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Epidemiology of *Xanthomonas euvesicatoria* in Tokat Province

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Abstract: This study was carried out to determine the life span of *Xanthomonas euvesicatoria* (*Xeu*) in seeds, soil and plant residues and the first inoculum source that cause the onset of the disease. The study was carried out in the province of Tokat. Rifampicin-resistant *Xeu* isolate was inoculated into soil, seeds and plant residues. As a result of the studies, it was determined that *Xeu* remained viable in inoculated seeds until the 220th day (7 months), and lived in the soil for 105 days in summer and 14 days in winter. In plant residues, it was determined that the the life span in summer period was 60 days at 0 cm depth, 30 days at 20 cm depth; 30 days at 0 cm depth and 15 days at 20 cm depth in that of winter period. According to the results of this study, it was determined that the *Xeu* did not survive for a long time in plant residues and soil in Tokat province and thus was not carried to the next year, on the other hand the seed was the most important source of inoculum.

Keywords: *Xanthomonas euvesicatoria*; epidemiology; Tokat; bacteriology; pepper

1. Introduction

Xanthomonas euvesicatoria (*Xeu*), which causes bacterial spot disease in pepper and tomato plants, is one of the bacterial agents seen in pepper production areas. This disease was first described by [1] in 1921 and diagnosed *Bacterium vesicatorium*. As a result of the studies carried out to date, it is seen almost everywhere in the world and the presence of the plant at different rates is reported in the areas where it is grown [2]. In line with the studies carried out in recent years, it is known that four different Xanthomonas species cause the disease. Of these species, *Xanthomonas euvesicatoria* [3] (Group A; includes most isolates known as *Xanthomonas campestris* pv. *vesicatoria* or *Xanthomonas axonopodis* pv. *vesicatoria*), *Xanthomonas vesicatoria* [1–4], (Group B)), *Xanthomonas perforans* [3] (Group C) and *Xanthomonas gardneri* [3–5] (Group D). There are three races of the disease agent in tomato and these races are distributed worldwide. While the T2 strain is common in South America, it is not seen in Asia. The most dominant pepper strains in Asia are P1, P2 and P3, while the P2 strain is only available in the Americas [6]. However, today, there are four races in tomato groups and eleven races in pepper groups [7]. In addition, it has been stated that P7, P8 and P10 of the races in the pepper groups are available in our country [8]. It reduces the production and market value of pepper, which is very important economically. This agent, which is seen in tomato and pepper plants, can be found almost everywhere in the world and its presence is reported in areas where cultivation is carried out at different rates [9]. In Turkey, the disease has been reported in tomato production areas in Çanakkale [10], Western Anatolia [11], and the Western Mediterranean region [12]. The presence of this disease in pepper plants was also recorded in Antalya [12,13], Erzurum, Erzincan, Yusufeli [13], Adana and Osmaniye [8,14]. The province of Tokat, where the study was conducted, is in the twelfth place in the province ranking in Turkey with 68,646 tons of pepper production [15]. The province of Tokat has a hot and humid climate, thus, the pathogen can easily develop and cause product losses. In pepper production, which is generally carried out on large lands throughout the province, this disease can be found

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almost every area. While the incidence of the disease in the field was 35.80% in 2016, it was determined as 42.9% in 2017 [16].There are many disease factors that cause product loss in most cultivated plants in the region [17]. Pepper Bacterial Spot Disease (*Xanthomonas euvesicatoria*, *Xanthomonas vesicatoria*, *Xanthomonas gardneri* and *Xanthomonas perforans*) is one of the important diseases that cause more crop loss in pepper plants in hot and humid areas [18].

