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INVESTIGATION OF RESISTANCE OF SOME LETTUCE VARIETIES AGAINST LETTUCE BACTERIAL LEAF SPOT DISEASE AGENT *Xanthomonas hortorum* pv. *vitians* AND THE SUSCEPTIBILITY OF THE DISEASE AGENT TO COPPER

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Abstract: Bacterial Leaf Spot (BLS), caused by *Xanthomonas hortorum* pv. *vitians* (*Xhv*), is a significant disease affecting lettuce cultivation and causing crop losses. In this study, eight lettuce varieties were tested against the most virulent strain (HBXcv1) of *X. hortorum* pv. *vitians* using two inoculation methods: hand sprayer and needle syringe. The symptoms appearing on lettuce plants ten days after bacterial inoculation using the spraying method were evaluated. The varieties' tolerance levels were determined using the Duncan Multiple Range Test (DMRT), with Carmesi RZ Lolo being the most tolerant variety (DMRT mean of 0.25 at 5% significance level) and Cospirina being the most susceptible variety (DMRT mean of 216.55 at 5% significance level). After bacterial inoculations using a syringe with a needle, similar reactions were observed in lettuce as those obtained from spray inoculations. Among different lettuce varieties, Carmesi RZ Lolo showed the most tolerance to the effect of HBXcv1. No symptoms were found in lettuce leaves after 10 days of inoculation. On the other hand, Cospirina, Nun 06118, and Presidential lettuce varieties were determined to be sensitive to HBXcv1. In the second part of the study, the susceptible level of *Xhv* HBXcv1 strain to different copper sulfate ($CuSO_4$) concentrations was investigated using Nutrient Agar and Casitone Yeast extract agar. HBXcv1 strain was found to be resistant to 50ppm CuSO4 and susceptible to 100ppm and 200ppm. These results contribute to understanding the response of lettuce varieties to BLS. It has been found that copper-containing mixtures may be effective in the chemical control of *Xhv* infections.

Keywords: *Xanthomonas hortorum* pv. *vitians,* Copper sulfate, Resistance, Susceptible, Lettuce

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1. Introduction

Lettuce (*Lactuca sativa* L.), is one of the most important leafy vegetables worldwide due to its fast growth and commercial value, providing essential vitamins and minerals to human diets. In Türkiye and most parts of the world, lettuce is widely consumed raw in salads and in sandwiches, while in some other countries, its stem or leaves are cooked. Lettuce, which is rich in vitamins and minerals, is beneficial for health, especially when consumed fresh. According to Pierce (1987) and Ryder (1979), 100 grams of lettuce contains 96% water, 13 calorie energy, 0.9 g protein, 0.1 g fat, 2.9 g carbohydrate, 6 mg vitamin C, 0.3 mg niacin, 0.06 mg thiamine (B1), 0.06 mg riboflavin (B2), 20 mg calcium, 22 mg phosphorus, 9 mg sodium, 0.5 mg iron and 175 mg potassium.

However, the growth and productivity of lettuce are often hampered by various plant diseases, including Bacterial Leaf Spot (BLS) caused by the pathogen *Xanthomonas campestris* pv. *vitians*, formally named *Xanthomonas hortorum* pv. *vitians* (*X. hortorum* pv. *virtians*) (Koike et al. 2018; Morinière et al. 2020). In 1916, a bacterium was initially discovered in South Carolina and identified as *Bacterium vitians* (Brown, 1918). However, when the genus was established, it was later renamed *Xanthomonas vitians* (Dowson, 1943). The disease thrives in warmer temperatures and high relative humidity. These conditions create an environment where the disease outbreaks can occur unpredictably, leading to a potential total crop loss. According to Pernezny et al. (1995) and Koike et al. (2018), a single outbreak of BLS can cause up to 100% crop loss.

To prevent the spread of BLS and protect lettuce crops, it is important to understand the defense responses of different lettuce varieties to *X. hortorum* pv. *vitians*. Host resistance is considered the most effective and recommended approach for managing BLS and can be used alongside other methods for integrated pest management (Şahin and Miller, 1998; Nicolas et al., 2018). The defense responses of plants against pathogenic bacteria are a complex process that involves a range of molecular, physiological, and biochemical changes. Many studies have investigated the defense mechanisms of lettuce against *X. hortorum* pv. *vitians* during the past few years and have shown remarkable outcomes (Robinson, 2003; Al-Saleh and Ibrahim, 2009; Zhang et al., 2018). In recent years, advances in molecular biology and genomics have enabled researchers to gain a deeper understanding of the molecular mechanisms underlying the defense response of plants against pathogenic bacteria. Studies have shown that plant hormones, such as salicylic acid and jasmonic acid, play a role in the defense response of lettuce against *X. hortorum* pv. *vitians* (Bari and Jones, 2009).

