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Effectiveness of Phosphorous acid, *Bacillus subtilis* and Copper Compounds on Apple cv. Gala with M9 Rootstock in the Control of Fire Blight

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ABSTRACT

Necrogenic Gram-negative bacterium *Erwinia amylovora*, which causes economic losses especially in pome fruits such as apple, pear, quince and loquat, some berries and many ornamental plants, causes fire blight disease. Copper compounds are used extensively in disease control programs and they can cause toxicproblems in terms of plant and environmental health. In addition, the formation of resistance to copper in the pathogen is frequently observed. In this study, plant activator phosphorous acid and biological control agent *Bacillus subtilis*, and 3 different copper compounds, Copper sulfate, Copper oxychloride and Copper hydroxide which are commonly used against *E. amylovora*, on apple cv. Gala with M9 rootstock were evaluated comparatively. When the new season shoot lengths of 3-year-old plants with homogeneous growth reached 20-25 cm, chemicals and *B. subtilis* were applied first time before one week ago from the pathogen inoculation, and after 2 times with 1 week intervals. The youngest two leaves at tips of actively growing terminal plant shoots were inoculated by cutting off using scissors dipped in suspension of *E. amylovora* str. EaARADY5 containing 10^8 CFU ml^{-1}. Disease assessments were made after the disease severity (%) was determined on the basis of shoot blight after symptom development of the disease stopped, and the results were found to be statistically significant ($p<0.05$). According to the findings, while the highest effect was obtained by copper oxychloride with 69% effectiveness among all applications, the lowest effectiveness was obtained with 43.5% copper sulfate. It has been revealed that the use of the most effective of the copper compounds in field applications will result in less exposure to chemicals in terms of human and environmental health, and that *B. subtilis* and phosphorous acid can be used significantly in the integrated control of fire blight.

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Introduction

Apple (*Malus* x *domestica* L.) is the third most widely produced fruit worldwide, following bananas and grapes (FAO, 2018). In Turkey, it accounts for approximately 4% of global apple production, with a total of 3.6 million tons (Anonymous, 2020). The highest production area in Turkey is in Niğde, with 235,150 hectares dedicated to apple cultivation. Niğde, Isparta, Karaman, Antalya, and Konya provinces collectively constitute 53.4% of Turkey's total apple production areas, covering approximately 912,000 hectares (Anonymous, 2020).

Diseases and pests lead to significant yield losses in apple production. Fire blight disease, caused by *Erwinia amylovora*, was first observed in the United States in 1780. The pathogen is a highly serious and destructive disease reported in more than 50 countries worldwide, causing significant issues, especially in apple trees and many ornamental plants belonging to the Rosaceae family (Zhao et al., 2019). The disease also causes symptoms of wilting and cankers in plants, in addition to shoot, leaf, and fruit blight. Fire blight leads to significant economic losses worldwide. While a permanent effective method for combating the disease has not been identified, every year, the size of infected apple and pear orchards decreases due to quarantine-related measures (Deckers and Schoofs, 2007). Controlling this disease is only possible by discovering and implementing the most suitable control methods.

Copper compounds, especially in countries like the European Union where antibiotic use is restricted, are still widely used in the fight against fire blight disease. This application is one of the most common methods to control bacterial plant diseases, but it has led to the development of various strategies by many bacteria against copper ions. To date, there is very little information about the interaction between *E. amylovora* and Cu^{2+} ions (Ordax et al., 2006).

Globally, various copper-based preparations with different compositions are used in the current chemical control programs for the disease. The limited use of effective copper compounds against the disease is becoming important due to issues such as the rapid development of resistance to copper preparations, resulting in a loss of effectiveness, harm to beneficial bacteria, and phytotoxicity (Saygılı, 2008).

Plant activators also provide protection against bacterial diseases in addition to fungal diseases (Yüce et al., 2020). Phosphorous acid is rapidly absorbed by the plant and can be transported to all parts of the plant through the phloem and xylem, thereby strengthening the plant's immune system.

Bacillus subtilis is a commonly used biological control agent in the management of bacterial diseases (Abbasi and Weselowski, 2014; Fousia et al., 2016; Ibrahim et al., 2016). It has been reported that *B. subtilis* induces systemic acquired resistance (SAR) in plants (Borriss, 2011).

