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Protection Efficacy of Antibacterial Strains against Fire Blight Caused by *Erwinia amylovora* on Apple Blossom

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Fire blight caused by *Erwinia amylovora* is one of the destructive diseases in the family of Rosaceae plants, including apple and pear, in the world. Since the first report in 2015, the number of infected farms and area steadily increased in Korea. In case of eradication failure against this disease, protection strategies using both chemicals and biocontrol agents should be established. In this study, to select an effective antibacterial agent against fire blight on apple trees, four bacterial strains isolated from Jeju Island were investigated. Among the bacterial strains, *Bacillus circulans* BRH433-2 showed bactericidal effects against *E. amylovora* Ea385 forming inhibition zone on an artificial medium. The other bacterial strains such as *Pseudomonas fluorescens* THJ609-3, *Micrococcus luteus* TRK2-2 and *P. fluorescens* TRH415-2 showed bacteriostatic activity preventing growth of *E. amylovora* Ea385 in shaken cultures as well as on detached apple blossoms inoculated with *E. amylovora* Ea385, as measured with quantitative PCR. Bio-tests on detached blossoms showed that the treatment with all bacterial strains caused strong suppression of bacterial ooze formation, indicating inhibition of disease incidence of fire blight, which was similar to blossoms treated with streptomycin sulfate. Therefore, it was suggested that these bacterial strains may be useful in organic apple orchards to control fire blight where chemical use is limited.

Keywords **:** bio-bactericide, PGPR, quarantine

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Fire blight is one of the most destructive diseases in the family of Rosaceae plants, including apple and pear, in the world. Since its first report in Korea in 2015 (Park et al., 2016) this disease has been rapidly spread to other provinces of Korea (Choi et al., 2022; Ham et al., 2024), leading to many losses of entire apple orchards by activation of the eradication program (Cho et al., 2023). Therefore, to avoid eradication, it is necessary to establish a strategy to control the disease. Prevention of epiphytic growth of the pathogen in blossoms will reduce infections and symptom development of fire blight on apple trees.

Like other plants, which belong to Rosaceae, apple trees can be infected by *Erwinia amylovora*, which is the causal bacterium of fire blight. Although this bacterium can invade plant tissue through wounds caused by insects or strong wind, flowers are the main site of infection (Pedroncelli and Puopolo, 2023). When the bacteria enter the plant, they spread systematically through the parenchyma cells and break epidermal cells leading formation of ooze which become secondary inoculum (Slack et al., 2017). In the early stages, various environmental factors may influence the epiphytic survival of the pathogen whereas genetic factors from both host and pathogen may influence the successful endophytic infection and symptom development (Zeng et

al., 2021).

Since this disease is very difficult to control after infection took place, it is important to implement control measures before invasion of the pathogens (Ham et al., 2022). Many practices have been employed to control fire blight such as spraying chemicals, antibiotics, or biological agents as well as removing the infected parts of host plants. Especially, the use of antibiotics is the most effective measurement to control fire blight in many countries, except Europe where the use of antibiotics is prohibited. However, overuse of antibiotics may cause occurrence of antibiotic-resistant strains among the bacterial pathogens. Indeed, it is well known that resistant strains of *E. amylovora* against antibiotics including streptomycin have been reported in many countries (Ham et al., 2022).

Using biological agents, such as effective microorganisms as an alternative strategy for fire blight control, should be focused on. Recently, microorganisms have been newly reported, which were able to reduce the disease incidence of fire blight on apple trees. For example, some bacterial strains like *Pseudomonas* sp. CT-1059 or *Pantoea* sp. CT-1039 isolated from apple stigma could suppress disease incidence of fire blight on apple flowers (Cui et al., 2021). Also, the yeast-like fungal strains *Aureobasidium pullulans* DSM14940 and DSM14941 were effective in reducing fire blight incidence in apple and pear orchards. The effectiveness was enhanced by combining the two synergistic strains of *A. pullulans* with a citric acid-based buffer (Kunz et al., 2023). In Korea some effective bacterial strains were found by laboratory experiments (Jeon et al., 2022), without field studies because *E. amylovora* is a quarantine pathogen, making field experiments impossible.