Xanthomonas species can survive on the seed epiphytically and the pathogen maintains its viability for up to 18 months. In addition, the agent overwinter in plant residues and diseased leaves in areas where pepper and tomato are planted and where the disease is present [19]. *Xeu* and *Xanthomonas vesicatoria* spead over long distance through diseased seeds. In addition, the agent spreads from areas with epiphytic populations of the disease to areas where the disease does not exist, with the effect of appropriate humidity, temperature and wind [20,21]. Humidity on the plant surface plays an important role in the emergence and spread of the disease, typical disease symptoms can be observed within one or two days at a humidity of 85% and above [22], temperatures between 24–30 °C and relative humidity above 70% humidity has been reported to promote the development of infection and disease symptoms [23,24]. In particular, it was stated that the agent was moved to a distance of 32 m with the effect of wind and precipitation, and the severity of the disease reached 100% [25]. It has been stated that *Xanthomonas* spp. live epiphytically on the above-ground parts of tomato plants and weeds and can cause latent infections under suitable conditions. Plants with disease during the seedling period also have the disease in the field throughout the production season [26,27]. This agent also exist on the plants in the *Solanaceae* family, *Nicotiana rustica*, *Physalis minima*, *Solanum nigrum*, *S. dulcamara*, *S. rostratum*, *S. tuberosum*, *S. melongene*, *Hyoscyamus niger*, *H. aureum*, *Lycium chinense* and *L. halimifolium* [28]. The disease can easily spread from one plant to another with the tools and equipment used during cultural processes. Studies have shown that the pathogen can maintain its viability for ten years in dried pepper seeds and for one year in soil and plant residues [29], and the disease can be seen again after three or six months in pathogen-infected fields [30].

In this study, it was aimed to determine the life span of *Xeu* (seed, soil and plant residues) infected with the first inoculum sources that caused the onset of the disease in the province of Tokat in Turkey. Studies on the epidemiology of *Xeu* in the world are limited. No studies have been conducted on the epidemiology of *Xeu* in Turkey. This is the first study on *Xeu* epidemiology in Turkey

2. Material and Method

Pathogen isolate: *Xeu* isolate, which was isolated from infected pepper plants from pepper production areas in Tokat province, identified as *Xeu* by various biochemical and molecular analyzes and made resistant to rifampicin antibiotic, was used.

Pepper seeds and seedlings: In the studies carried out under laboratory and greenhouse conditions, Istek F1 pepper variety seed and seedling were used.

Medium: King B medium (Proteose peptone 20 g, K_2HPO_4, 1.5 g, $MgSO_47H_2O$ 1.5 g, Glycerin 10.0 mL, Agar 15.0 g, Distilled Water 1000 mL) and Nutrient Broth Yeast Extract (NBY; 8 g Nutrient Broth, 2 g Yeast Extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 2.5 g Glucose, 1000 mL Distilled Water) media were used.

In the first stage of the study, the *Xeu* isolate was resistant to the rifampicin antibiotic. For this process, the method specified by the rifampicin resistant bacterial isolate was modified to the medium in which *Xeu* was grown. A loopful of *Xanthomonas euvesicatoria* regional isolate grown in King B medium for 48 h was transferred to 20 mL of Nutrient Broth Yeast Extract (NBY) broth and developed in a shaker operating at 150 rpm for 72 h. A 25 mg/L dose of rifampicin prepared using 70% alcohol was added to the bacterial suspension by cold sterilization. After 48 h, this suspension was drawn on King B medium containing 25 mg/L rifampicin. It was taken from the developing colonies and redeveloped in 20 mL NBY medium. 50 mg/L rifampicin was added to the bacterial suspension, which

was incubated in this medium for two days, and incubation was continued until the fourth day. At the end of the fourth day, the bacterial suspension taken from here was transferred to King B medium containing 50 mg/L rifampicin. These procedures were repeated for 75 and 100 mg/L rifampicin doses [31].

2.1. Determination of the Lifespan of Xanthomonas euvesicatoria in Inoculated Seed

Firstly, the seeds of the Istek F1 pepper variety were surface disinfected in 1% NaOCl solution. Then, the seeds were inoculated with 100 mg/L rifampicin-resistant pathogen isolate at a density of 5.32×10^{-8} cells/mL using vacuum (15 min) followed by immersion (30 min) method [32]. Inoculated seeds were dried in a sterile cabinet overnight and stored in paper bags at room temperature. King B medium containing 100 mg/L rifampicin on the 33, 66, 100, 129, 162, 193, 220, 249, 280, 310, 340, 370, 400 and 430 days following the first inoculation day. Infected seeds were placed on the medium. The percentage of inoculated seeds was determined by counting the colonies that developed around the seed [33].

2.2. Determination of the Lifespan of Xanthomonas euvesicatoria in Inoculated Soil

The trial was carried out on the land of Tokat Gaziosmanpaşa University, Agricultural Application and Research Center. In the study, sifted soil without plant residues was used. The soil was inoculated with a 10^{-8} cell/mL suspension prepared from 100 mg/L rifampicin-resistant *Xeu* pathogenic bacteria grown in King B medium for 48 h. The soil was then placed in 25 L cans and buried in the field. During this time, the soil was not irrigated at all. Isolations were continued until no pathogen could be obtained from the soil in weekly periods from the date of inoculation. Isolations were made according to the dilution method from the samples taken from 0–15 cm depth of the contaminated soil. The life span of the pathogen in inoculated soil during the summer and winter periods was investigated for one year [31–34].

