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Antagonistic Activity of *Bacillus* spp. Against Fire Blight Disease *In vitro* and *In planta*[#]

Haris Butt[1, a], Kubilay Kurtulus Bastas[1,b,*]

[1]*Department of Plant Protection, Faculty of Agriculture Selçuk University, 42250 Konya, Turkey*
[*]*Corresponding author*

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ABSTRACT

Fire blight, affecting more than one hundred and thirty species in the *Rosaceae*, is probably the most destructive disease affecting pear and apple cultivars in many countries. Currently, there are no effective synthetic compounds with systemic properties. Other major problem is the occurrence and spread of strains of *Erwinia amylovora* with resistance to streptomycin and copper. Taken into consideration the human and environmental health, the use of biocontrol agents either as an alternative or as a supplement within an integrated fire blight management strategy has attracted worldwide attention. In this study, *E. amylovora* solution of 10^7 CFU ml^{-1} was treated with bio-control agents, *Bacillus subtilis* str. QST 713, *B. amyloliquefaciens* str. MBI 600 and their mixture (at solution densities of 10^6, 10^7 and 10^8 CFU ml^{-1} for each one) on Petri dishes, containing King's B medium and, compared with positive (streptomycin sulphate) and negative (sterile distilled water) controls. *In vivo* studies were performed on two-year-old apple cv. Gala seedlings grown in 45-cm-diameter pots containing a sterilized mix of soil–sand–peat under controlled greenhouse conditions (85% relative humidity, 25°C temperature and 16h of day light). The plants were irrigated as needed by drip-irrigation and each pot received a mineral solution (NPK: 20–20–20) at 2 g l^{-1} twice. When plant shoots reached a length of 30-35 cm, bio-control agents, individually and their mixture, were applied to the plants by a hand-sprayer. Obtaining the data, 10^8 CFU ml^{-1} of *Bacillus* spp. suspension mixture showed strongest *in vitro* antibacterial effect (26mm) among the tested treatments after positive control streptomycin (28.6mm). Parallel to *in vitro* findings, the mixture was most effective against the pathogen on cv. Gala (66.03%). Findings show that the use of mixture of beneficial microorganisms with individual antagonistic properties against the pathogen can be an effective strategy as a natural alternative to agrochemicals in the scope of good agriculture practices.

[a] *harisbutt23@hotmail.com* *https://orcid.org/0000-0003-3068-2332* | [b] *kbastas@selcuk.edu.tr* *https://orcid.org/0000-0002-2367-1849*

Introduction

Major factors contributing to global crop losses include biotic stresses such as plant pathogens and pests. Devastating epidemics occur as a result of these pathogens proliferating in plants which severely affect food production especially in developing countries with limited resources. Currently, plant pathogens account for an alarming 8-40% of global yield losses (Ntushelo et al., 2019). Among such economically important pathogens is the Gram-negative enterobacterium *Erwinia amylovora* (Burr.), responsible for a devastating fire blight disease of apple and pear and also infecting other genera of the Rosaceae family. *E. amylovora* was the first bacterium to be identified as a plant pathogen (Bastas, 2016) and it ranks among the most destructive top 10 plant pathogenic bacteria in the world (Borruso et al., 2017). In Turkey, fire blight was first reported in the year 1985 in a pear orchard in Sultandağı district of Afyon province (Öktem and Benlioğlu, 1988) and rapidly spread to other regions within a single growing season. Primarily, the bacterium enters the host through flowers, natural openings or injuries on vegetative parts and moves internally to other sites causing blossom, shoot, and rootstock infections or blight (Peil et al., 2009). Either blossoms, shoots, or the rootstock can show blight symptoms leading to severe economic losses to varying extents. Unintended trade of latently infected plants is responsible for its spread to distant ecological areas, while wind, rain, and pollinating insects disseminate the pathogen to nearby regions (Pester et al., 2012).

The susceptibility of commercial cultivars of the domesticated apple (*Malus domestica* Borkh.) is very high with some cultivars showing low susceptibility (Sobiczewski et al., 2011). In the early 2000s, $20-$100

million were reported in economic losses due to fire blight outbreaks in pome fruit species (Müge et al., 2020). Destruction of trees to minimize the spread of the pathogen and application of antibiotics in controlling disease in the United States were responsible for such huge losses. Since the use of antibiotics in managing plant diseases is either completely forbidden or regulated strictly in Europe, production costs are mainly responsible for such economic damages (Emeriewen et al., 2019).

