (frost protection or non-protection) of six plots each (for various bactericide treatments). Thermo-regulation fans (NK-607YD, Matsushita Electric Co., Japan) were used from 16 October to 31 December 2007, and the fans were set to activate at 4°C on the tea canopy surface for 17 October to 13 November, 2°C for 14 November to 30 November, 1°C for 1 December to 14 December, and 0°C for 15 December to 30 December, and the stop temperature to inactivate the fans was set to the start temperature plus 2°C. 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 , Japan) at a dilution ratio of 1/500 and application dose of 4 ml/m^2 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. The area of each plot was 7.2 m^2 and each treatment was replicated three times. Temperature on the tea canopy surface in the center of each block was monitored automatically with the thermo recorder RTR-53 (T&D Corp., Japan).

Supplementary figure legends

Supplementary Figure 1

Thermo-regulative and frost-protective fan. **a** and **b**, Thermo-regulative and frost-protective fans are present in half of the tea fields in Japan. **c**, Leaves of tea plants in a field with thermo-regulative and frost-protective fans. **d**, Leaves of tea plants in a field without frost protection. These photographs were taken early in the frosted morning at 10 December 2007.


a
b
c

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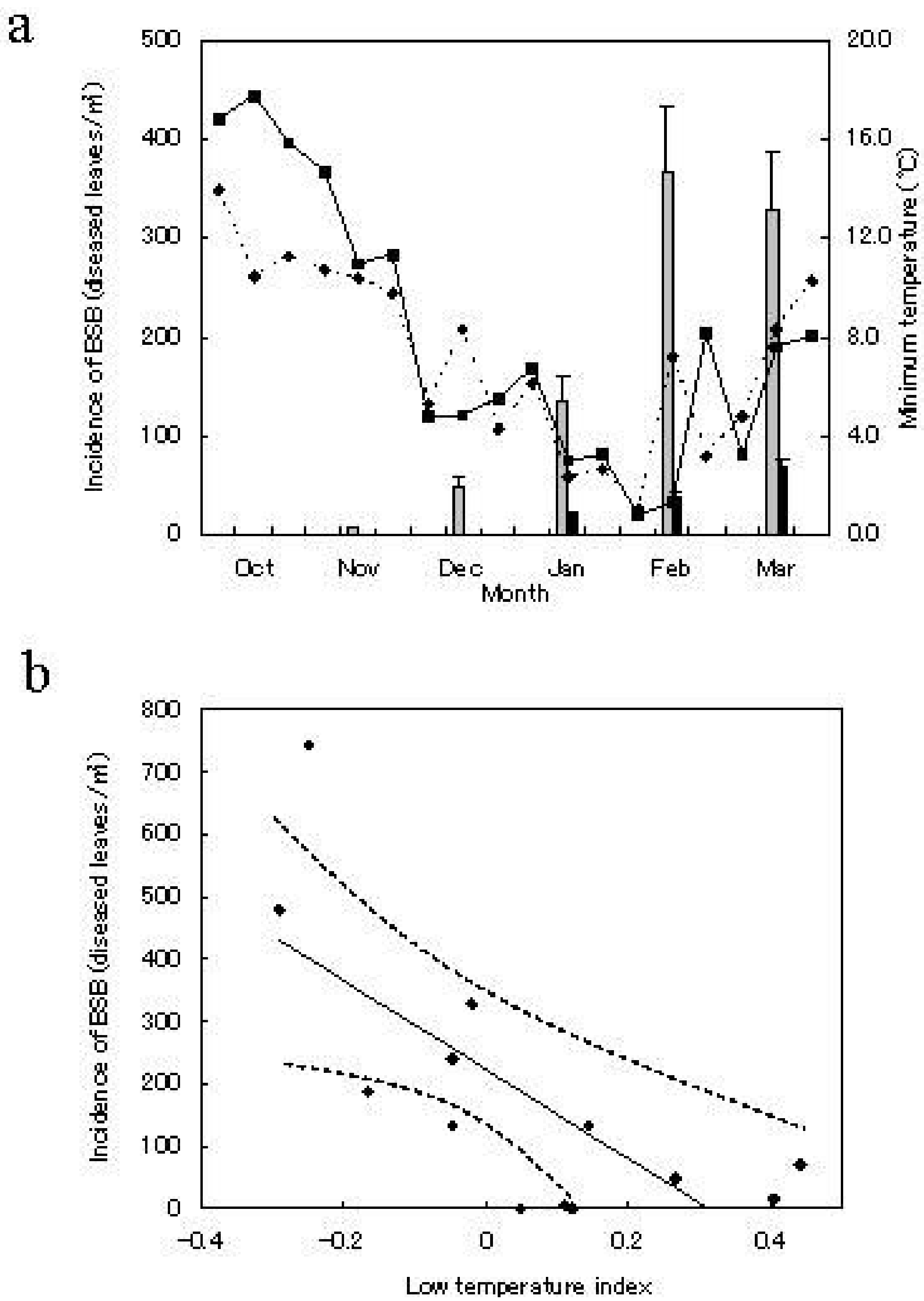