2.3. Determination of the Lifespan of Xanthomonas euvesicatoria in Plant Residues in Soil

Pepper plants with 4–5 leaves grown under greenhouse conditions were inoculated with the pathogen resistant to 100 mg/L rifampicin at a density of 10^8 cells/mL. After inoculation by spraying, when disease symptoms were observed on the leaves, the infected leaves were taken and to the laboratory. *Xeu*-infected leaves, which were cut into small pieces were weighed 10 g and placed in cheesecloth. Cheesecloths containing plant residues were buried at 0 and 20 cm depths of the soil. Isolation was made by removing two of the buried samples from 0 and 20 cm in fifteen-day periods. The life span of the pathogen in plant residues was determined by isolations made on King B medium [31–34]. In the one-year study, the populations of the pathogen in contaminated plant residues during the summer and winter months were determined.

3. Results

3.1. Lifespan of Xanthomonas euvesicatoria in Inoculated Seed

In the study, according to the method used, 100 seeds inoculated with *Xeu* pathogen isolate at a density of 5.32×10^{-8} cells/mL were randomly selected and placed in King B medium containing 100 mg l-1 rifampicin and the seeds around which *Xeu* colonies developed were counted. In the study, which lasted for 249 days, yellow colony development was observed around all the seeds on the first day (day 0) of the incubation (Figure 1). Inoculated seed percentage was 100% (Table 1). At the end of 33rd, 66th, 78th, 100th, 129th, 162nd, 193rd and 220th day of incubation, infected seed rates were 86%, 78%, 66%, 58%, 40%, 4%, and 1% respectively. Bacterial growth was observed and isolation was continued throughout these days. However, no bacterial growth was observed on 249th day of incubation. According to these results, it was concluded that *Xeu* can survive in inoculated pepper seeds up to 220 days (7 months). It is quite a long process for the pathogen to have a seven-month life span in the seed. this time will easily be carried over to the following season when the seed will be planted. For this reason, the disease appears in the seedling

period and causes crop losses. This makes it difficult to control the disease. It is necessary to start control applications in the early period. This situation negatively affects both the economy and the workforce.

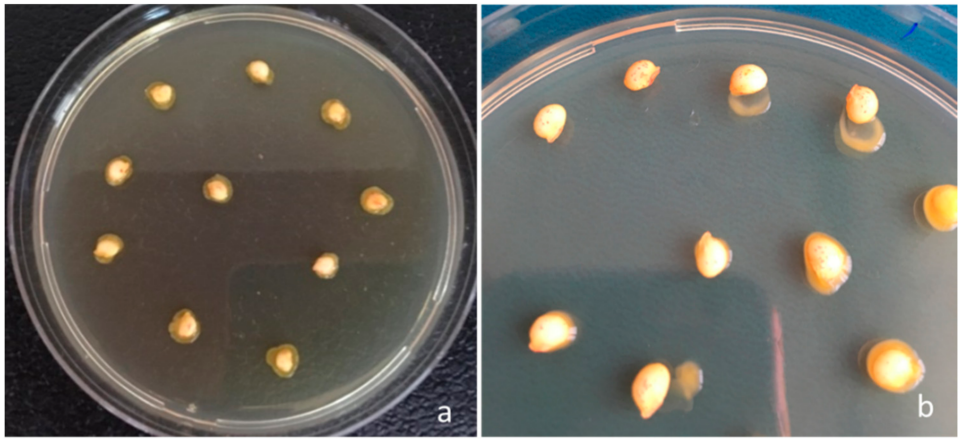

Figure 1. Colonies growing around seeds infested with *Xeu* (**a**) Image of a bacterial colony developing around the seeds on the first day (**b**) Image of a bacterial colony developing around the seeds on one hundred ninety third day.

Table 1. Life span of pathogen in pepper seeds infested with pathogen.