Copper compounds are mainly used to control plant disease but the effectiveness of these compounds is insufficient in apple disease management and are known to have negative effects on human and animal health, on the environment and are also phytotoxic to plants. Streptomycin is the most effective chemical against fire blight disease, but it has a short effectiveness period because of the development of resistant strains (Bastas, 2020). Europe has banned the use of antibiotics in apple and pear orchards to eliminate the traces of antibiotics contaminating foods and to minimize the resistance development in bacteria. In addition, its use has been banned by National Organic Standards Board in the United States in organic pear and apple orchards in 2014 (Dagher et al., 2020). To combat fire blight disease, the development of alternative strategies is inevitable that should be eco-friendly and ethically responsible in comparison to the use of hazardous copper compounds and antibiotics. Biological control of plant diseases is one of such practices that is achieved by the use of beneficial microorganisms with antagonistic properties against pathogens, and such microorganisms are referred to as the biological control agent (BCA). The use of BCA either as an alternative to synthetic chemicals or as a supplement has attracted the world to be included in integrated pathogen management programs in different food systems. Various species of different microorganisms such as bacteria, fungi, and viruses have been reported to be beneficial against pathogens. The genus Bacillus among bacterial antagonists has many species reported for their antagonistic relationship with the pathogens, and the number is increasing rapidly (Shafi et al., 2017). Secretion of various cell-wall degrading enzymes by *Bacillus* spp. such as protease, cellulase, chitosanase, glucanases, lipopeptides, and hydrogen cyanide holds the key to inhibit the growth and development of many plant pathogenic bacteria, fungi, and viruses. *Bacillus*-induced physiological changes, such as the activation of the antioxidant and defense systems resembles the harmful effects of pathogens on crops. Interaction between *Bacillus* and plants triggers plant defenses against infections by influencing resistance genes, proteins, phytohormones and metabolites (Butt and Bastas, 2022). Due to their biofertilizer and biocontrol properties, the popularity and importance of these BCA is gradually increasing to be used as a natural alternative to synthetic pesticides and other agrochemicals (Qiao et al., 2014). Commercially available *B. subtilis* based biopesticides include Avogreen, Ballad, Bio safe, Biosubtilin, Cease, Companion, Ecoshot, FZB 24WG, HiStick N/T/Subtilex/Pro-Mix, Kodiak, Rhapsody, Rhizo Plus, Serenade and *B. amyloliquefaciens* based products are RhizoVital 42, RhizoVital 42 TB (Fira et al., 2018) and Serifel.

Synergistic interactions between more than one lipopeptide family produced by many strains of the *Bacillus* species can enhance their biocontrol capacity. Investigations regarding additive, antagonistic, or synergistic effects of different lipopeptides produced simultaneously from particular *Bacillus* spp. are scarce and need further studies as the mixture of different lipopeptides is considered beneficial in plant protection (Butt and Bastas, 2022). Hence the aim of this study was to evaluate the *in vivo* and *in vitro* effect of individual and in mixture applications of the strains *B. subtilis* QST 713 and *B. amyloliquefaciens* MBI 600 against *E. amylovora* on apple cv. Gala.

Material and Methods

Plant Material

Two-year-old apple cv. Gala seedlings were grown in a greenhouse in 45-cm-diameter pots containing a sterilized mix of soil–sand–peat (2:1:1 by volume) under controlled greenhouse conditions (85% relative humidity, 25±2°C temperature and 16h of day light). The plants were irrigated as needed by drip-irrigation and each pot received a mineral solution (NPK: 20–20–20) at 2 g L^{-1} twice (Bastas, 2020).

Pathogen Strains and Growth Conditions

Highly virulent *E. amylovora* isolates, ArAdy5 (93%) and ArAdy7 (86%) used in the assays were obtained from culture collection of the Molecular Plant Bacteriology Laboratory, Department of Plant Protection, Selcuk University. The pathogens were grown in nutrient broth (NB) at 25±2°C. Bacterial suspensions were prepared with ArAdy5 and ArAdy7 isolates, 48-hour-old adjusted to 10^7 CFU ml^{-1} concentrations in a spectrophotometer (Eppendorph Bioplus, OD_{600}: 0.15). A mixture of these isolates was used in the experiment and maintained on the ice during the inoculations.