In our previous studies, some effective bacterial strains were isolated from Jeju Island. Bacterial strain *Pseudomonas fluorescens* THJ609-3 and *Bacillus circulans* BRH433-2 suppressed the mycelium growth of *Phytophthora citrophthora* on citrus fruit after *in vivo* wound inoculation (Kang and Jeun, 2010). Furthermore, pre-treatment with *P. fluorescens* THJ609-3 and TRH415-2 showed disease suppression of citrus melanose caused by *Diaporthe citri* on citrus leaves (Ko et al., 2012). Also, pre-treatment with *Micrococcus luteus* TRK2-2 reduced effectively the disease severity of late blight caused by *Phytophthora infestans* in the potato plants (Kim and Jeun, 2006) and anthracnose by *Colletotrichum orbiculare* in cucumber plants (Jeun et al., 2004).

In this study, to select an effective antibacterial agent against fire blight on apple trees the characteristic of four bacterial strains isolated from Jeju Island showing antifungal activity were investigated. Bactericidal activity of the bacterial strains was tested by observing inhibition zones, bacteriostatic activity by evaluating growth of *E. amylovora* in shaken cultures. Furthermore, apple blossoms were inoculated with *E. amylovora* and treated with the bacterial strains. On these blossoms, the population development of *E. amylovora* was measured using a quantitative PCR and the symptom development was evaluated, by counting blossoms with bacterial ooze, to determine whether the bacterial strains have efficacy to suppress the disease development of fire blight.

Materials and Methods

Bacterial strains. *Erwinia amylovora* strain Ea385 was obtained from the strain collection of Bio-Protect, Konstanz, Germany. *E. amylovora* Ea385 was isolated from an infected apple tree in 2003 and used as a reference in several studies (Hinze et al., 2016; Kunz, 2006). The reference bacterium was grown on nutrient broth with sucrose agar (NBSA) medium (8 g/l nutrient broth, 50 g/l sucrose, 20 g/l Agar-Agar Kobe I) at 25°C for 48 h. Bacterial cells were removed from the agar-plates with a pipette tip and suspended in 0.6% NaCl. Concentration of the bacterial cell suspensions were determined by correlation with the optical density at 660 nm, given by the equation [cells/ml = OD660 × 1.3E+09].

Four bacterial strains *Pseudomonas fluoescens* THJ609-3, *Bacillus circulans* BRH433-2, *Micrococcus luteus* TRK2-2, and *Pseudomonas fluoescens* TRH415-2, which showed antifungal effect against various plant pathogens, were obtain from Plant Pathology Lab in Jeju University. The bacterial strains were grown in tryptic soy agar medium at 28°C for 48 h and the bacterial suspension was prepared with its concentration of 1.0×10^8 cfu/ml, each in 0.6% NaCl solution. The bacterial suspension was used for either the bio-test on the apple blossom or the direct antibacterial test.

Inhibition zone test with paper disc. The direct antibacterial effect of the bacterial strains against *E. amylovora* Ea385 was tested using an artificial medium. On the NBSA medium 50 µl of the prepared *E. amylovora* Ea385 suspension (1.0×10^7 cfu/ml) was inoculated and spread broadly using a loop. Subsequently, a paper disc was laid on the middle of the plate and 30 µl of the bacterial strain suspension was dropped on the disc. The plates were incubated in an incubator (LABWIT, ZWYR-240, Shanghai, China) at 25°C for 48 h and the diameter of the inhibition zone around the paper disc was measured with a ruler. As negative control H_2O and as positive control 0.025% streptomy-

cin sulfate solution was used instead of the suspension of the bacterial strains, respectively.