Day	Percentage of İnfected Seeds with *Xeu* (%)
0. Day	100 ± 0.33 a
33. Day	86 ± 0.58 b
66. Day	78 ± 0.67 b
100. Day	66 ± 0.00 c
129. Day	58 ± 0.33 c
160. Day	40 ± 0.00 d
193. Day	4 ± 0.00 e
220. Day	1 ± 0.33 e
249. Day	0 ± 0.00 e

Viability of *Xanthomonas euvesicatoria*. Means followed by the same letter on the same. Column are insignificantly different at $p = 0.05$ using ANOVA and Tukey for means comparison.

3.2. Lifespan of Xanthomonas euvesicatoria in Inoculated Soil

In the soil survival study, weekly isolations were made from the samples at 0 and 15 cm depth of the soil in the tin cans buried in the trial area (Figure 2). The isolation process was continued from July to September in the summer period and from September to July in the winter period until no pathogen could be obtained. The soil placed in the tin cans was inoculated with the pathogen at a density of 7.97×10^{-8} cells/mL in the summer period and 8.60×10^{-8} cells/mL in the winter period. In the study, which was carried out in three repetitions, the pathogen survived in the soil until the 105th day in the summer period and until the 14th day in the winter period (Table 2). As a result of the study, the summer period lived longer in the soil inoculated with pathogen. As a result of the isolations made from the soil, bacteria could not be obtained for some weeks. The presence of bacteria was detected the following week. This is due to the random selection of the sample taken from the soil. Bacteria do not grow homogeneously in the soil.

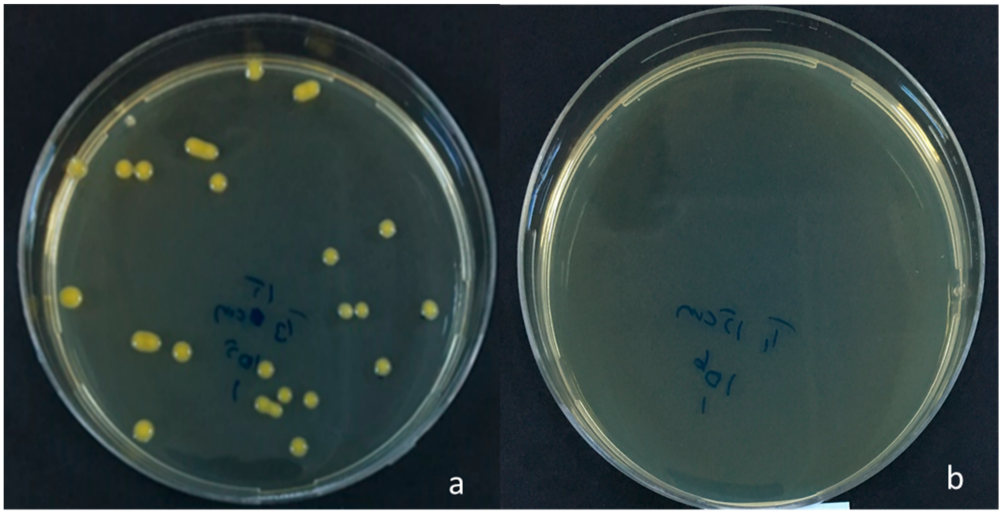

Figure 2. Colony growth from summer *Xeu*-infected soil isolation (**a**) Initial isolation (**b**) Final isolation.

Table 2. Determination of the life time of *Xeu* in inoculated soil.

Days	Summer Term Bacterial Density		Winter Term Bacterial Density	
	0 cm	**15 cm**	**0 cm**	**15 cm**
0. day	7.9×10^{-8}	7.9×10^{-8}	8.6×10^{-8}	8.6×10^{-8}
7. days	2×10^{-4}	2×10^{-4}	4.6×10^{-4}	5.0×10^{-4}
14. days	2×10^{-4}	2×10^{-4}	0.5×10^{-4}	0
21. days	0	2×10^{-4}	0	0
28. days	1.5×10^{-4}	2.5×10^{-4}	0	0
35. days	1×10^{-4}	2.5×10^{-4}	0	0
42. days	0	0	0	0
49. days	2.5×10^{-4}	1×10^{-4}	0	0
56. days	1.5×10^{-4}	2×10^{-4}	0	0
63. days	0	1.5×10^{-4}	0	0
70. days	1×10^{-4}	1.0×10^{-4}	0	0
77. days	0	0	0	0
84. days	0	0	0	0
91. days	0	0	0	0
98. days	0	0	0	0
105. days	1.0×10^{-4}	1.0×10^{-4}	0	0
112. days	0	0	0	0