Bacillus Strains and Growth Conditions

B. subtilis QST 713 and *B. amyloliquefaciens* MBI 600 were obtained from culture collection of the Molecular Plant Bacteriology laboratory, Department of Plant Protection, Selcuk University and were grown in LB agar medium for 24hr at 30±2°C before any experimental use.

In vitro studies

Evaluation of the pathogen inhibition by Bacillus spp.

The antibacterial effect of *B. subtilis* str. QST 713 and *B. amyloliquefaciens* str. MBI 600 on *E. amylovora* was investigated *in vitro* using dual culture method described by Almoneafy et al. (2012). The suspension of *E. amylovora* containing 10^7 CFU ml^{-1} populations was spread to 9.0 cm diameter petri dishes containing King's B medium. Individual and mixed suspension of test antagonist strains at 10^6, 10^7 and 10^8 CFU ml^{-1} dilutions were transferred to the center of agar plates using sterile toothpicks. Streptomycin sulphate and sterile water were used as positive and negative control, respectively. Three replications per treatment were setup and the petri dishes containing the pathogen, *Bacillus* spp. and controls were incubated at 26±2°C for 48 hours. The inhibition zones formed around tested isolates as clear haloes were measured and noted in mm.

In planta experiments

Most effective concentration (10^8 CFU ml^{-1}) of *Bacillus* spp. observed in *in vitro* assays in preventing *E. amylovora* was used in *in planta* experiments. When plant shoots reached a length of 30-35 cm, bio-control agents, individually and their mixture, were applied to the plants by a hand-sprayer. The apple seedlings were sprayed with bacterial suspensions twice prior to pathogen inoculation with 7 days interval. The plants were kept at 85% RH, 25±2°C and 16h of day light in greenhouse. Twenty-four hours after the second application, the density (CFU ml^{-1}) of *Bacillus* spp. on host leaves were determined according to Yadav et al. (2010).

Inoculum was prepared from 48-h-old pathogen cultures grown on LB agar medium at 25±2°C. The inoculum density was adjusted to 10^7 CFU ml^{-1} in sterile distilled water (SDW) using spectrophotometer (Eppendorph Bioplus, OD_{600}: 0.15). Shoots were inoculated, after 24 hours of second treatment with *Bacillus* strains, by transversally bisecting the two youngest actively growing leaves and dipped for 30 sec in suspension mixture of isolates ArAdy5 and ArAdy7. The treated shoots were labeled with flagging tape for evaluation purposes (Bastas, 2020).

Experimental Design

In planta experiment was set up in a completely randomized block design with three replicates. A single replicate was a mean from nine shoots on three saplings.

Evaluation of Disease Severity and Treatment Effectiveness On Apple Cv. Gala

When the fire blight lesions ceased to extend, the length of visible necrosis and healthy shoot lengths were recorded. Percent disease severity (DS) was calculated using the following formula:

$$DS\ (\%) = (a / b) \times 100$$

where 'a' is the length of the blighted part of the shoot (cm), and 'b' is the whole length of the shoot (cm) (Aldwinckle et al., 2001; Fernando and Jones, 1999).

Percent effectiveness of the treatments (A) was calculated according to the following formula (Abbott, 1925):

$$A\ (\%) = (B-C) / B \times 100$$

where 'B' is the percent disease severity in the controls, and 'C' is the percent disease severity in treated shoots.

Statistical Analysis

MINITAB ver. 18 program was used for variance analysis and statistical evaluations were done with Tukey multiple comparison test in the MSTAT program to analyze the data obtained (Düzgüneş et al., 1987). Significant differences between the two mean values were computed by their significant levels at $P \leq 0.05$.

Results

In vitro Studies

Obtaining the data, the mean values of the inhibition zones ranged from 2.5 mm to 26 mm (Table 1). The inhibition zones expanded with increasing concentration of the *Bacillus* spp. and the maximum inhibition zone (26 mm) were obtained with 10^8 CFU ml^{-1} solution mixture of both *Bacillus* strains with no significant difference to 28.6 mm inhibition zone formed by streptomycin (Table 1 and Figure 1). It was followed by *B. subtilis* str. QST 713 (19.6 mm) at 10^8 CFU ml^{-1}. The lowest inhibition zones of 2.5 mm, 3.8 mm and 4 mm were obtained by 10^6, 10^7 and 10^8 CFU ml^{-1} concentrations of *B. amyloliquefaciens* MBI 600, respectively.