In this study, in addition to determining the most effective copper compound in Gala apple variety with M9 rootstock against fire blight disease and allowing the use of a small number of copper compounds in terms of human and environmental health, the usability of *B. subtilis* and phosphorous acid applications in integrated fire blight control programs was investigated.

Materials and Methods

Materials

Three-year-old apple cv. Gala seedlings with M9 rootstock, known to be susceptible to *Erwinia amylovora*, were used as the plant material for this study.

The *E. amylovora* str. EaARADY5 was provided from the Molecular Bacteriology Laboratory collection of Selçuk University, Department of Plant Protection. The biological agent, copper compounds and phosphorous acid used in the experiment were obtained from commercial companies (Table 1).

Methods

In vitro experiments

The chemicals provided in Table 1 were added to Nutrient Agar (NA) medium at the specified doses and intensities after autoclaving and cooling. The pathogen suspensions were prepared from 48-hour fresh cultures of *E. amylovora* str. EaARADY5 at a concentration of 10^8 CFU ml^{-1}, determined by measuring an optical density of 0.15 at 600 nm using a spectrophotometer (Eppendorph Bioplus). These suspensions were then spread on NA medium containing chemicals compounds used in the experiments.

The application of *B. subtilis* was carried out after streaking the *E. amylovora* str. EaARADY5 onto Petri dishes.

After incubating the Petri dishes at 27°C for 48-72 hours, the bacterial population densities were determined by counting the colonies that developed on the medium following the method described by Klement et al. (1990).

Bacterial population densities (cell ml^{-1}) = NC × DS × 10

(NC; number of the colony, DS; dilution series)

In vivo experiments

The experiments were conducted under controlled greenhouse conditions with a temperature of 23-25°C and 80% relative humidity, following a 16-hour light / 8-hour dark cycle. The potting soil for the plants was prepared as a mixture of soil, composted animal manure, and peat in a 1:1:1 ratio. During the growing season; the plants were fertilized with NPK (20,20,20) once time to growth healthy, and sulfur (WP, 40g L^{-1}) was applied twice to protect against powdery mildew disease.

Application of chemicals and biological agent

The chemicals and B subtilis used in the experiments were applied twice; first when the new season shoots of homogeneous growing Gala apples with M9 rootstock plants reached 20-25 cm in length, and the second three days after *E. amylovora* inoculation. The inoculation of *B. subtilis* was carried out by spraying the leaves of the plants with a pressurized hand sprayer, prepared from a 48-hour fresh culture with a concentration of 10^8 CFU ml^{-1}, as measured by a biophotometer (Eppendorph bioplus, OD: 660 nm; 0.15). Chemicals were applied to the leaves of the plants using a pressurized hand sprayer at the recommended doses by the manufacturer.

Pathogen inoculation

Inoculation of the pathogen was performed by cutting the two youngest leaves at the tips of actively growing terminal plant shoots, using sterilized scissors dipped in a suspension of *E. amylovora* str. EaArady5 containing 10^8 CFU ml^{-1}. Inoculations were carried out one week after the initial applications. The surfaces of the inoculated shoots were covered with a polyethylene bag for the first 24 hours following inoculation (Bonasera et al., 2006).

Re-isolation of the pathogen

Before disease assessments were made, in accordance with Koch's postulates, pathogen re-isolation and diagnosis were conducted using samples taken from shoots displaying disease symptoms (Koch, 1884). The diagnosis of the pathogen was carried out through biochemical, morphological, and physiological tests according to Schaad et al. (2001).

Table 1. Chemicals, biological agent, formulations, application method, and usage rates used in the experiments

Application	Commercial Name	Formulation	Application	Usage Dosage
Erwinia amylovora	-	S	leaf	10^8 CFU ml^{-1}
Copper sulfate	Mastergold	SC	leaf	125 ml / 100 liters of water
Copper oxychloride	Cuprocol	SC	leaf	150 ml / 100 liters of water
Copper hydroxide	Kocide 2000	WG	leaf	175 g/ 100 liters of water
Phosphorous acid	Fosfogard	SC	leaf	200 ml / 100 liters of water
Bacillus subtilis QST713	Serenade	S	leaf	10^8 CFU ml^{-1}

Table 2. The effects of copper compounds (copper hydroxide, copper oxychloride and copper sulfate), phosphorous acid, and *Bacillus subtilis* QST713on bacterial populations *in vitro*.

