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Novel Detection Protocol for *Erwinia amylovora* in Orchard Soil after Removal of Infected Trees

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Fire blight is a bacterial disease caused by *Erwinia amylovora*. In Korea, fire blight was first reported in 2015 in an orchard. If the infection is confirmed, all trees in the orchard must be removed and the orchard must remain closed for 3 years. Since 2020, if the number of trees infected with fire blight is less than 5% of the total trees in the orchard, only the infected tree and adjacent trees are removed in Korea. Three years after removal, the trees can be replanted after confirming that the orchard soil is free from *E. amylovora*. In this study, a protocol was established for detecting *E. amylovora* in soil via selective enrichment, using tryptic soy broth with 0.05% bile salts and 50 µg/ml cycloheximide, and real-time polymerase chain reaction. This protocol resulted in a 1,000-times improved detection limit for *E. amylovora* in soil samples compared to that in unenriched samples. Soil monitoring was performed for orchards where fire blight–infected trees had been removed 3-27 months prior; the selected orchards were monitored every 3 months. Monitoring confirmed that *E. amylovora* was not present in the soil at any site in any of the orchards. A new detection protocol facilitates the monitoring of *E. amylovora* in soil and could help permit the replanting of trees in orchards. Also monitoring results provide evidence that trees can be planted earlier.

Keywords **:** detection, *Erwinia amylovora*, soil

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Fire blight is a deadly disease caused by the gram-negative bacterium *Erwinia amylovora*. This disease causes significant economic losses by affecting crops in the *Rosaceae* family, including apples and pears, which serve as its hosts worldwide (Bonn and van der Zwet, 2000; Momol and Aldwinckle, 2000). Infection is initiated through flowers and shoots (Steiner, 2000), and the entire fruit tree becomes withered (van der Zwet and Keil, 1979). The first fire blight outbreak in Korea was reported in 2015 in apple and pear orchards in Anseong and Jecheon (Myung et al., 2016; Park et al., 2016); 43 cases occurred in 2015. Subsequently, cases have increased annually, with 67 cases in 2018 and 744 in 2020 (unpublished). *E. amylovora* has been registered as a quarantine pathogen by the Animal and Plant Quarantine Agency (APQA) of Korea, based on the Plant Protection Act of 1962 (Park et al., 2017).

After the first outbreak in 2015, yearly observation and control of fire blight have been conducted by the government. If fire blight was found, even on only one tree in an orchard, the orchard was closed after digging up and removing the roots of all trees. In accordance with the guidelines for observation and control of fire blight provided by the Rural Development Administration (RDA) in 2020, a new removal method has been implemented, in which only infected trees are removed when they comprise less than 5% of the total trees in an orchard (Ham et al., 2020). Currently, if less than 5% of the total trees in an orchard

are affected by fire blight, infected trees, and adjacent trees are removed in apple orchards, whereas only infected trees are removed in pear orchards. Orchards that have removed trees affected by fire blight are banned from replanting the trees for 3 years. To replant after the ban period, the soil must be confirmed to be free of *E. amylovora.* However, studies that confirm safety from re-infection through soil insects or soil containing surviving *E. amylovora* from removed infected host plants are lacking, and farmers continue to express anxiety and concerns about re-infection in their fields (Kim et al., 2019).

A protocol for detecting *E. amylovora* in soil was first included in the guidelines for observation and control of fire blight provided by the RDA in 2022. This protocol involves enrichment of soil samples, followed by polymerase chain reaction (PCR) using two separate sets of primers and electrophoresis. However, many microbes exist in the soil (Fierer, 2017); hence, predicting the maximum effects of enrichment is difficult because the use of a general nutrient medium provides a favorable environment for both *E. amylovora* and other bacteria. In addition, PCR is time-consuming because electrophoresis is essential and false-positive bands (Powney et al., 2011) may appear when bacteria with target sequences similar to those of *E. amylovora* exist in the microbial communities in the soil. In such cases, additional sequencing is required. Thus, this study aimed to remedy the problems in the detection protocol in the guidelines and establish an efficient protocol for detecting *E. amylovora* in soil. The proposed protocol in this study is expected to help ensure soil safety for farmers who want to plant trees after the ban.