In planta Experiments

After two treatments of the leaves of cv. Gala with *Bacillus* spp., the highest bacterial population density was obtained with the mixture of *Bacillus* strains (1.2×10^9 CFU ml^{-1}), followed by str. QST713 (8.5×10^7 CFU ml^{-1}) and str. MBI600 (2.9×10^7 CFU ml^{-1}) (Table 2 and Fig. 2).

Parallel to *in vitro* findings, the mixture of *Bacillus* strains was most effective against the pathogen when applied to the apple cv. Gala (66.03%) followed by individual treatments with *Bacillus subtilis* str. QST713 (54.75%) and *B. amyloliquefaciens* str. MBI 600 (47.01%) (Table 2 and Figure 3). All the treatments were significantly different from each other at $P \leq 0.01$.

Table 1. *In vitro* antibacterial effects of *Bacillus* spp. against *E. amylovora* by dual culture method

Treatments	Concentration (CFU ml^{-1})	Inhibition Zone (mm)
Positive Control (Streptomycin)	-	28.6^{a}
Negative Control (SDW)	-	0.0^{e}
Bacillus subtilis QST 713	10^6	10.16^{c}
	10^7	12.6^{c}
	10^8	19.6^{b}
B. amyloliquefaciens MBI 600	10^6	2.5^{de}
	10^7	3.8^{d}
	10^8	4.0^{d}
Bacillus subtilis QST 713+ *B. amyloliquefaciens* MBI 600	10^6	13.3^{c}
	10^7	18.3^{b}
	10^8	26.0^{a}

*The same letters next to the mean values (n = 3) in the column indicate that the difference between the applications is not statistically significant (Tukey multiple comp. test, $P \leq 0.05$)

Table 2. Disease severity (%) and the efficacy (%) of *Bacillus* spp. against fire blight disease on apple cv. Gala seedlings

Treatments	*Bacillus* spp. on apple cv. Gala leaves after 2nd application (CFU ml^{-1})	Disease Severity (%)	Effectiveness of Treatment (%)
Bacillus subtilis QST713	8.5×10^{7b}	45.25^{b}	54.75^{c}
B. amyloliquefaciens MBI600	2.9×10^{7c}	52.99^{b}	47.01^{c}
B. subtilis QST713+*B. amyloliquefaciens* MBI600	1.2×10^{9a}	33.97^{c}	66.03^{b}
Control (Streptomycin)	-	19.86^{d}	80.14^{a}
Control (Water)	-	100.0^{a}	-

*The same letters next to the mean values (n = 3) in the column indicate that the difference between the applications is not statistically significant (Tukey multiple comp. test, P≤0.05)

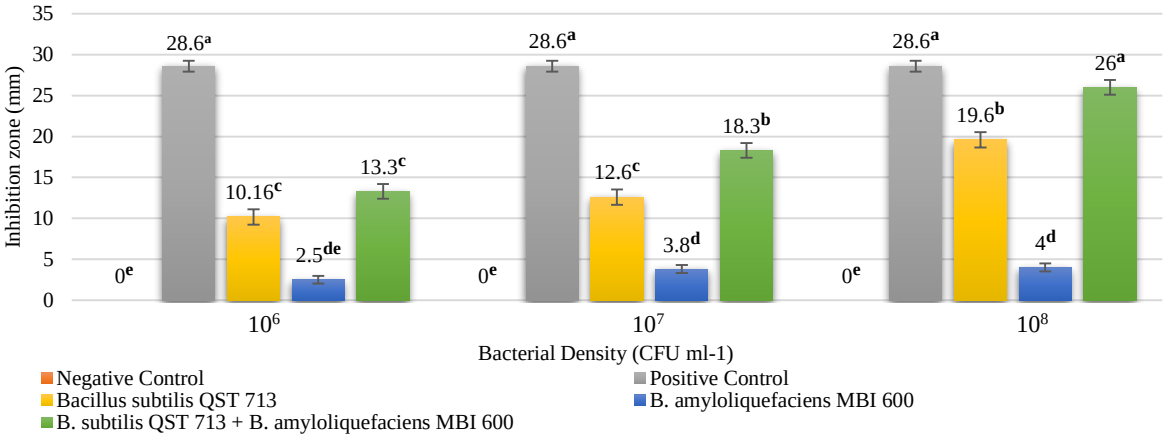

Figure 1. *In vitro* antagonistic effects of *Bacillus* spp. against *E. amylovora* by dual culture method