Application	Bacterial growth (CFU ml^{-1})
Erwinia amylovora (Control)	1.60×10^6
Phosphorous acid	1.50×10^4
Bacillus subtilis QST713	1.40×10^2
Copper sulfate	1.20×10^1
Copper hydroxide	0.20×10^1
Copper oxychloride	0.19×10^1

Table 3. Determination of disease severity and effectiveness of phosphorous acid, *Bacillus subtilis*, and commonly used copper compounds against *Erwinia amylovora*.

Application	Disease Severity (%)	Effectiveness of the Treatment (%)
Erwinia amylovora (Control)	70.67 a	-
Copper sulfate	39.86 ab	43.59
Bacillus subtilis QST713	38.94 ab	44.89
Phosphorous acid	32.75 ab	53.65
Copper hydroxide	32.18 ab	54.46
Copper oxychloride	21.72 b	69.26

These tests included growth at 36°C, gelatin hydrolysis test, KOH test, growth in 5% SNA, fluorescence pigment formation on King B medium, oxidative-fermentative test, reduction of compounds from sucrose, esculin hydrolysis, cysteine H2S production, and acid production from carbohydrates.

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

(DS = disease severity, a = length of the blighted of the shoot (cm), b = total length of the shoot (cm))

The percentage effectiveness of the applications used in the experiments was determined using the Abbott (1925) formula, shown in the following lines:

$$E\ (\%) = (K – U / K) \times 100,$$

(E = effectiveness, K = percentage disease severity of the control plant, U = percentage disease severity of the treated plant).

Molecular diagnosis of the pathogen using specific primer base pairs A/B (A: 5'- CGGTTTTTAACGCTGGG-3' and B: 5'- GGGCAAATACTCGGATT- 3') and using the PCR protocol (at 95°C for 3 sec (1 cycle), followed by 94°C for 1 sec, 52°C for 1 sec, and 72°C for 2 sec (35 cycles), a final extension at 72°C for 10 sec (1 cycle)) suggested by Bereswill et al. (1992) was performed by a thermal cycler (Eppendorph Personal). The PCR products were evaluated by using a Prizma imaging device after electrophoretic seperation (Russell and Sambrook, 2001).

Evaluation of treatments

After disease symptoms ceased (on the 30th day after the pathogen inoculation), the effects of the treatments on the disease severity percentage (DS, %) were determined by dividing the length of necrotic tissue by the total shoot length, as shown in the following formula (Fernando and Jones, 1999; Aldwinckle et al., 2002).

Statistical Analyses

The data obtained from the study were subjected to variance analysis using the MINITAB version 14 program, and statistical evaluations were conducted using the MSTAT program, where the Tukey multiple comparison test was applied to determine the interactions between the treatments and the disease (Düzgüneş et al., 1983).