Incubation of *E. amylovora* Ea385 and the bacterial strains in shaken cultures. Growth suppression of *E. amylovora* Ea385 by the bacterial strains was evaluated *in vitro* after co-inoculation of shaken cultures (Kunz, 2006). The concentration of inoculum of *E. amylovora* Ea385 was adjusted to 1×10^7 cfu/ml in 25 ml nutrient broth with sucrose (NBS) medium in a 100 ml Erlenmeyer flask containing the testing bacterial strains with 1×10^8 cfu/ml, each. 4 and 24 h after incubation on a rotary shaker at 27°C, the concentration of *E. amylovora* Ea385 in the culture was analyzed using a quantitative PCR (Bio-Rad, CFX Duet, Feldkirchen, Germany). For controls, cultures with only *E. amylovora* Ea385 and cultures with addition of 0.025% of streptomycin sulfate to *E. amylovora* Ea385 were used.

Bio-test on detached apple blossoms. An *in vivo* test system with detached apple blossoms was established according to Pusey (1997) and adjusted by Kunz (2006). Freshly opened blossoms were taken from commercial apple orchards nearby Konstanz from the varieties 'Gala' or 'Pinova'. The blossoms were cut and maintained with the pedicel submerged in 10% sucrose in plastic racks. Blossoms were sprayed with a suspension of *E. amylovora* Ea385 adjusted to 1.0×10^7 cfu/ml in water until run-off.

After 1 h drying at room temperature, the blossoms were treated with the suspension of bacterial strains with the same method. Blossoms were allowed to dry for 3 h at room temperature in open boxes. Afterwards, the boxes were closed to maintain a high relative humidity and were incubated at 21-24°C until appearance of fire blight symptoms on untreated blossoms. The number of blossoms with bacterial ooze at the pedicel was counted 5-8 days following inoculation, to evaluate the disease incidence as (Number of blossoms with bacterial ooze/Number of blossoms without bacterial ooze) × 100 (%).

The experiments were replicated 3 times with 24 blossoms per treatment and replicate. For analysis of the population development of *E. amylovora* Ea385 subsamples (4 blossoms/subsample) of the inoculated blossoms were taken at 1, 24, and 48 h after inoculation and proceeded for analyses with the quantitative PCR.

Sample preparation for quantitative PCR. A blossom sample consisted of four apple blossoms without petals. Eight ml bi-deist water (Carl Roth GmbH + Co.KG, Karlsruhe, Germany) (2 ml per blossom) were used to wash apple blossom. One ml washing water per sample was removed. From shaken cultures, 1 ml of culture broth was used per sample. Samples were centrifuged at 20,238 ×*g* for 5 min at room temperature. Supernatant of blossom samples as well as those of culture samples was discarded and pellets were resuspended in 1 ml sterile distilled water (bioscience grade, DEPC-treated, Carl Roth GmbH + Co.KG).

Quantitative PCR. The number of *E. amylovora* Ea385 cells in the samples was analyzed using quantitative PCR. CFX Duet (Bio-Rad) and QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany) were used to perform the analyses using a threshold set to 3,000 rfu (relative fluorescence units). Primer set of p29TF 5′-CAC TGA TGG TGC CGT TG-3′ and p29TR 5′-CGC CAG GAT AGT CGC ATA-3′ (Salm and Geider, 2004) were used according to the protocol by Hinze et al. (2016). Total volume of reaction was 25 µl containing a final concentration of 0.5 µM of each primer, with a sample volume of 10 µl. Samples consisted of washing fluids containing bacterial cells, no DNA extraction was performed. The PCR process consisted of an initial denaturation step of 5 min at 95°C; followed by 40 cycles of denaturation at 95°C for 10 s, and a combined annealing and elongation step at 60°C for 30 s. To verify identity of the amplicon (112 bp) a melt curve analysis was performed showing a melting point of the amplicon of 83.5 ± 0.5°C, if *E. amylovora* Ea385 was amplified. For quantification of *E. amylovora* Ea385 cells per sample an external standard curve (*E. amylovora* cells/10 µl = $10^{(-0,2938*\text{ct-value} + 10,878)}$) was used.

Statistical analysis. Data of disease incidence on apple blossoms untreated and treated with streptomycin sulfate and the bacterial strains were analyzed with Duncan's multiple range test using statistical analysis system (SAS) program version 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance was considered at $P < 0.05$.