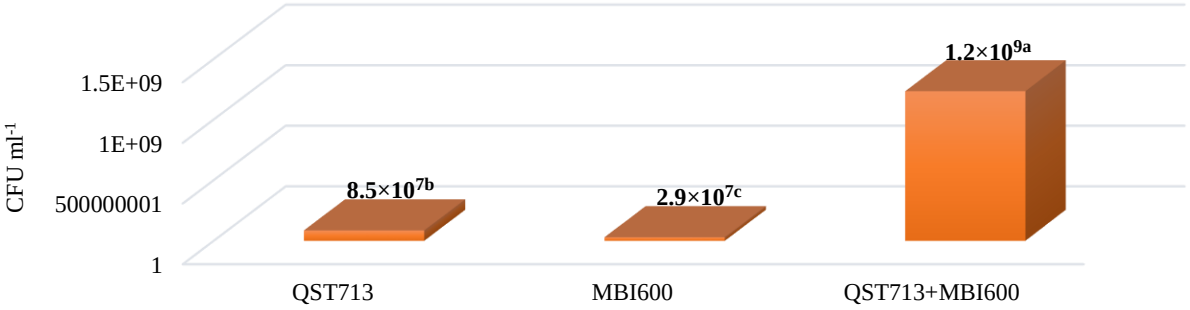

Figure 2. *Bacillus* densities (CFU ml^{-1}) recovered from apple cv. Gala leaves after second application.

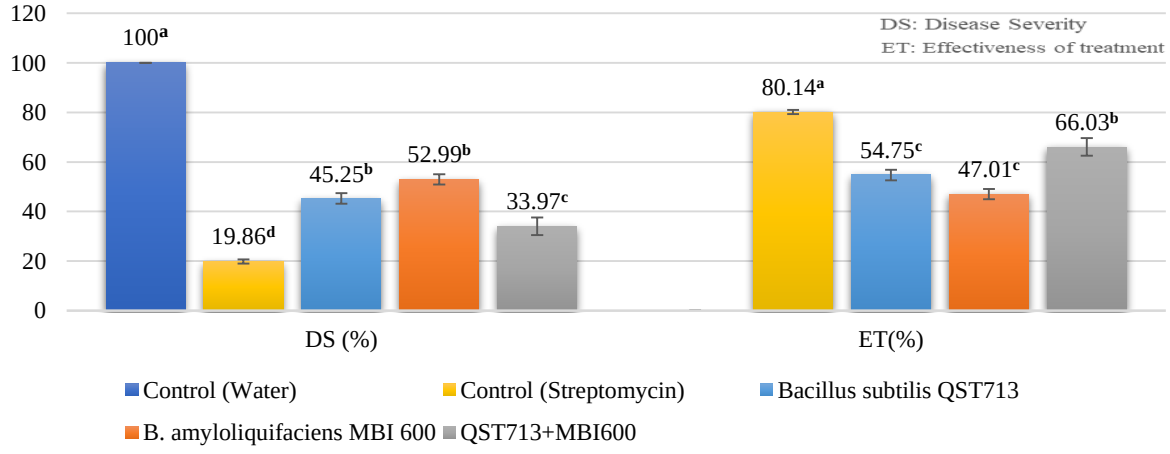

Figure 3. Efficacy of *Bacillus* spp. against *E. amylovora* and disease severity on apple cv. Gala

Discussion

E. amylovora ranks among the top 10 plant pathogenic bacteria in the world (Borruso et al., 2017), and it cause losses of more than $100 million per year (Norelli et al., 2003; Müge et al., 2020). Commercial production of apple and pear is still challenged by this devastating pathogen throughout the world (Ngugi et al., 2011). As the current control practices are ineffective or insufficient and are not reliable, fight against fire blight outbreak is still very difficult (Sundin et al., 2009). The use of bacteria as biological control agents (BCAs) has received more attention due to their versatile modes of action in protecting plants and their potential to be a part of an integrated control strategy (Bahadou et al., 2017). Having the capability to minimize the applications of hazardous chemical compounds, this alternative can provide a powerful and eco-friendly control of this bacterial threat (Finkel et al., 2017: Bahadou et al., 2017).