3.3. Lifespan of Xanthomonas euvesicatoria in Plant Residues

The study to determine the life span of *Xeu* in inoculated plant residues started in July 2018 and was carried out during the summer period July-September while the winter period was from September to July. Plant residues inoculated with the pathogen were buried in the soil at a density of 6.3×10^{-8} cells/mL in summer studies and 5.4×10^{-8} cells/mL in winter studies. As a result of the isolations made from plant residues at 0 and 20 cm depth in the time period covering the summer period (Figure 3), it was observed that the bacterium lived until the 60th day at 0 cm depth and until the 30th day at 20 cm depth. In the winter period, it was observed that the bacteria lived until the 30th day at 0 cm depth

and until the 15th day at 20 cm depth (Table 3) as a result of the isolation made from the buried plant residues. As a result of the study, it was determined that the plant residues of pathogenic bacteria had a longer life span at 0 cm depth in the soil in summer and winter periods. As the depth of the soil increases, the life of the bacteria shortens.

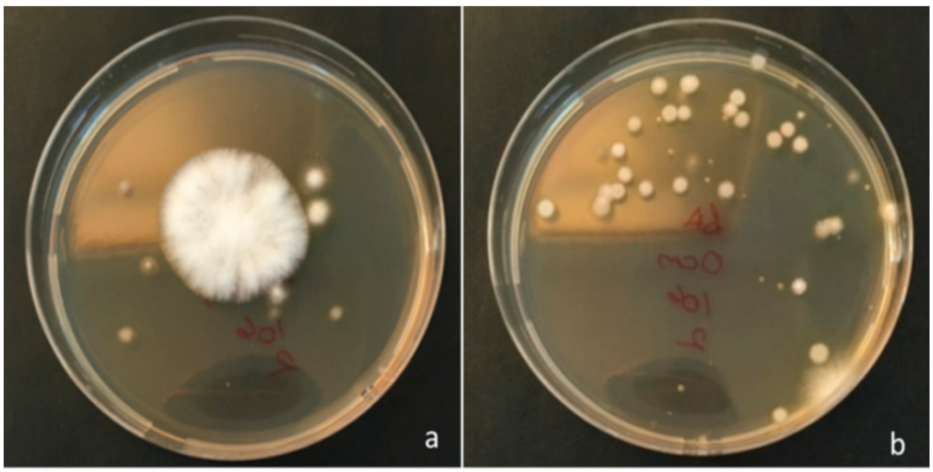

Figure 3. Growth in the medium of 10^6 diluted solution of winter isolation from soil (**a**) Sample taken from 20 cm depth (**b**) Sample taken from 0 cm depth.

Table 3. Determination of life span of *Xeu* in plant residues.

Days	Summer Term Bacterial Density		Winter Term Bacterial Density	
	0 cm Depth (Cell/g)	20 cm Depth (Cell/g)	0 cm Depth (Cell/g)	20 cm Depth (Cell/g)
0. day	$6.3 \times 10^{-8} \pm 0.58$ a	$6.3 \times 10^{-8} \pm 0.58$ a	$5.4 \times 10^{-8} \pm 0.58$ a	$5.4 \times 10^{-8} \pm 0.58$ a
15. days	$15 \times 10^{-6} \pm 0.00$ b	$5 \times 10^{-6} \pm 0.58$ b	$2 \times 10^{-6} \pm 0.00$ b	$1 \times 10^{-6} \pm 0.00$ b
30. days	$10 \times 10^{-6} \pm 0.58$ c	$1 \times 10^{-6} \pm 0.00$ c	$1 \times 10^{-6} \pm 0.00$ b	0 ± 0.00 c
45. days	$1 \times 10^{-6} \pm 0.00$ d	0 ± 0.00 d	0 ± 0.00 c	0 ± 0.00 c
60. days	$1 \times 10^{-6} \pm 0.00$ d	0 ± 0.00 d	0 ± 0.00 c	0 ± 0.00 c
75. days	0 ± 0.00 e	0 ± 0.00 d	0 ± 0.00 c	0 ± 0.00 c
90. days	0 ± 0.00 e	0 ± 0.00 d	0 ± 0.00 c	0 ± 0.00 c

Means followed by the same letter on the same column are insignificantly different at $p = 0.05$ using ANOVA and Tukey test for means comparison.