In lettuce, more than 30 polygenic resistance loci have been identified, and several major genes have been cloned and sequenced in recent years (Simko 2013; Christopoulou et al., 2015). The sequencing of the lettuce genome has led to the identification of a large number of genes encoding NB-LRR proteins, known as Resistance Gene Candidates (RGCs) (McHale et al., 2009). These RGCs are responsible for various responses to lettuce diseases such as BYL. Most major genes and quantitative trait loci for resistance are localized in regions of the lettuce genome containing clusters of RGCs. In lettuce, RGCs can be divided into 20 families based on sequence similarity. Among these, the RGC2 family is one of the largest, containing over 40 members located on chromosome 2 and providing resistance to many diseases (McHale, 2006). In a study by Hayes et al. (2014), a single dominant gene located on chromosome 2, called *Xar1*, was identified in the lettuce varieties La Brillante, Little Gem, and Pavane. This resistance gene was found to confer a high level of resistance to strain 1 of *X. hortorum* pv. *vitians*. Sandoya et al. (2019) also identified a quantitative trait locus (QTL) named *qXCR2.1* associated with resistance to *X. hortorum* pv. *vitians* in lettuce.

Chemical controls for BLS in lettuce fields are not practical or completely cost-effective due to the unpredictable nature of the disease (Hayes et al., 2014). Although growers may use combinations of chemicals, including copper biocides, these do not provide a substantial cure. The continuous use of copper compounds has caused most bacterial pathogens to mutate and develop resistance genes against antibiotics over the years. A study done by Richard et al. (2017) using a long-read PacBio RSII technology to fully sequence six strains, using one single-molecule real-time (SMRT) cell for each strain, proves that the bacteria involved in copper tolerance are generally located in plasmids of *Xanthomonas* strains. They discovered that two copper resistance systems, the copper-inducible chromosomal copABCD system, and a plasmid-born copLAB system, carried on a conjugative plasmid are primarily responsible for facilitating the spread of copper resistance traits within the cell and offering redundancy in copper detoxification pathways, ensuring the bacterium's survival in environments with varying copper levels (Richard et al., 2017). In natural environments, integrative conjugative elements (ICEs) may also facilitate the acquisition of copper resistance genes in *Xanthomonas* (Fan et al., 2022).

Recent studies have delved into gaining a better understanding of *X. hortorum* pv *vitians* resistance and susceptibility to copper across different vegetable crops by employing various agar media and laboratory protocols. Pohronezny et al. (1992) used plates of nutrient agar amended with 200 µg/mL of $CuSO_4{\cdot}5H_2O$ to examine the resistance of pepper strains of *X. axonopodis* pv. *vesicatoria* to copper. Employing this method, they discovered that 114 out of 118 strains collected from commercial pepper fields in Florida were resistant to copper.

A similar approach was adopted by Pernezny et al. (2008) to evaluate the copper resistance of *X. hortorium* pv. *vitians* strains. In their experiments, none of the 19 *X. hortorium* pv. *vitians* strains showed growth on modified Casitone-Yeast Extract (CYE) agar as well as on Nutrient Agar (NA) both supplemented with CuSO4 (aq).

The importance of this research lies in its potential to enhance the productivity of lettuce cultivation and reduce economic losses, particularly in Türkiye, by identifying lettuce varieties resistant to BLS disease. The high costs and limited effectiveness of chemical control measures against this disease emphasize that identifying and utilizing resistant lettuce varieties is the most effective and sustainable management approach. Thus, this research which was undertaken to evaluate the resistance of eight different lettuce cultivars against BLS caused by *X. hortorium* pv. *vitians* and the sensitivity of *X. hortorium* pv. *vitians* to copper sulfate can contribute to strategies aimed at preventing the spread of BLS and combating lettuce diseases. The findings obtained provide valuable information for lettuce growers, plant breeders, and researchers, aiding in the development of BLS control strategies and the appropriate use of copper mixtures in disease management. This research provides critical information for the effective management of BLS disease by addressing the potential for bacterial resistance development.

2. Material and Methods

2.1. Bacteria Strain Preparation

In a study conducted by Basim et al. (2017), bacterial leaf spot was observed on some leaves of lettuce seedlings grown in a field in Antalya and various PCR tests were performed to identify the pathogen. Seven bacterial strains were observed and isolated from "Cresidential" and "Caipira" lettuce varieties namely; HBXcv1, HBXcv2, HBXcv3, HBXcv4, HBXcv5, HBXcv6, and HBXcv7.

For this research, the most virulent bacteria strain HBXcv1 was stored in the laboratory of the Plant Protection Department of Akdeniz University Agriculture Faculty at an optimum temperature of -80°C. To test the pathogenic ability, the bacteria strain was inoculated on three different three-week-old lettuce varieties for one week and later extracted and stored in 30% aqueous glycerol at a temperature of -80°C. After a few weeks, the same process was repeated for the second consecutive time. On these two separate occasions, it was observed that the lettuce varieties inoculated displayed symptoms of BLS lesions.