Results

Inhibition zone forming by bacterial strain. Growth inhibition efficacy by the bacterial strains was revealed on the NBSA medium inoculated with *E. amylovora* Ea385 and the bacterial strains together. Inhibition zone was formed surround the paper disc, in which streptomycin sulfate was dropped, indicating growth of *E. amylovora* Ea385 was suppressed by the antibiotic (Fig. 1B). Similarly, *B. circulans* BRH433-2 showed antibacterial effect against *E. amylovora* Ea385 forming an inhibition zone on the NBSA medium (Fig. 1D). However, other bacterial

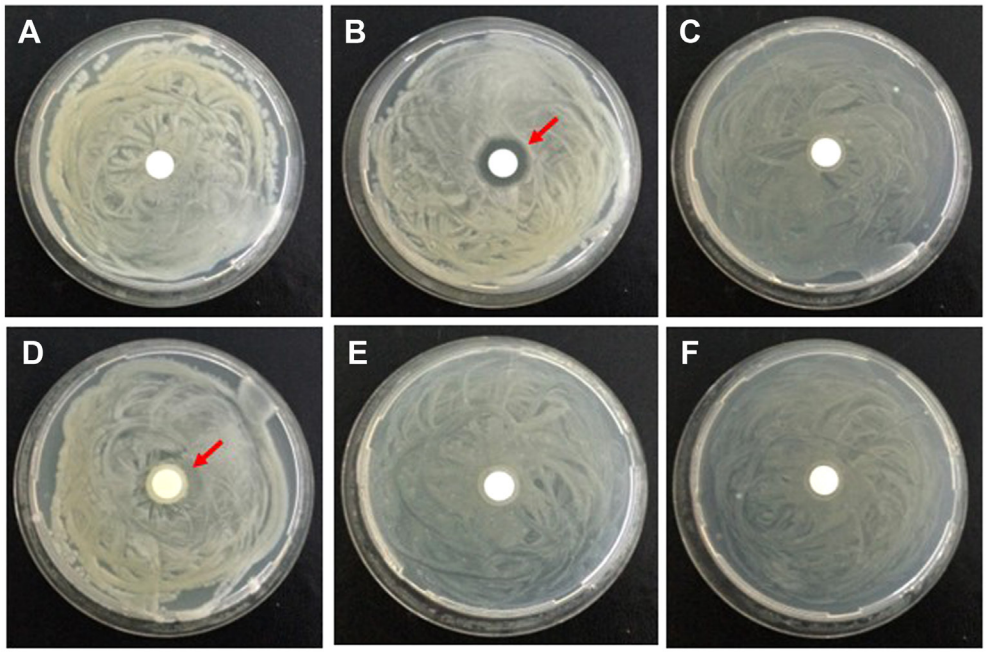

Fig. 1. Inhibition zones caused by bacterial strains in *Erwinia amylovora* Ea385 cultures on NBSA medium. The presented plates were untreated (A), treated with streptomycin sulfate (B), *Pseudomonas fluorescens* THJ609-3 (C), *Bacillus circulans* BRH433-2 (D), *Micrococcus luteus* TRK2-2 (E), *P. fluorescens* TRH415-2 (F). The inhibition zone was formed by 0.025% streptomycin sulfate and *B. circulans* BRH433-2 (arrow). The concentration of the bacterial strains was 10^8 cfu/ml each.

strains such as *P. fluorescens* THJ609-3, *M. luteus* TRK2-2 and *P. fluorescens* TRH415-2 could not inhibit the growth of *E. amylovora* Ea385 on the NBSA medium, like those of H_2O dropped paper disc (Fig. 1A, C, E, and F).