Materials and Methods

Bacterial strains and culture conditions. *E. amylovora* TS3128 was isolated from a pear orchard in Anseong (Myung et al., 2016), and *Bacillus pumilus* SR073 was isolated from soil from a tomato farm. Bacterial cultures were incubated with tryptic soy broth (TSB) (Difco, Franklin Lakes, NJ, USA) at 28°C and 150 rpm for 16 h.

Selective enrichment conditions. To establish selective enrichment conditions, the growth of the bacterial strains was compared across five different media and in the presence of six inhibitors. Growth was measured by calculating the optical density (OD) at 600 nm and 27°C using a microplate reader (Hidex Co., Turku, Finland) at 1 h intervals for 24 h. The assay was performed with an initial bacterial concentration of 10^5 colony forming unit per ml (cfu/ml). The five media were TSB, M9 minimal salts (Difco), nutrient broth (NB; Difco), King's B broth (KB) (MBcell, Seoul, Korea), and mannitol glutamic acid yeast broth (MGY) (comprising 10 g of D-mannitol, 2 g of L-glutamic acid, 0.5 g of KH_2PO_4, 0.2 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 g of yeast extract per 1 liter; pH 7.0). The inhibitors used 50 μg/ml rifampicin (Biosesang, Seongnam, Korea), 20 μg/ml novobiocin (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml erythromycin (Sigma-Aldrich), bile salts at concentration of 0.01%, 0.05%, and 0.15% (Oxoid, Basingstoke, UK), 0.4% Niaproof 4 (Sigma-Aldrich), and 50 μg/ml cycloheximide (Sigma-Aldrich).

***E. amylovora* detection in spiked soil samples.** To confirm the effects of selective enrichment and determine the incubation time necessary for the detection of *E. amylovora* in soil, real-time PCR was performed using isolated DNA from *E. amylovora*–spiked soil cultured on the selective enrichment medium. Soil samples were collected from an apple orchard that had never been infected with fire blight. The soil samples were divided into six groups, each inoculated with 10^1 to 10^6 cfu/g of *E. amylovora*, and enriched under three conditions: TSB only, TSB with 0.05% bile salts, and TSB with 0.05% bile salts 10^2 and 50 μg/ml cycloheximide. Microbial DNA was isolated from enriched soil samples, which were collected at 15 h and 24 h, and used as templates for real-time PCR.

DNA isolation. The enriched soil samples with TSB only, TSB with 0.05% bile salts, and TSB with 0.05% bile salts and 50 μg/ml cycloheximide for 15 h and 24 h at 28°C were homogenized by vortexing for 10 min. The homogenized sample (800 μl), including soil particles, was centrifuged (15,000 ×*g*, 5 min), and the supernatant was discarded. Total DNA was isolated from the soil samples using the DNeasy Power Soil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Real-time PCR. Real-time PCR amplification was performed using a CFX96 real-time PCR system (Bio-Rad Inc., Hercules, CA, USA) with a HelixDtec EAD-T100 kit (Nanohelix, Daejeon, Korea). The probes for the two target genes were labeled with FAM (6-carboxyfluorescein) and VIC (2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein). The cycling conditions were 95°C for 5 min for initial denaturation, followed by 40 cycles of 10 s at 95°C, and 30 s at 60°C. A Ct value of ≤35 for both target genes was considered a positive result, based on the instructions for the kit.

Soil sampling for *E. amylovora* monitoring. Soil samples were collected from 13 orchards where fire blight–infected trees were removed between 2020 and 2022 and subsequently tested. Orchards were separated into three zones: center zone (up to 1 m from the position of the removed tree), inner zone (between 1 m and 5 m from the position of the removed tree), and outer zone (the remainder of the orchard). Using an auger, 100 g of soil to a depth of 50 cm was sampled from three sites in each of the three zones between March 2022 and September 2022. Soil samples were stored in a refrigerator prior to the experiments. The samples were incubated in selective enrichment media containing 0.05% bile salts and 50 µg/ml cycloheximide for 15 h, and the extracted DNA were used as a template for real-time PCR.