The preparation of inoculum was similar to that described by Nicolas et al. (2018). The bacteria was streaked on a nutrient agar in a petri dish and grown at 27°C in for 72 hours. Bacteria from the incubation plates were scraped off and suspended in phosphate buffer. The culture was diluted to a final concentration of 10^8 colony-forming units (cfu) /mL (OD_{600} ve $A_{0.2}$) in phosphate buffer.

2.2. Plant Growth Process

Eight different lettuce varieties were used in this research which includes: Carmesi RZ Lolo, Batraz, Melina, Cospirina, Presidential, Nun 06118, Boratal, and Green Moon (Table 1). Two weeks old lettuce seedlings were sowed in 0.3-liter plastic pots containing peat moss as growth substrate and the entire growth process was similar to that described by Nicolas et al. (2019). The pots were placed in a growth chamber for two weeks between 24°C-28°C (day/night), and 70% relative humidity with a 16-h photoperiod.

Table 1. Different lettuce varieties and types used to evaluate their response to *X. hortorum* pv. *Vitians*

Lettuce Types	Lettuce Varieties
Curly	Batraz, Carmesi RZ Lolo, Melina
Romaine	Cospirina, Nun 06118, Presidential, Boratal
Iceberg	Green Moon

2.3. Lettuce Inoculation Procedure

Four-week-old lettuce plants were inoculated with the HBXcv1 strain using a method similar to that described by Bull et al. (2007). A pressurized handheld sprayer was used to apply about 1 mL of bacterial suspension per plant so that it would run off the abaxial and adaxial sides of the plant leaves. The second inoculation method was similar to that described by Hebert et al. (2021). To introduce HBXcv1 bacterial suspensions into the abaxial side of young lettuce leaves, a volume of 1 mL was gently administered using a syringe until the solution infiltrated an area measuring approximately 1.5 cm in diameter. To promote disease development, 5 minutes of overhead irrigation using sterile water was administered 30 minutes before the inoculation process. Control plants were sprayed with sterile pure water, and all plants were incubated in a growth chamber and covered with thin plastic sheets for a day. Plants subjected to inoculation were watered daily over a 10-day period.

2.4. Evaluation of Resistance of Lettuce Varieties Against *Xanthomonas hortorum* pv. *vitians*

To evaluate disease severity, each treatment was replicated 5 times with each replicate containing the eight lettuce varieties. The total number of lettuce plants used during the experiment was 120 plants (8 cultivars, 5 replicates, 2 treatments, and a control). Ten days post-inoculation, BLS symptoms were assessed. Visual analysis showed that there was no lesion spotted on any plant within the control experiment. On plants within the inoculation by hand sprayer, lesions were counted on four leaves, and ratings were assigned based on the number of individual lesions and patches that were formed as a result of combined individual lesions. The score was given on a scale of 0 to 4. 0 = Plant with no lesion; 1 = Plant with a few lesions ranging from 1 to 10 lesions; 2 = Plant with numerous lesions of 11 to 20 lesions; 3 = Plant with 21 to 100 individual lesions and small patches of combined lesions; and 4 = Plant with more than 100 individual lesions and small patches of combined lesions. Also, Duncan's Multiple Range Test (DMRT) and Analysis of Variance (ANOVA) were performed to assess the significance of differences in disease incidence among various lettuce cultivars. Plants subjected to inoculation using a syringe underwent visual analysis and were either labeled as visible symptoms plants or hypersensitive or absence of symptoms plants.

2.5. Determination of *Xanthomonas hortorum* pv. *vitians* Sensitivity to Copper

Nutrient agar (NA) (Oxoid) made of 4 g nutrient agar, 25 g of glucose, 11 g of bacteriological agar, and 0.5 l distilled water as well as Casitone Yeast extract (CYEA) (Merck) made of 0.85 g of casitone, 0.1 g of yeast extract, 1 g of glucose, and 11 g of bacteriological agar (Sigma) and 0.5 l distilled water were used to compared growth of the bacteria strain with and without copper amendment at different concentration. Copper sulfate at appropriate concentrations of 50ppm, 100ppm, and 200ppm was added to each medium and autoclaved at 121 °C for 2 hours and cooled at 55 °C for 1 h. *Erwinia amylovora* (Ea2), a disease-causing agent of fire blight in pears served as a control during the experiment due to its known susceptibility to copper sulfate. Its sensitivity rate was evaluated alongside the HBXcv1 strain for comparative analysis. The research consisted of eight treatments: NA, NA+ 50ppm $CuSO_4$, NA+ 100ppm $CuSO_4$, NA+ 200 ppm $CuSO_4$, CYEA, CYEA+ 50ppm $CuSO_4$, CYEA+ 100ppm $CuSO_4$ and CYEA+ 200ppm $CuSO_4$. Each medium was replicated two times.