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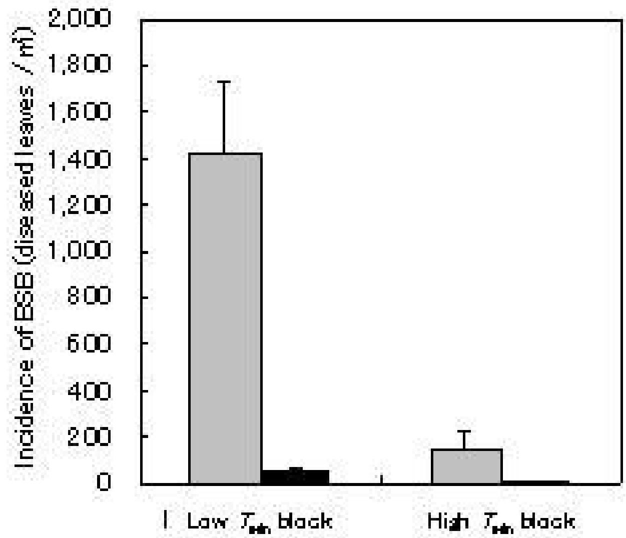
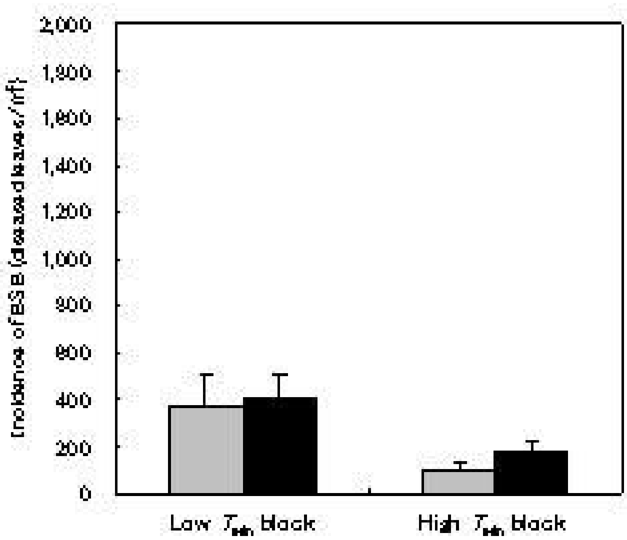

Incidence of BSB (diseased leaves / m²)
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
Low T_min block
High T_min block

b


Incidence of BSB (diseased leaves / m²)
2,000
1,800
1,600
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1,200
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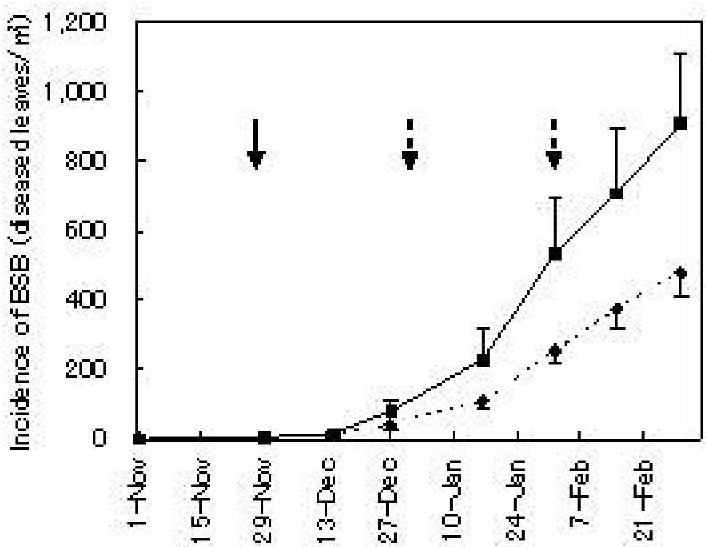
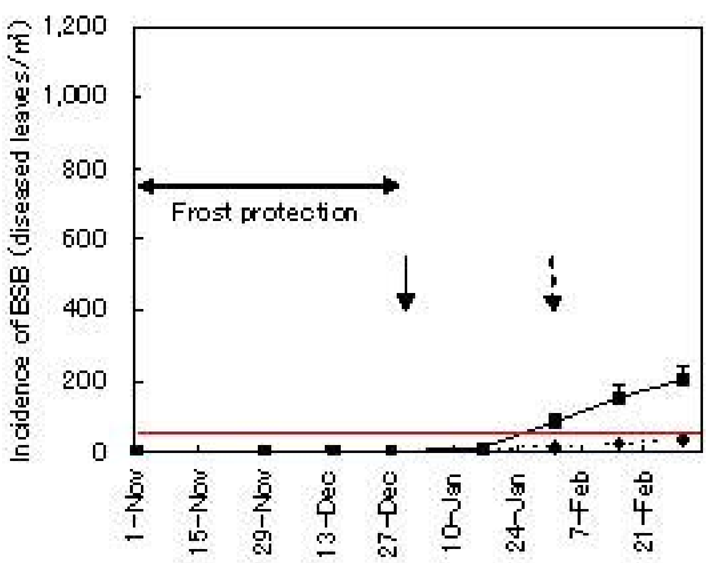
Low T_min block
High T_min block

a


Incidence of BSB (diseased leaves/m²)
1,200
1,000
800
600
400
200
0
1-Nov
15-Nov
29-Nov
13-Dec
27-Dec
10-Jan
24-Jan
7-Feb
21-Feb

b


Incidence of BSB (diseased leaves/m²)
1,200
1,000
800
600
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Frost protection
1-Nov
15-Nov
29-Nov
13-Dec
27-Dec
10-Jan
24-Jan
7-Feb
21-Feb