Results and Discussion

Selective enrichment conditions for *E. amylovora.* PCR and real-time PCR methods for detecting *E. amylovora* have been reported in several previous studies (Bereswill et al., 1992, 1995; Salm and Geider, 2004). These molecular detection protocols were confirmed using isolated DNA or cell cultures of *E. amylovora.* PCR can be inhibited by plant-derived phenolic compounds; nevertheless, plant extracts obtained by grinding the tissue or tissue suspensions of host plants with distinct symptoms can be used as templates for real-time PCR (Jin et al., 2022). In general, real-time PCR provides advantages over traditional PCR; these advantages include accurate detection, immediate information availability, and reduced time. However, detecting specific microbes using soil samples as templates for PCR or real-time PCR is difficult because they may contain substances that can inhibit DNA polymerase, such as humic acid and fulvic acid (Schneegurt et al., 2003). In addition, many microbial communities are present in the soil; hence, isolating specific bacteria with a low population is challenging. Therefore, to detect *E. amylovora* in soil samples using PCR or real-time PCR, the enrichment of *E. amylovora* and subsequent DNA isolation should be performed.

E. amylovora grows on commonly used general-purpose media like nutrient agar and broth and tryptic soy broth and agar. The optimal temperature for its growth is 27°C, and cell division occurs at temperatures ranging from 5°C to 31°C (Johnson, 2015). However, these conditions are also beneficial for the growth of other soil microbes. Thus, several types of selective media have been used to isolate *E. amylovora*. Colonies are cream-colored on NB medium supplemented with 5% sucrose (Billing et al., 1961) and translucent with dark orange centers on Miller-Schroth (MS) medium (Miller and Schroth, 1972). Cream-colored, round colonies have been identified on KB medium (Paulin and Sampson, 1973). On levan medium, the colonies are white, round, domed, smooth, and mucilaginous. Colonies on crystal violet-cycloheximide-tergitol (CCT) medium (Ishimaru and Klos, 1984) are pale purple, round, highly convex to domed, smooth, and mucilaginous and grow slower than those on KB or levan medium (International Plant Protection Convention, 2016). Distinguishing *E. amylovora* from other bacterial species is possible based on typical characteristics, such as the color and shape of colonies on these media. However, the selectivity on KB, MS, NB, CCT, and levan media is relies on the morphology of *E. amylovora* colonies and not the inhibitory properties of the media on the growth of other bacteria. Thus, to establish growth and enrichment conditions for the detection of *E. amylovora* in soil, growth curves were compared for various media, antibiotics, and growth inhibitors.

The growth of *E. amylovora* was measured at 27°C at 1 h intervals for 24 h using a microplate reader. *B. pumilus* SR073 was used as a representative gram-positive soil bacterium for comparison with the gram-negative *E. amylovora* across TSB, NB, KB, M9, and MGY media. After 24 h, the OD values obtained following *E. amylovora* culture in different media were as follows: 0.99 on TSB, 0.51 on KB, 0.41 on MGY, and 0.28 on NB (Fig. 1A). In contrast, the OD values obtained following *B. pumilus* culture were as follows: 0.96 on TSB, 0.72 on KB, 0.31 on MGY, and 0.09 on NB (Fig. 1B). Neither species showed significant growth on the M9 minimal medium during the 24 h period. Upon comparing the growth of both bacteria among the five types of media, the growth of both species was the highest in TSB, followed by KB, MGY, NB, and M9. Moreover, the level of growth for both species was similar in all media except for NB, in which *B. pumilus* growth was lower than that of *E. amylovora*.

As TSB was the most effective medium for the growth of *E. amylovora* but also promoted the growth of *B. pumilus,* various supplements were added to TSB to identify conditions that inhibited the growth of *B. pumilus.* The following six inhibitors were tested: three antibiotics (novobiocin, erythromycin, and rifampicin), two surfactants (Niaproof 4 and bile salts), and one antifungal agent (cycloheximide). The three antibiotics tested have different mechanisms of action and their activity against gram-positive and gram-negative bacteria can be different. But the result is both *E. amylovora* and *B. pumilus* were inhibited by rifampicin,