Medium was divided into two equivalent parts and both *HBXcv1* and *Ea2* streaked in an S-pattern on their respective halves of the agar in the petri dishes. This allows for easier comparison between the two strains and facilitates the easier investigation of copper susceptibility mechanisms. A loopful of each strain was taken from 3-day-old growth on NA plates. Plates were incubated at 27 °C for 72 h. A visual assessment was made to determine the growth of HBXcv1 isolate in copper-containing media and was evaluated in comparison with the amount of bacterial growth in control petri dishes without added copper and the growth of the copper-sensitive *E. amylovora* isolate using a scale of 0 to 3 with 0 = (No visible growth /Susceptible, 1 = (Very minimal growth), 2 = (Moderate growth) and 3 = Abundant growth /Resistant.

3. Results

In the first part of this study, eight lettuce cultivars were all tested for their reactions against *X. hortorum* pv. *vitians* a disease-causing agent of bacterial leaf spot. The mean and percentage ratios of the eight varieties were well calculated, compared and displayed with each variety accounting for its unique value 10-days after being inoculated with the bacterial pathogen using a hand sprayer (Table 2, 3 and Figure 1).

The same cultivars tested in five uniform replicates for their resistance reaction against the same bacterial strain after being inoculated using a syringe were subjected to visual analysis. The necrosis scores of each plant were calculated and the result was displayed as either visible symptomatic plants or hypersensitive or absence of symptoms plants (Table 4).

Table 2. ANOVA table for the response of eight lettuce varieties response against BLS

Source of variation	df	Sum of Squares	Affected leaves	F. value	P. value
Model	39	1387512.100	35577.23***	26.40	<0.0001
Replicate	4	36597.2875	9149.32***	6.79	<0.0001
Variety	7	880288.2000	25755.46***	93.33	<0.0001
Variety*Replicate	39	470626.6125	16808.0933 `	12.47	<0.0001
Error	120	161691.000	1347.43	-	-
Corrected Total	159	1549203.100			
R^2	-		0.90	-	-

Values in the table are shown as mean squares. *, **, *** are significant at (p≤0.05), (p≤0.01), (p≤0.001) level respectively. R2 : R-square.

Table 3. Mean separation using duncan's multiple range test (DMRT) for lettuce varieties subjected to inoculation using Hand Sprayer

Varieties	Affected leaves
Cospirina	216.55[a]
Presidential	141.90[b]
Boratal	112.75[c]
Nun	63.75[d]
Green Moon	23.90[e]
Batraz	6.35[e]
Melina	3.15[e]
Carmesi RZ Lolo	0.25[e]

The means followed by the same letter(s) in the same column are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% significance level.

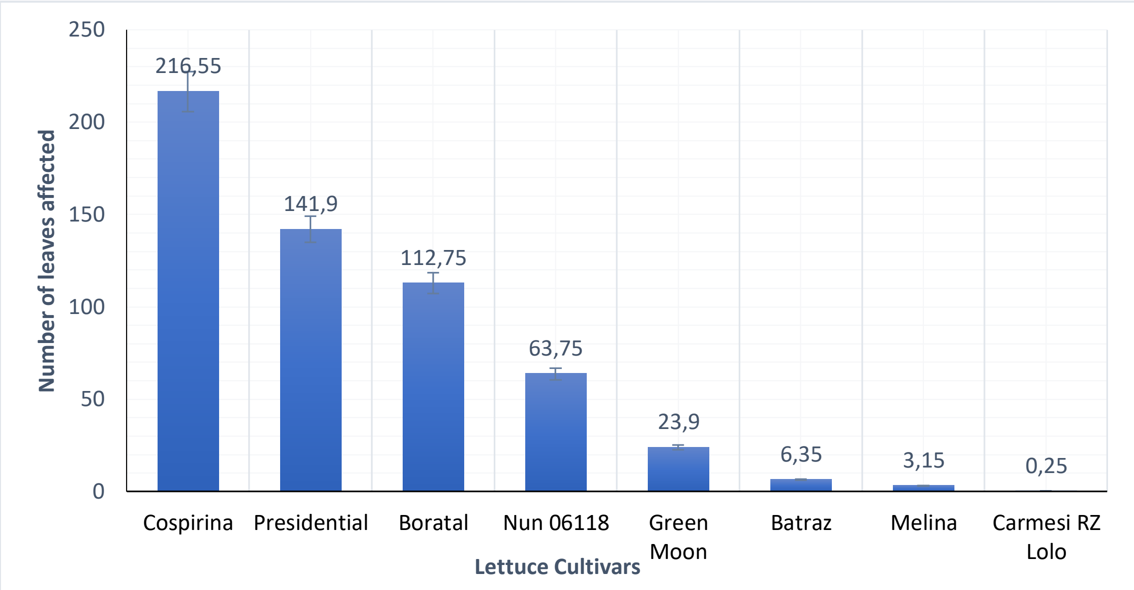

Figure 1. Means separation for the response of eight lettuce varieties against BLS.