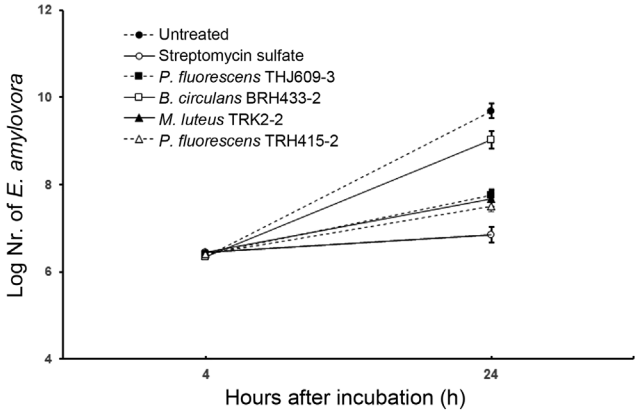

Fig. 2. Growth of *Erwinia amylovora* Ea385 cells determined by quantitative PCR in NBS medium which were untreated, treated with 0.025% streptomycin sulfate or bacterial strains *Pseudomonas fluorescens* THJ609-3, *Bacillus circulans* BRH433-2, *Micrococcus luteus* TRK2-2 and *P. fluorescens* TRH415-2 incubation at 27°C. The concentration of the bacterial strains was 10^8 cfu/ml each.

Growth suppression of *E. amylovora* by the bacterial strains in shaken cultures. In order to investigate direct bacteriostatic efficacy of the bacterial strains, concentration of *E. amylovora* Ea385 was measured using a quantitative PCR after co-culture with *E. amylovora* Ea385 and the bacterial isolates in NBS broth. Without the bacterial isolate concentration of *E. amylovora* Ea385 rose over 1,000 times after 24 h (Fig. 2). However, the bacterial strains such as *P. fluorescens* THJ609-3, *M. luteus* TRK2-2 and *P. fluorescens* TRH415-2 limited the growth of *E. amylovora* Ea385 in comparison to *E. amylovora* Ea385 alone (Fig. 2), indicating a significant bacteriostatic efficacy by these bacterial strains. Unexpectedly, the growth suppression by *B. circulans* BRH433-2, which formed an inhibition zone in NBSA medium, was very weak (Fig. 2). The strongest bacteriostatic effect was shown in the streptomycin sulfate treated medium (Fig. 2).

Reduction of fire blight incidence on apple blossoms. Once the bacteria have moved into the flower nectary and initiated blossom blight infection, they will begin to spread systemically through the tree and break epidermal cells leading to formation of ooze which becomes secondary inoculum (Slack et al., 2017) and this causes epidemics in the apple orchards (Pedroncelli and Puopolo, 2023). To