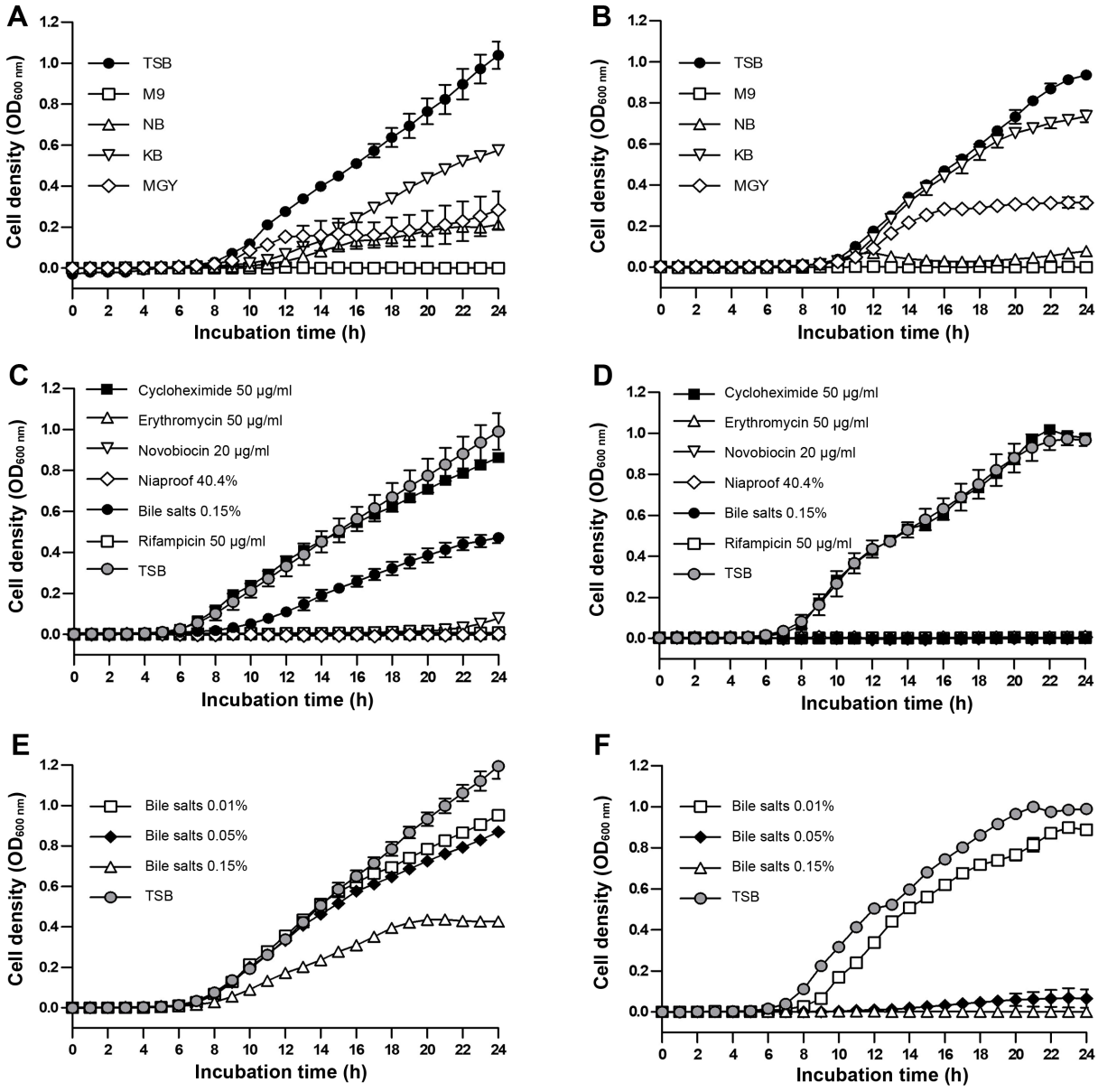

Fig. 1. Comparison of *Erwinia* and *Bacillus* growth in liquid medium with different supplements. (A, C, E) The growth curves of *E. amylovora*. (B, D, F) The growth curves of *B. pumilus*. (A, B) Growth curves with various medium comparisons: TSB (●), NB (△), KB (▽), M9 (□), and MGY (◇). (C, D) Growth curves with TSB containing 50 µg/ml rifampicin (□), 50 µg/ml erythromycin (△), 20 µg/ml novobiocin (▽), 0.4% Niaproof 4 (◇), 50 µg/ml cycloheximide (■), and 0.15% bile salts (●), respectively. (E, F) Growth curves with TSB containing 0.01% (□), 0.05% (◆), and 0.15% bile salts (△), respectively. TSB, tryptic soy broth; NB, nutrient broth; KB, King's B broth; MGY, mannitol glutamic acid yeast broth. Mean values for three independent experiments are plotted for each time point with error bars showing standard deviation.