Table 4. Disease severity scale of lettuce varieties inoculated with *X. hortorum* pv. *vitians*

Varieties	R1	R2	R3	R4	R5
Carmesi RZ Lolo	0	1	1	0	0
Melina	1	1	1	1	1
Batraz	1	1	1	2	1
Cospirina	4	4	4	4	4
Presidential	4	3	4	4	4
Boratal	2	4	4	3	4
Nun 06118	4	4	4	3	3
Green Moon	3	2	3	3	3

0= no lesion, 1= 1 to 10 lesions, 2= 11 to 20 lesions, 3= 21 to 100 lesions and small patches of combined lesions, 4= more than 100 lesions and small patches of combined lesions. R= replicate.

Bacterial isolate HBXcv1 injected using a syringe on lettuce plants induced dark brown lesions surrounded with a yellow halo on some varieties ten days after inoculation. Other varieties showed hypersensitive reactions while others repealed the effect of the bacterial pathogen. Symptomatic leaves that succumbed to the effect of the bacterial pathogen were cut off the plants and transferred to the laboratory for further evaluation (Figure 2).

Immediately after inoculation by syringe, a wound site on lettuce plant leaves was initiated causing minor physical injury to the plants. Within a span of ten days, Carmesi RZ Lolo was fast to gradually repel the tissue damage caused by the inoculation procedure. However, in replicate two, this defense response was delayed, as it struggled to repel the effect of both the effects of the inoculation procedure and the virulence of the disease pathogen. Other cultivars within the experiment apparently did not have the defense mechanism responsible for self-healing, rendering them incapable of resisting the effects of the inoculation procedure and the virulence of the pathogen (Table 5).

Figure 2. (From top left to bottom right) Carmesi RZ Lolo, Melina, Batraz, Green Moon, Nun 06118, Boratal, Presidential and Cospirina respectively showing symptoms of BLS 10-days after infiltration using a hand sprayer.

Table 5. Necrosis score frequencies of the BLS at 10-days after Inoculation with *X. hortorum* pv. *vitians* using a syringe

Varieties	Necrosis score of the BLS				
	REP[z] 1	REP[z] 2	REP[z] 3	REP[z] 4	REP[z] 5
Carmesi RZ Lolo	-	+	-		-
Batraz	+	+	-	-	+
Melina	-	-	+	-	-
Cospirina	+	+	+	+	+
Presidential	+	+	-	+	+
Boratal	+	+	-	+	+
Nun 06118	+	+	+	+	+
Green Moon	-	+	+	+	-

[z] Replicate, (+) visible symptoms; (−) HR or absence of symptoms.

In the second part of the research which aimed to investigate the sensitivity of *X. hortorum* pv. *vitians* to copper supplements, both HBXcv1 and a strain of *E. amylovora* (Ea2), a disease-causing agent of fire blight of pear, were tested for susceptible to copper. The Ea2 strain was used as a control due to its sensitivity to copper sulfate. Nutrient agar and Casitone Yeast extract agar were used to compare the growth of bacterial isolates in a medium with and without copper (Table 6).

Application of populations of *Xanthomonas* bacterial strains to NA amended with $CuSo_4$ suspensions tends to support the widespread growth of pathogen incidence as a result of copper resistant gene compared to CYE amended with $CuSo_4$ (Pernezny et al., 2008). Both HBXcv1 and *Ea2* were replicated twice and began showing visible response after two days and data collected after three days indicated that the bacteria pathogens can grow on both control medium of NA and CYE agar (without copper supplements) (Figure 3 and 4). The presence of genetic mechanisms for copper resistance within *Xanthomonas* species underscores their remarkable adaptability and resilience in challenging environmental conditions. Some *Xanthomonas* strains displayed growth on NA amended with copper, while others are completely inhibited due to specific genes and proteins involved (Strayer-Scherer et al., 2018).

Table 6. Reaction of *X. hortorum* pv. *vitians* HBXcv1 to copper supplements

Pathogen	Host of Origin	Medium	Susceptibility Score
		NA	3
		NA+50 ppm $CuSO_4$	3
		NA+100 ppm $CuSO_4$	2
		NA+200 ppm $CuSO_4$	1
HBXcv1	Lettuce	CYEA	3
		CYE agar+50 ppm $CuSO_4$	3
		CYE agar+100 ppm $CuSO_4$	0
		CYE agar+200 ppm $CuSO_4$	0
		NA	3
		NA+50 ppm $CuSO_4$	3
		NA+100 ppm $CuSO_4$	1
		NA+200 ppm $CuSO_4$	1
Ea2	Pear	CYEA	3
		CYE agar+50 ppm $CuSO_4$	1
		CYE agar+100 ppm $CuSO_4$	0
		CYE agar+200 ppm $CuSO_4$	0

0: No visible growth /Sensitive, 1: Very minimal growth, 2: Moderate growth, 3: Abundant growth /Resistant