Results

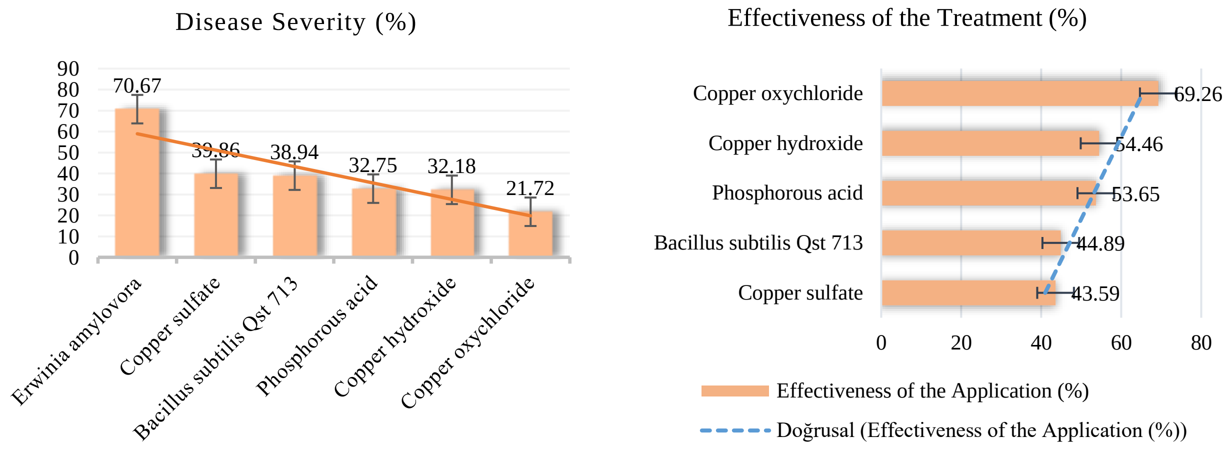
In this study, the effectiveness of copper sulfate, copper oxychloride, copper hydroxide, phosphorous acid, and *Bacillus subtilis* QST 713 against fire blight disease, which significantly impacts the yield and quality of Gala apple trees on M9 rootstock, was determined under *in vitro* and *in vivo* conditions.

In vitro experiments

The copper compounds (copper hydroxide, copper oxychloride, and copper sulfate) used in the experiments, phosphoric acid and *B. subtilis* were used at commercial application doses to determine the development of *E. amylovora* bacterial populations on NA medium and data were shown in Table 2. Accordingly, the most successful copper compounds were identified as copper hydroxide and copper oxychloride, while the lowest effectiveness was obtained by phosphorous acid on fire blight.

In vivo experiments

Copper-based compounds, phosphorous acid and *B. subtilis* were applied under in vivo conditions according to the dosages recommended by commercial companies, and the percentage of disease severity caused by *E. amylovora* on apple cv Gala with M9 rootstock plants was determined. According to the findings, the highest efficacy, with a 69% effectiveness, was achieved with copper oxychloride, statistically, all treatments (copper sulfate, copper hydroxide, phosphorous acid and *B. subtilis* QST713) had the close to effectiveness, despite numerical differences (Table 3; Figure 1; Figure 2).



a b

Figure 1. Disease severity and effectiveness of phosphorous acid, *Bacillus subtilis* QST713, and commonly used copper compounds (Copper sulfate, Copper oxychloride, Copper hydroxide) against *Erwinia amylovora* (a) disease severity (%) and (b) effectiveness of the treatments (%).

a b c

d e f

Figure 2. Shoot blight caused by *Erwinia amylovora* on apple plants cv. Gala with M9 rootstock, a) Control plants with only pathogen inoculation, Plants treated with b) Copper oxychloride, c) Copper sulfate, d) Copper hydroxide, e) Phosphorous acid, f) *Bacillus subtilis* QST713.

Table 4. Results of biochemical and molecular diagnostic tests conducted for the re-isolates.

Tests	RI	RZ
Gram reaction	G(-)	G(-)
Growth at 36°C	-	-
Hydrolysis of gelatin	+	+
Levan formation	+	+
Fluorescent pigment on KB agar	-	-
Oxidative-Fermentative Test	+	+
Esculin hydrolysis	-	-
H_2S production from Cysteine	-	-
Acid production from carbohydrates		
Sorbitol	+	+
Mannitol	-	-
Maltose	-	-
Tobacco hypersensitivity reaction (HR) test	+	+
PCR assay with specific A/B primers (1 kb)	+	+

RI: Reference isolate (ARADY5), RZ: Isolates obtained through re-isolation, (+) positive reaction, (-) negative reaction

Re-Isolation and Diagnosis of the pathogen

Bacterial isolations were conducted to confirm that the disease symptoms in the treated and control plants were caused by *E. amylovora*. The pathogen responsible for the disease was identified as *E. amylovora* through the biochemical, morphological, physiological, and molecular diagnostic tests provided in Table 4.

Discussion

One of the major quality and economic losses in apple production is caused by fire blight disease, which is attributed to *E. amylovora* (Deckers and Schoofs, 2007).