erythromycin, novobiocin, and Niaproof 4. When cycloheximide was included, *E. amylovora* and *B. pumilus* showed an OD value change of only 0.01-0.13 compared to that in the control groups. Therefore, it was concluded that the antifungal agent did not affect these bacterial strains. However, *E. amylovora* and *B. pumilus* showed significant differences in growth in TSB medium containing bile salts (Fig. 1C and D).

Bile salts are produced from cholesterol in the liver, especially in the pericentral hepatocytes. Seventeen enzymes convert cholesterol into bile acids, which are then converted to bile salts by reaction with Na^+ or K^+ ions (Urdaneta

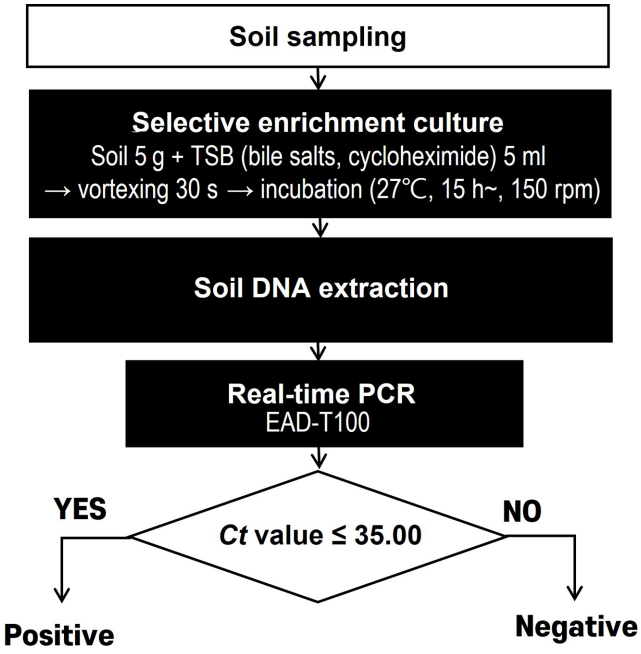

Fig. 2. Overall workflow of the protocol to detect *Erwinia amylovora* in soil sample.

and Casadesús, 2017). Bile salts play an important role in food digestion, exhibit antibacterial properties, and regulate the growth of intestinal bacteria. Previously, bile salts at a concentration of 0.15% have been used in selective media for the isolation of enteric gram-negative bacteria, such as *Escherichia coli* in food, because they selectively support the growth of gram-negative intestinal bacteria while inhibiting the growth of most gram-positive bacteria. This selective advantage is thought to be largely due to a component of the outer membrane of gram-negative bacteria that reduces its permeability to bile salts, thus improving bacterial survival (Cremers et al., 2014; Provenzano et al., 2000). The present results showed that bile salts inhibited the growth of the gram-positive bacterium *B. pumilus*. Therefore, a test was designed to determine the necessary concentration to inhibit the growth of gram-positive bacteria without affecting the growth of *E. amylovora*. The growth of *E. amylovora* and *B. pumilus* was measured in TSB containing three concentrations of bile salts (0.01%, 0.05%, and 0.15%). After 24 h, 0.01% bile salts had not inhibited either species. In the medium with 0.15% bile salts, *B. pumilus* was completely inhibited, but the OD of the *E. amylovora* culture medium decreased from 1.19 to 0.42. In the medium with 0.05% bile salts, *E. amylovora* was inhibited, with a corresponding OD of 0.87, whereas the growth of *B. pumilus* was completely inhibited, as shown by an OD of 0.06 (Fig. 1E and F). Therefore, a bile salt concentration of 0.05% was chosen to inhibit the growth of gram-positive bacteria while having a minimal effect on the growth of *E. amylovora*. Cycloheximide (50 μg/ml), which was also confirmed to have a minimal effect on the growth of *E. amylovora*, was added to inhibit the multiplication of fungi that could compete for nutrients during the enrichment process and was used together with 0.05% bile salts for the selective medium. This selective enrichment medium for *E. amylovora* in soil is expected to improve detection limits by providing a relatively favorable environment for the growth of *E. amylovora* among the numerous soil microbes.