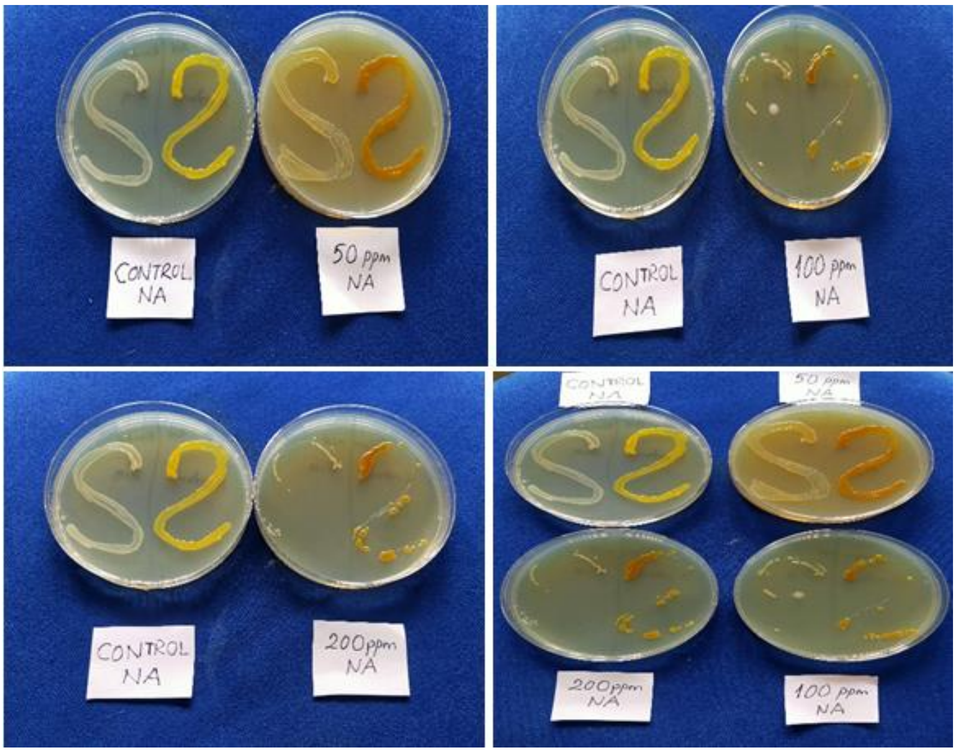

Figure 3. Reaction of HBXcv1 and Ea2 to NA amended with different copper supplements

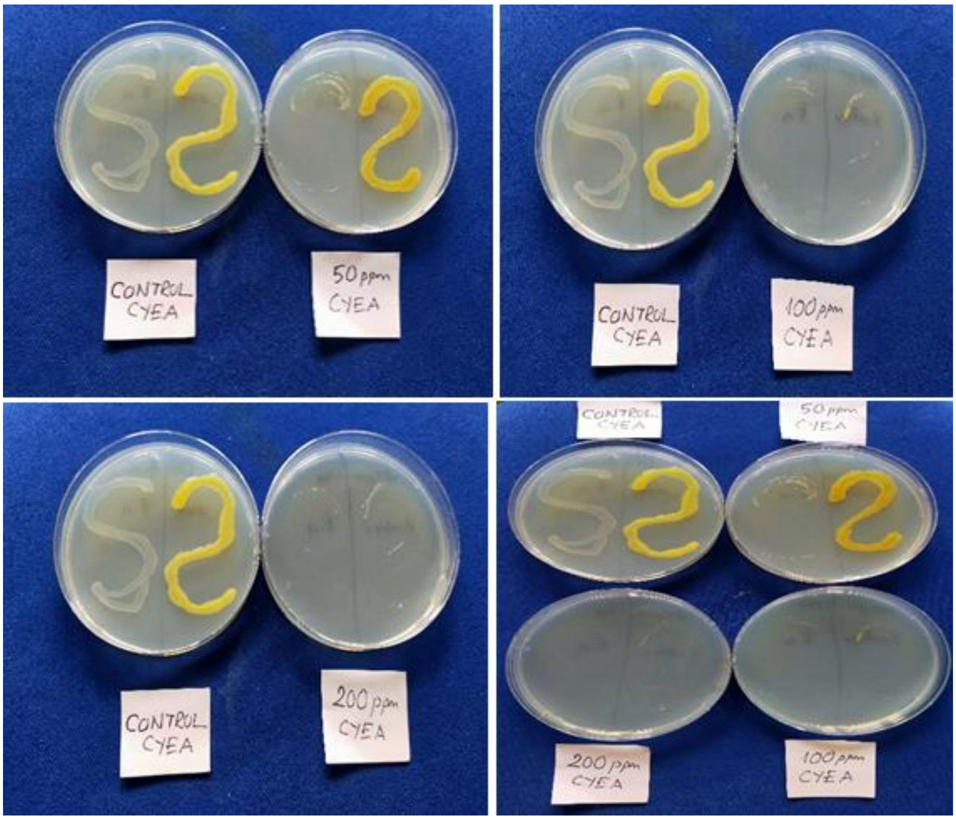

Figure 4. Reaction of HBXcv1 and Ea2 to CYE agar amended with different copper supplements

The selection of protocol, environment, and particularly, the culture medium for screening bacterial strains can significantly impact the research outcomes regarding the classification of strains as either copper-resistant or copper-susceptible.