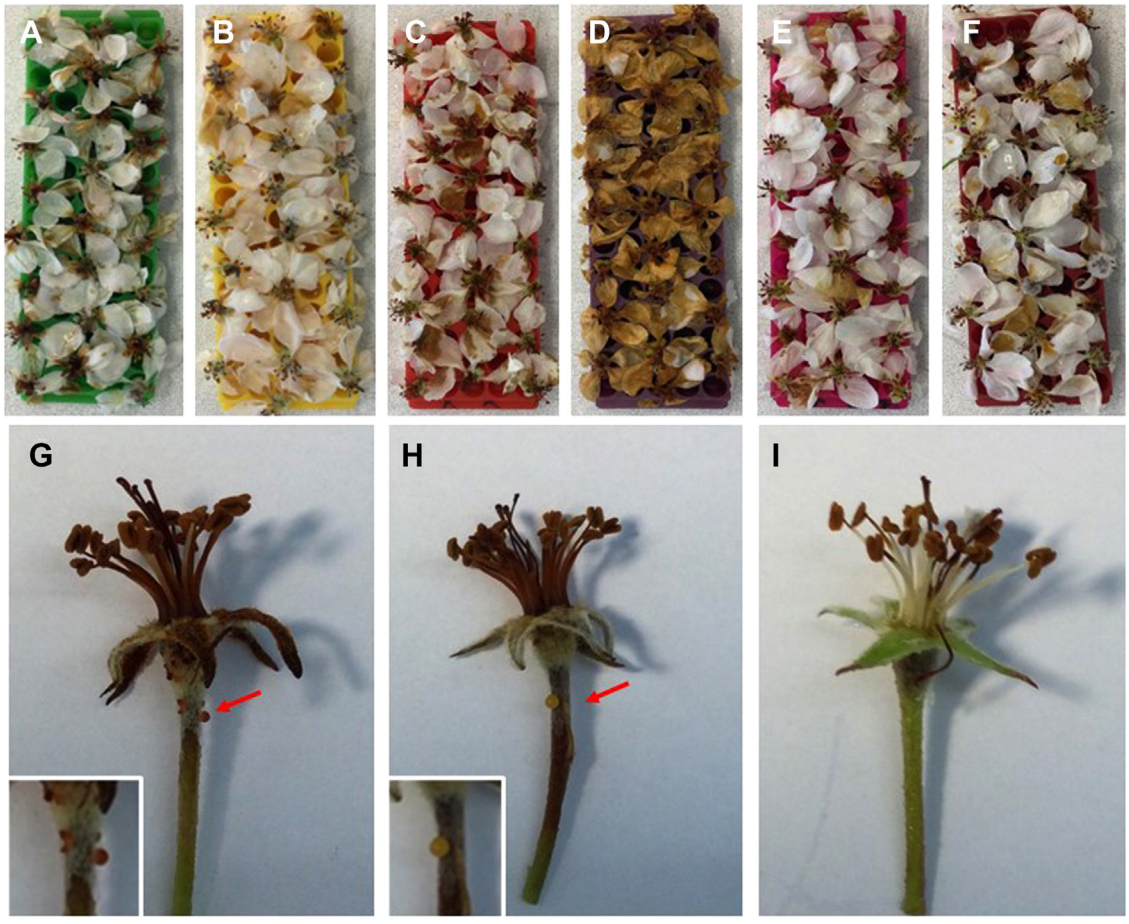

Fig. 3. Photographs of apple blossoms inoculated with 5.0×10^6 cfu/ml of *Erwinia amylovora* Ea385. The photographs were presented 7 days after inoculation. The blossoms were untreated (A), treated with 0.025% streptomycin sulfate (B), *Pseudomonas fluorescens* THJ609-3 (C), *Bacillus circulans* BRH433-2 (D), *Micrococcus luteus* TRK2-2 (E), and *P. fluorescens* TRH415-2 (F). The bacterial ooze droplets represented on the symptomatic apple blossoms with red (G, arrow) or white (H, arrow), whereas no ooze on the asymptomatic ones (I). The square boxes represent the bacterial oozes with magnification. The concentration of the bacterial strains was 10^8 cfu/ml each.

investigate whether the bacterial strains can protect apple blossoms against fire blight, bio-tests were carried out with inoculation of detached blossoms. Looking on the top of the blossoms 7 days after inoculation with *E. amylovora* Ea385, it seemed that all the blossom in all treatments showed similar (Fig. 3A-F). Exceptionally, blossom treated with *M. luteus* TRK2-2 showed brown petals (Fig. 3D). However, the typical symptom of fire blight could be identified, when droplets with bacterial ooze were observed on the peduncle or calyx (Fig. 3G and H), which were not observed on asymptomatic blossoms (Fig. 3I).

In average, 72% of the untreated apple blossoms showed droplets of bacterial ooze after inoculation with *E. amylovora* Ea385. However, the treatment with the bacterial strains *P. fluoescens* THJ609-3, *B. circulans* BRH433-2 and *M. luteus* TRK2-2 significantly reduced fire blight incidence on inoculated blossoms and showed ooze formation, indicating suppression of disease incidence by fire blight, comparable to streptomycin-sulfate (Fig. 4). *P. fluoescens* TRH415-2 showed a medium reduction of fire blight incidence.

Suppression of *E. amylovora* Ea385 population development by the bacterial strains. To illustrate the epiphytic growth suppression of *E. amylovora* Ea385 by the bacterial strains, the population of *E. amylovora* Ea385 was measured using a quantitative PCR. On the untreated apple blossoms the abundance of *E. amylovora* Ea385 increased more than 1,000 times 48 h after inoculation. However, in the apple blossoms treated with the bacterial strains *B.*