Detection protocol for *E. amylovora* in soil. To confirm the effects of selective enrichment and determine the incubation time necessary for the detection of *E. amylovora* in soil, real-time PCR was performed using DNA isolated from *E. amylovora*–spiked soil that was cultured with the selective enrichment medium. An EAD-T100 real-time PCR kit was used to detect *E. amylovora*; this kit was designed to amplify two *E. amylovora*–specific target genes, thereby improving accuracy. In the absence of enrichment, the detection limit was 10^4 cfu/g. However, the detection limit improved 10-fold for *E. amylovora* in soil samples

Table 1. Ct values of FAM channel of dual qPCR (EAD-T100) using soil samples cultured under various conditions

Erwinia amylovora (cfu/g)	Incubation time without supplements			Incubation time with bile salts		Incubation time with bile salts and cycloheximide	
	0 h	15 h	24 h	15 h	24 h	15 h	24 h
1×10^6	25.45 ± 0.20	22.35 ± 0.09	23.20 ± 0.27	16.26 ± 0.10	16.14 ± 0.14	15.09 ± 0.33	15.78 ± 0.15
1×10^5	30.31 ± 0.28	22.91 ± 0.07	23.64 ± 0.48	16.72 ± 0.17	16.44 ± 0.03	15.00 ± 0.04	15.50 ± 0.19
1×10^4	33.58 ± 0.24	31.85 ± 0.41	31.43 ± 0.27	22.87 ± 0.24	22.75 ± 0.21	21.78 ± 0.39	21.69 ± 0.10
1×10^3	-	34.27 ± 0.34	33.48 ± 0.34	27.92 ± 0.23	28.15 ± 0.13	26.02 ± 0.22	26.34 ± 0.33
1×10^2	-	-	-	30.61 ± 0.09	30.12 ± 0.51	30.97 ± 0.27	30.76 ± 0.09
1×10^1	-	-	-	-	34.23 ± 0.20	34.53 ± 0.43	33.63 ± 0.54

Table 2. Ct values of VIC channel of dual qPCR (EDA-T100) using soil samples cultured under various conditions

Erwinia amylovora (cfu/g)	Incubation time without supplements			Incubation time with bile salts		Incubation time with bile salts and cycloheximide	
	0 h	15 h	24 h	15 h	24 h	15 h	24 h
1×10^6	24.87 ± 0.32	22.40 ± 0.09	21.79 ± 0.05	15.63 ± 0.20	15.98 ± 0.21	14.83 ± 0.16	15.72 ± 0.14
1×10^5	29.30 ± 0.06	23.78 ± 0.28	21.01 ± 0.04	16.03 ± 0.28	16.28 ± 0.12	14.76 ± 0.23	15.60 ± 0.13
1×10^4	32.46 ± 0.28	31.72 ± 0.38	29.15 ± 0.11	21.99 ± 0.26	22.18 ± 0.07	21.07 ± 0.14	21.52 ± 0.17
1×10^3	-	33.55 ± 0.08	30.93 ± 0.11	26.50 ± 0.01	26.90 ± 0.41	25.08 ± 0.13	26.78 ± 0.17
1×10^2	-	-	-	31.33 ± 0.13	29.06 ± 0.11	30.43 ± 0.22	28.68 ± 0.10
1×10^1	-	-	-	-	34.00 ± 0.25	33.80 ± 0.10	33.31 ± 0.22