4. Discussion

The Duncan's Multiple Range Test (DMRT) result showed that Cospirina, Presidential, Boratal, and Nun varieties were significantly affected by 38.08%, 24.96%, 19.83, and 11.21%, respectively as compared to Green Moon, Batraz, Melina and Carmesi RZ Lolo varieties which were affected by 4.20%, 1.12%, 0.55% and 0.04% respectively, which were not significantly affected by the effect of BLS (Table 3). This shows that each Romaine lettuce varieties used in the research (Cospirina, Presidential, Boratal, and Nun cultivars) was susceptible to BLS while the curly lettuce varieties (Batraz, Melina, and Carmesi RZ Lolo) and the Iceberg lettuce variety (Green Moon) are relatively tolerant to BLS.

Ten days post-inoculation by sprayer, varieties Cospirina and Presidential consistently had the highest disease severity in the experiments in which they were tested (Table 4). In the five replicates in which they were compared, disease severity levels for Cospirina and Presidential did not differ from each other based on the scale of measurement used by the researcher (Table 4) even though Presidential variety slightly differed in the second replicate. Boratal and Nun 06118 varieties were also highly susceptible to BLS disease and as well did not differ from Cospirina and Presidential varieties (P= 0.05).

Carmesi RZ Lolo was the most tolerant variety during the experiment with a DMRT mean of 0.25^{e} at 5% significance level (Table 3). When data from experiments were pooled, the disease incidence and severity rating for Carmesi RZ Lolo at 5% significance level was significantly lower than all other varieties and had disease severity significantly lower than the levels for all varieties. Followed by variety Carmesi RZ Lolo, both Melina and Batraz variety which is of the same variety groups (Curly Lettuce), showed resistant reactions to BLS which were not significantly different from Carmesi RZ Lolo. In Duncan's Multiple Range Test analysis, Carmesi RZ Lolo, Batraz and Melina showed no significant difference at 5% significance level. This result clearly shows that the lettuce of the curly group used in the experiment had high resistance to repel the effect of the disease pathogen.

Green Moon had relatively low disease incidence accounting for the variety that was neither highly susceptible nor tolerant to BLS (Figure 2). The relative susceptibility of all the varieties that were tested varied somewhat among experiments, but all consistently ranked between the most susceptible (Cospirina and Presidential and Nun 06118) and the moderate varieties (Melina, Batraz, and Green Moon respectively) and the tolerant variety (Carmesi RZ Lolo).

It was observed that Carmesi RZ Lolo lettuce variety of replicates 1, 3, 4 and 5 (Table 5) were completely tolerant to the effect of HBXcv1 isolate, showing no physical damage 10 days after infiltration with the bacterial pathogen, while in plants within replicate 2 displayed minimum dark brown spot on leaves injected. HBXcv1 was more aggressive on Cospirina, Nun 06118 and Presidential than any other variety. While, on average, HBXcv1was had an intermediate reaction on variety Green Moon and Batraz.

The second part of the research which investigated the susceptibility of HBXcv1 to copper sulfate showed an astonishing result. HBXcv1 streaked on both NA and NA +50 ppm showed abundant growth (3) and was rated resistant to $CuSO_4$ while HBXcv1 streaked on NA +100 ppm and NA+ 200 ppm showed minimum growth of 2 and 1 on the rating scale respectively and were considered to be sensitive to medium for both strains (Table 6).

Results of HBXcv1 on $CuSO_4$ amended on CYE agar were resistant at 50 ppm level and sensitive at 100 ppm and 200 ppm levels displaying a score of 3, 0, and 0 respectively. This finding suggests that despite CYE agar providing a suitable environment for HBXcv1 growth, the higher the concentrations of $CuSO_4$, the greater cell death occurs for HBXcv1 on the medium.

In Figure 4, HBXcv1 exhibited a significant reduction in colony number on NA and CYE supplemented with copper sulfate at 100 ppm and 200 ppm levels respectively compared to plain NA and CYE and 50 ppm of $CuSO_4$ on both media (Cooksey, 1994). Visual examination reveals that NA and CYE with no copper sulfate facilitated adequate growth of both the HBXcv1 and *Ea2* strains, resulting in both strains being resistant to the two culture media. In contrast, the assessment demonstrates that the inclusion of a higher concentration of 200 ppm $CuSO_4$ on NA and 100 ppm and 200 ppm on CYE creates an environment where there is a higher number of $CuSO_4$ molecules on the agar plates than that of the copper-resistant gene of the bacteria strain. This hostile environment hindered the development of both HBXcv1 and *Ea2* strains, consequently making them susceptible to the medium. This becomes particularly clear that both strains exhibit varying reactions to copper present in the two types of media and they become more susceptible when copper sulfate concentration increases. This suggests that despite NA and CYE providing a suitable environment for bacteria growth, various concentrations of copper sulfate disrupt essential cellular processes for HBXcv1 growth. This finding highlights the potential of copper sulfate as a control measure against *X. hortorum* pv. *vitians* infections.