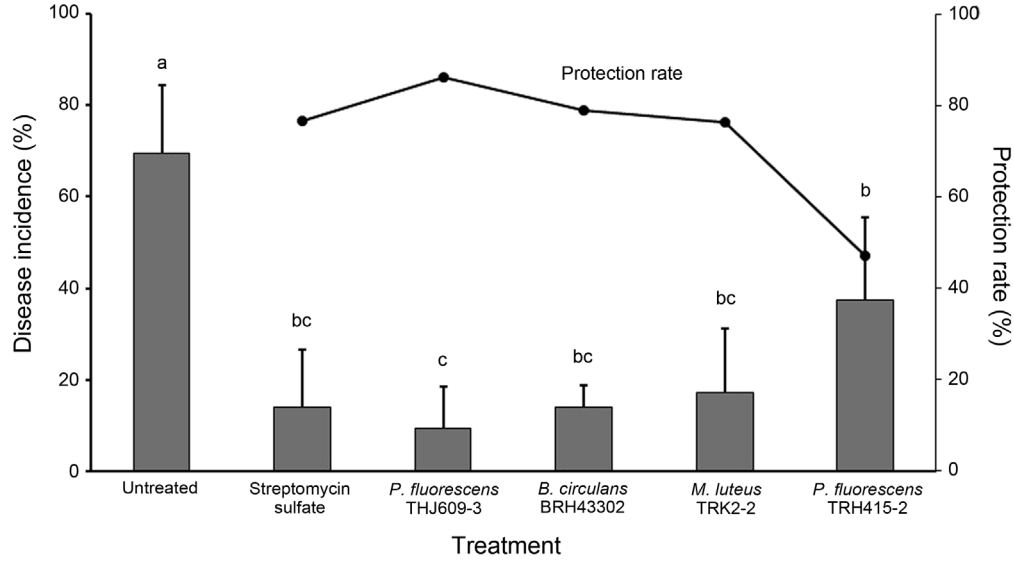

Fig. 4. Disease incidence of *Erwinia amylovora* Ea385 on apple blossoms untreated, treated with 0.025% streptomycin sulfate, bacterial strains *Pseudomonas fluorescens* THJ609-3, *Bacillus circulans* BRH433-2, *Micrococcus luteus* TRK2-2, and *P. fluorescens* TRH415-2 at 7 days after inoculation. The line in the graph indicates protection rate by each treatment. The concentration of the bacterial strains was 10^8 cfu/ml each. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

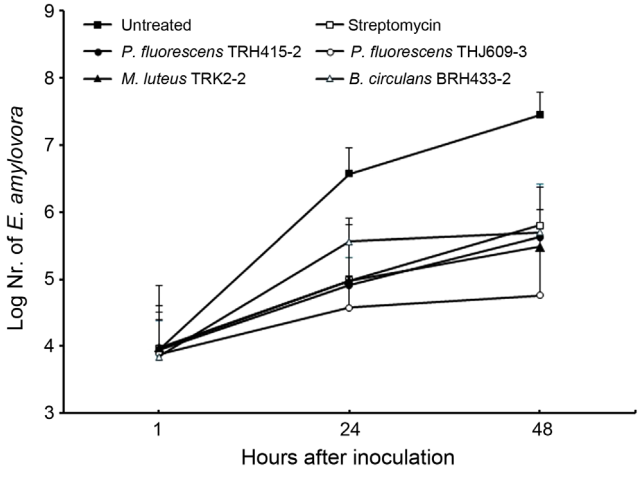

Fig. 5. Growth of *Erwinia amylovora* Ea385 on the apple blossoms, determined by quantitative PCR at different times after inoculation. The blossoms were untreated, treated with 0.025% streptomycin sulfate, *Pseudomonas fluorescens* THJ609-3, *Bacillus circulans* BRH433-2, *Micrococcus luteus* TRK2-2 and *P. fluorescens* TRH415-2. The concentration of the bacterial strains was 10^8 cfu/ml each.

circulans BRH433-2, *M. luteus* TRK2-2, and *P. fluoescens* TRH415-2, as well as on blossoms treated with streptomycin sulfate, the growth of *E. amylovora* Ea385 was limited to 10 to 100 times. Remarkably, *P. fluoescens* THJ609-3 suppressed the growth of *E. amylovora* Ea385 to less than 10 times multiplication (Fig. 5), which coincided with the low disease incidence in the bio-test on detached blossoms with this treatment (Fig. 4).