The management of the disease is quite challenging due to the lack of specific synthetic compounds that directly affect the pathogen (Myung et al., 2016). Additionally, *E. amylovora* can rapidly spread and develop resistance to antibiotics (McGhee and Sundin, 2011). Antibiotics not only have adverse effects on the environment and soil but also pose challenges in clinical medicine since commensal microorganisms serve as reservoirs for antibiotic resistance genes (McManus, 2014; Lamichhane et al., 2018). For these reasons, their use is banned in a lot of country.

It has been reported that the use of copper-based preparations in disease management can lead to the development of resistance in the pathogen and, at the same time, cause phytotoxicity in the plant (Saygılı, 2008). In Turkey, copper-based preparations, biological agents, and plant growth regulators are commonly used. Therefore, it is of great importance to limit the use of effective copper compounds against the disease to a minimum.

Copper compounds are effective bactericides in some areas, but they can lead to phytotoxic effects on leaves and fruits. Therefore, they are generally used in strategies combined with other compounds or reduced spraying programs, often applied before flowering (Ninot et al., 2002). Momol et al. (1991) reported that a combination of maneb and copper allowed for successful results without causing any phytotoxicity in plants when applied following the flowering period.

Saygılı and Üstün (1995) conducted research on the effectiveness of various chemicals against *E. amylovora in vitro* and on pear fruits. They determined that the most effective chemicals were, in order, streptomycin, copper salts + mancozeb, kasugamycin, and copper oxychloride + maneb. They also found that copper hydroxide was more effective than copper oxychloride, and copper sulfate showed lower activity compared to other copper compounds. In our study, we tested on apple plant shoots, and copper oxychloride was identified as the most effective copper compound with an effectiveness rate of 69.26%, while copper sulfate showed the lowest effectiveness at 43.59%. The difference in the results obtained with the copper hydroxide used in our study, which shows parallels with Saygılı and Üstün (1995), suggests that different results can be achieved by using different formulations of the chemical in different plants and different climates.

Recently, Butt and Bastas (2021) determined the antagonistic effect of Bacillus spp against Erwinia amylovora. They found that Bacillus subtilis QST713 had a success rate of 54.75%, while *B. amyloliquefaciens* MBI600 had a success rate of 47.01%. *Bacillus subtilis* QST713 has been reported to provide an average of 60% control and to yield the best results when used as part of an integrated program (Johnson and Temple 2013; Smith 2012, 2015). According to Aldwinckle et al. (2002), the commercial strain Bacillus subtilis QST713 had a success rate of 64.3% in controlling flower infections of fire blight disease. In our study, the Bacillus subtilis QST713 has become a success rate of 44.84%.

Considering all the results, it is thought that it may be beneficial to use *B. subtilis* together with other antagonistic factors after preliminary trials to increase the success of *B. subtilis* in the combat against fire blight.

Phosphorous acid, also known as phosphonic acid with the chemical formula H_3PO_3, is one of the colorless oxyacids of phosphorus. It is rapidly absorbed by plants and can be transported throughout the plant's various parts via the phloem and xylem, strengthening the plant's immune system. It can be used to prevent fungal diseases as well as bacterial diseases (Yüce et al., 2020).

Norman et al. (2006) tested the effectiveness of phosphorous acid against *Ralstonia solanacearum*, which causes bacterial wilt in geranium (*Pelargonium hortorum*). They found that commonly used phosphorus-containing products in the industry, such as phosphorus pentoxide (P_2O_5) and phosphoric acid (H_3PO_4), were unable to protect the plant from infection. In contrast, they determined that phosphorous acid (H_3PO_3) was successful (Norman et al., 2006).

The phosphorous acid used in our study caused similar successful results with some copper compounds and *B. subtilis* applications. These results suggest that phosphorous acid will be included in programs that can be applied both in plant development and in the combat against fire blight disease.

This study has demonstrated that copper oxychloride and copper hydroxide stand out due to their success in field conditions and they can be used alternately and fewer in numerical terms in combat against the disease. Additionally, it highlights that *B. subtilis* and phosphorous acid can be significantly employed in the integrated and eco-friendly control of fire blight.

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