Many strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*, and the others have been reported to interact with host plants and produce beneficial effects including fire blight disease suppression (Choudhary and Johri, 2009; Kabeil et al., 2010; Gerami et al., 2013; Rosello et al., 2013). *B. subtilis* is the most studied biocontrol agent, which has been reported as a growth promoter and as antagonistic to a variety of pathogens *in vitro* and in greenhouse and field studies. *B. subtilis*, as a disease suppressor, adopt various mechanisms such as antibiosis, lysis of pathogen hyphae, competition for space and nutrients, plant growth promotion (PGP), and induced systemic resistance (ISR). *Bacillus subtilis* str. QST713 (Serenade) is one of the registered and currently available biopesticide to protect apple and pear trees during blooming (Mikicinski et al., 2016). *B. amyloliquefaciens* associated with plants is known for their indirect support to plant growth as it can suppress the disease by producing a variety of enzymes and secondary metabolites such as chitinase involved in microbial antagonism. In addition, development of plant resistance was enhanced by *B. amyloliquefaciens* FZB24 against competitive pathogenic bacteria and fungi (Jamal et al., 2018).

Strains of *B. amyloliquefaciens* have been reported to be 95% effective against *E. amylovora* in some apple cultivars (Bahadou et al., 2018). In another work, commercial strain of *B. subtilis* str. QST713 was 64.3% efficient in controlling blossom blight against fire blight (Aldwinckle et al., 2018). Chen et al. (2009) has proposed the use of *B. amyloliquefaciens* strains with enhanced synthesis of difficidin and/or bacilysin for development of efficient biocontrol agents against fire blight disease. Antagonistic activity was shown by *B. amyloliquefaciens* subsp. *plantarum* str. FL50S against *E. amylovora* both *in vitro* and *in planta* (Dagher et al., 2021). Synthesis of several cyclic lipopeptides (cLPs) genes like bmyB (bacillomycin), ituC (iturin), srfAA (surfactin), and fenD (fengycin) by strains of *B. subtilis* and *B. amyloliquefaciens* is key to their strong antibacterial activity against *E. amylovora* and several other pathogens (Mora et al., 2015).

In our experiment, *in vitro* and *in planta* effectiveness of two different *Bacillus* strains, individually and in mixture, was evaluated for the first time against fire blight disease in Turkey. In addition, to the best of our knowledge, there is no report in the literature of strains of *B. subtilis* and *B. amyloliquefaciens* being used as a mixture against *E. amylovora*.

Our results indicate that the effectiveness of the mixture of the *Bacillus* strains against *E. amylovora* is significantly high, in both *in vitro* and *in planta* experiments, then the individual treatments. Interestingly, the population density of *Bacillus* spp. recovered from the leaves of mixture treated apple cv. Gala was greatly higher than the individual treatments. This might be the result of possible synergistic relationship between the two that help them thrive way more efficiently in comparison to their individual survival. Furthermore, *in planta* effectiveness of str. QST 713 against the pathogen was in parallel to the *in vitro* findings as expected. On the other hand, an unexpected high *in planta* effectiveness was shown by the str. MBI 600 as compared to its very low ability to inhibit the pathogen growth *in vitro*. Probable reason can be the activation of host plant defenses against *E. amylovora* by the str. MBI 600 as it came in contact with the host.

The application of BCAs, at least 24 h before the arrival of the pathogen has always been more successful. This gives enough time to the antagonist to fight the pathogen by establishing its own niche first in the site of infection. However, eco-physiological traits of BCAs must be powerful enough to survive under varying field conditions and competitors' indigenous microflora as various factors are continuously altering the environmental conditions. Consequently, numerous works have emphasized the importance of assessing the performance of BCAs under conditions close to field in order to improve their fitness and effectiveness (Daranas et al., 2018). In order to give sufficient amount of time to the *Bacillus* species to colonize the plant, pathogen inoculation was carried out 24 hours after the last application of test bacteria, and in accordance with the literature report, it obtained more successful results than the pathogen and Bacillus applications at the same time (data not given in this study).

It is known that isolates of the pathogen used in our study as a mixture were highly virulent. In connection with this, it seems natural that our results in preventing fire blight using *Bacillus* spp. have lower results than other studies. Whereas higher and more successful results are expected under natural infection conditions. This should be tested in regions with different climatic and ecological conditions and with different strains of the pathogen.

Investigations regarding additive, antagonistic, or synergistic effects of different *Bacillus* spp. are scarce and need further studies as the mixture of different lipopeptides from *Bacillus* spp. is considered beneficial in plant protection (Butt and Bastas, 2022). Further studies are required using two or more different bacterial antagonists at different concentrations, under different ecological conditions, specially under field conditions in order to find more effective combinations of BCAs against different *E. amylovora* strains.

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