Discussion

To control fire blight, effective antibiotics like streptomycin sulfate, kasugamycin, and nalidixic acid have been applied resulting in successful results in many crops (Johnson et al., 2016). However, due to the side effects of antibiotics such as resistance of the pathogen or phytotoxicity in host plants, the application of antibiotics has been prohibited in many countries (Deckers and Schoofs, 2002; Sundin and Wang, 2018). Therefore, biological control using effective microorganisms has been risen as an alternative way instead of antibiotics (Gusberti et al., 2015; Temple et al., 2019). In this study, some bacterial strains were tested as candidates for biological control agents against fire blight in apple blossoms.

The antibacterial effect of *B. circulans* BRH433-2 was observed on NBSA medium by forming an inhibition zone surrounding the treated paper disc (Fig. 1D). Although the inhibition zone was smaller compared to those formed by streptomycin sulfate, the inhibition zone indicated the direct antibacterial effect. However, in the shaken culture *B. circulans* BRH433-2 could not significantly inhibit the growth of *E. amylovora* Ea385 showing direct bacterio-

static activity (Fig. 2). Conversely, bacteriostatic activity against *E. amylovora* Ea385 was apparently shown in the other bacterial strains *P. fluorescens* THJ609-3, *M. luteus* TRK2-2 and *P. fluorescens* TRH415-2 (Fig. 2), although they did not form inhibition zones on NBSA medium (Fig. 1). Consequently, these bacterial strains were further evaluated to determine their potential efficacy in controlling fire blight in apple blossoms. There are many studies showing suppression of disease severity caused by bacterial pathogen by effective bacterial strains such as plant growth promoting rhizobacteria (PGPR) including *Bacillus* spp., *Pseudomonas* spp. etc. (Benchlih et al., 2023; Fallahzadeh-Mamaghani et al., 2021). Many PGPR have an antimicrobial effect by forming siderophores or releasing some cell wall-degrading enzymes (Ali et al., 2020). However, not all bacterial strains showing antimicrobial effects could suppress disease severity in planta (Kim et al., 2008).

Most bacterial strains tested in this study could reduce disease incidence of fire blight in apple blossoms. Especially, three bacterial strains *P. fluorescens* THJ609-3, *M. luteus* TRK2-2, and *B. circulans* BRH433-2 showed strong suppression of fire blight, comparable or even better than streptomycin sulfate (Fig. 4), suggesting that these bacterial strains may be applicable in the field as an alternative agent instead of chemical bactericides or antibiotics. Furthermore, these bacterial strains were applied after the inoculation with *E. amylovora* Ea385, indicating a high competitiveness of the bacterial strains against *E. amylovora* Ea385. Indeed, in many cases, the reduction of yield loss by application of some effective bacterial strains has been reported (Karačić et al., 2024; Nigro et al., 2018).

Epiphytic colonization and the accumulation of a significant population of *E. amylovora* on apple blossoms are essential prerequisites for infection (Slack et al., 2017). Therefore, reduction of the abundance of *E. amylovora* on apple blossoms is a crucial point to establish the protection strategy. In our study, the abundance of *E. amylovora* Ea385 on the apple blossoms was compared between the bacterial strains treated and untreated blossoms. All bacterial strains could reduce the abundance of *E. amylovora* Ea385 on apple blossoms (Fig. 5), which was coincided with results of disease incidence in the bio-test (Figs. 4 and 5). Based on these results, it was suggested that the bacterial strains could reduce the population of *E. amylovora* on apple blossom which may lead to reduction of disease incidence of fire blight. In a summary of nine field trials in Germany with artificial inoculation in Germany, the abundance of *E. amylovora* in blossoms during bloom correlated with the fire blight incidence on blossom clusters in untreated plots (Kunz et al., 2012). This indicates that reduction of the *E. amylovora* population size on blossoms, as achieved with the bacterial strains in this study (Fig. 5), would result in a reduction of symptoms in the field.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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