cultured with TSB for more than 15 h. The addition of 0.05% bile salts decreased the detection limit for the 15 h cultured soil sample to 10^2 cfu/g and the detection limit for the 24 h cultured sample to 10^1 cfu/g. Additionally, a detection limit of 10^1 cfu/g was achieved by incubating for 15 h with TSB, 0.05% bile salts, and 50 μg/ml cycloheximide. The role of cycloheximide is to inhibit the growth of fungi, thus facilitating the utilization of nutrient sources by *E. amylovora* contained in soil samples. The addition of an antifungal agent led to a reduction in the incubation time by 9 hours, enabling the detection of up 10^1 cfu/g (Tables 1 and 2). In conclusion, the detection limits for *E. amylovora* in the soil under these selective enrichment conditions (that is, adding two supplements and incubating for over 15 h), was improved by more than 1,000 times compared to the unenriched condition. This protocol could help detect *E. amylovora* in the soil to obtain permission for replanting in orchards that had previously been affected by fire blight (Fig. 2).

Monitoring of orchards where infected trees have been removed. Thirteen orchards in which a small number of fire blight–infected trees were removed between 2020 and 2022 were selected for monitoring for *E. amylovora*. Information on the regions, fruit species, and number of trees infected with fire blight in these orchards is shown in Table 3. These orchards had 1-4 infected trees and a <5% incidence of fire blight; hence, they were classified as orchards

Table 3. List of orchards where fire blight diseased trees removed

Year	No.	Site	Fruit	Total trees	Diseased trees	Rate (%)	No. of positive sites/ tested sites in orchard		
							2022 Mar	2022 Jun	2022 Sep
2020	1	Hwadang-ri, Baegun-myeon, Jecheon-si	Apple	157	1	0.6	0/9	0/9	0/9
	2	Sinneung-ri, Seoun-myeon, Anseong-si	Pear	176	1	0.6	0/9	0/9	0/9
	3	Ochon-ri, Seoun-myeon, Anseong-si	Pear	414	3	0.7	0/9	0/9	0/9
2021	4	Hwadang-ri, Baegun-myeon, Jecheon-si	Apple	584	1	0.2	0/9	0/9	0/9
	5	Myeongseo-ri, Sancheok-myeon, Chungju-si	Apple	458	1	0.2	0/9	0/9	0/9
	6	Deoknyeon-ri, Judeok-eup, Chungju-si	Apple	65	1	1.5	0/9	0/9	0/9
	7	Osaekdang-ri, Seonggeo-eup, Seobuk-gu, Cheonan-si	Pear	93	1	1.1	0/9	0/9	0/9
2022	8	Yangbyeon-ri, Miyang-myeon, Anseong-si	Pear	866	2	0.2	-	-	0/9
	9	Nowa-ri, Paengseong-eup, Pyeongtaek-si	Pear	132	2	1.5	-	-	0/9
	10	Sango-ri, Sinpyeong-myeon, Dangjin-si	Apple	196	3	1.5	-	-	0/9
	11	Namsan-ri, Sinpyeong-myeon, Dangjin-si	Apple	325	4	1.2	-	-	0/9
	12	Yeongok-ri, Gonjiam-eup, Gwangju-si	Pear	265	2	0.8	-	-	0/9
	13	Gwangjiwon-ri, Namhansanseong-myeon, Gwangju-si	Apple	251	2	0.8	-	-	0/9

for which closing was not essential, according to the guidelines for observations and control of fire blight provided by RDA. Instead of closing the orchards, only infected trees were removed. Soil samples from the orchards were collected and analyzed as described above. *E. amylovora* was not detected in any sample, regardless of region, zone, or repetition. The elapsed period after removing infected trees from the orchards was 27 months at the longest and 3 months at the shortest. This non-detection of *E. amylovora* was likely owing to the difficulty in long-term survival of *E. amylovora* in soil. German researchers have reported that *E. amylovora* in non-sterile soil decreased below the detection limit after 5 weeks (Hildebrand et al., 2001), which supports our results regarding the detection of *E. amylovora* in orchard soil. According to the official guidelines, at least 3 years must pass before a new tree can be planted on a site where an infected tree has been removed, and the soil must be confirmed to be free of *E. amylovora*. However, the monitoring results confirmed that *E. amylovora* is unlikely to survive in the soil for a long time. Therefore, the period required prior to replanting could be shortened to less than 2 years. In addition, the protocol established for the detection of *E. amylovora* in soil in this study could contribute to the rapid and accurate determination of planting suitability.

Conflicts of Interest

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

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