Many studies have found that numerous *Xanthomonas* strains tend to be resistant when grown on NA with a certain concentration of added copper and susceptible when grown on CYE with a certain concentration of added copper (Pernezny et al., 2008; Griffin et al., 2017). Thus, the initial categorization of strains based on their copper resistance is more accurately determined when using NA as the base medium with copper sulfate supplementation. However, further testing on a broader range of strains from both genera is necessary before any conclusions can be drawn.

5. Conclusion

The bacterial leaf spot caused by the plant bacterial pathogen *X. hortorum* pv. *vitians* has become a pressing global issue for lettuce growers over the years, leading to post-harvest losses and a decline in the market value of commercial lettuce. The most effective approach to managing the disease, as identified by researchers and growers, is the use of resistant varieties. The use of chemical control measures is limited in many countries, and the unpredictable nature of the disease makes it difficult to achieve complete control over BYL outbreaks. In this study, when evaluating the responses of eight lettuce varieties to *X. hortorum* pv. *vitians*, it was found that the varieties Cospirina, Presidential, Boratal, and Nun, typically associated with the Romaine lettuce subgroup, exhibited susceptibility to the pathogen. The varieties Batraz, Melina, and Carmesi RZ Lolo, closely related to the "Little Gem" lettuce variety (Bull et al., 2015), showed the lowest disease severity, emerging as the most tolerant varieties and performing significantly better than others in terms of disease percentage and severity. This could be related to the innate genetic defense mechanisms that regulate their resistance to infections caused by the pathogen *X. hortorum* pv. *vitians.* In recent years, various polygenic resistance genes, such as *Xar1, Xcr,* and *qXCR2.1*, have been identified, particularly located on chromosome 2, in some curly lettuce varieties like La Brillante, Little Gem, and Pavane (Sandoya et al., 2019; Hayes et al., 2014). These resistance genes have been found to provide a high level of resistance to *X. hortorum* pv. *vitians* strain 1 (from which HBXcv1 is derived), which causes BYL. Additionally, the genomic sequencing of lettuce has led to the identification of a large number of genes encoding NB-LRR proteins, known as Resistance Gene Candidates (RGCs) (McHale et al., 2009). These RGCs are responsible for various responses to different lettuce diseases such as BYL and lettuce downy mildew. Symptomatological observations showed that the varieties responded differently to bacterial inoculation, with some displaying dark brown lesions while others exhibited hypersensitive reactions or resistance to the pathogen.

Investigating the sensitivity of *X. hortorum* pv. *vitians* to copper sulfate has provided valuable insights into potential control measures for BYL. The results showed that the growth of bacterial strains varied depending on the medium used and the concentration of copper. As agriculture develops, the use of chemical pesticides is being restricted or banned in some countries, and more importantly, some disease agents continue to adapt to chemical compounds. Bacteria exhibit a remarkable genetic adaptability that allows them to respond effectively to various environmental challenges, including the presence of antibiotic compounds that threaten their survival. They use two main genetic strategies to adapt to antibiotics: i) mutations in genes and ii) acquisition of foreign DNA encoding resistance determinants through Horizontal Gene Transfer (HGT) (Munita and Arias, 2016). Accordingly, the results of this research highlight the complexity of bacterial adaptation, emphasizing the importance of understanding the genetic mechanisms underlying bacterial resistance and sensitivity. The choice of culture medium, protocol, and environment can also significantly affect the classification of strains as resistant or sensitive to copper, underscoring the need for standardized protocols in future research efforts.

This research contributes to the understanding of the responses of lettuce varieties to BYL and the potential effectiveness of copper as a control measure against *X. hortorum* pv. *vitians* infections. It clarifies the different responses of varieties and bacterial strains to both pathogen inoculation and copper supplementation. The research findings lay the groundwork for further studies in bacterial leaf spot of lettuce management strategies and variety breeding programs. Moving forward, ongoing research efforts in this area will be effective in developing sustainable practices to reduce the impact of BYL on lettuce production not only in Türkiye but in the entire world.

Author Contributions

The percentage of the author(s) contributions is presented below. The author reviewed and approved the final version of the manuscript.

	RLK	HB
C	30	70
D	40	60
S	0	100
DCP	40	60
DAI	30	70
L	30	70
W	40	60
CR	0	100
SR	0	100
PM	20	80
FA	0	100

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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