4. Discussion

Xeu is the most important pathogen of pepper and tomato in the world. It causes significantly important losses. Also, the pathogen can survive on seed, plant residues, and soil epiphytically. So, this ability makes it more important to study the plant's pathology. In this study, which was aimed at determining the life cycle of *Xeu* on inoculated seed, plant residues, and soil was carried out during the summer period (July-September) and the winter period (September–July).

In a study performed at Gainesville, Florida, it was mentioned that inoculated seeds play a key role in spreading the pathogen over long distances [20–25]. The pathogen is known to survive in dry seeds for 10 years [29]. Moreover, it can survive both externally and internally in seeds [35]. According to our results, the pathogen was obtained from the seed for 7 months. similar to our findings, [19] stated that the population amount of the pathogen from the infected seed remained stable by decreasing up to 5 months and its viability on the seed was achieved for 10 months.

On the other hand, the plant residues of *Xeu* is important in terms of survival and reaching the next production season. Thus, plant residues become a source of inoculum for the following seasons [27–36]. The presented study showed that the pathogen can survive on plant remains at a depth of 0 cm for 60 days in summer and 30 days in winter. In addition, at a depth of 20 cm, the pathogen can survive for 30 days and 15 days in summer and winter period respectively. It is known that the pathogen can survive in suitable conditions such as hot and humid regions. Also, our results showed that the population of pathogens decreases due to weather conditions like summer and winter and the depth of soil. Similar studies have been carried out by other researchers and the results obtained are similar to our study [19]. It was observed that after freeze-drying or air-drying plant residues contaminated with *Xanthomonas axonopodis* pv. *vesicatoria,* the bacteria survived longer and maintained its virulence in dried pepper tissues, but after four months the pathogen's presence was reduced to stable levels. Also, ref. [37] rifampicin-resistant *Xav* persists for 10 months on the soil surface in diseased plant residues and 2 months on buried plant residues. Similarly, a two-year study conducted in Florida found that *Xav* plant residues remained alive for 74 days in the first year and 35 days in the second year. In addition, the disease symptoms were seen again after 3 to 6 months due to the infected plant residues remaining in the field [30]. Ref. [27] reported that the agent can live in plant residues for a long time, i.e., it can survive for about two years in temperate climatic conditions and only a few months in tropical and subtropical regions. At the same time, the study found that it can remain in the soil with plant residues, but it will begin to disappear within a few days as soon as decomposition starts.

The other part of the presented study investigated the survival of *Xeu* in the soil. The amount of pathogen population was evaluated from two different soil depths, 0 cm and 15 cm. Based on the weekly isolation after soil inoculation, the pathogen was obtained for 105 days in summer. But in winter, it can live up to 14 days. Soil temperature and soil type can affect *Xanthomonas* spp. therefore, it is thought that the survival of the pathogen in inoculated soil may vary depending on abiotic conditions such as temperature. According to our results, the pathogen can live from 14 days to 105 days. Ref. [19] reported that *Xav* can survive in soil for 16 days. Similar behavior has been observed with regard to the winter survival of *Xeu.*

5. Conclusions

In this study, which was carried out to determine the life span of *Xeu* in seeds, soil and plant residues and to determine the first inoculum sources, it was determined that the first inoculum source of *Xeu* was in pepper seeds. According to the methods used, *Xeu* maintained its viability in the seed until the 220th day. The pathogen survived soil until day 105 in summer and day 14 in winter. It was also found that the agent lived longer than 20 cm at 0 cm. In the infected plant residues, the agent could survive until the 60th day at a depth of 0 cm and up to the 30th day at a depth of 20 cm in summer. In winter, the agent survived in infected plant residues until the 30th day at a depth of 0 cm and until the 15th day at a depth of 20 cm. Determining the survival time of the pathogen in the seed is to provide easy control of the disease. It is very important to know how long the pathogen lives in the seed in the control of such seed-borne diseases. In such a case, seeds should not be taken from the regions where the disease is present. In areas where the disease is present, it is envisaged to be used alternately with products that are not hosts of the pathogen. Soil and plant debris inoculated with disease agents transmit the disease throughout the growing season and into subsequent seasons and also spread it to other areas. This reduces the chance of control. By inoculating soil and plant residues of disease agents, transmitting the disease throughout the growing season and into subsequent seasons, and also spreading it to other areas. The results of this study once again revealed the importance of this situation. According to the results of this study, it was observed that the *Xeu* disease agent could not survive for a long time in plant residues and soil in Tokat province and therefore it was not carried to the next year. In addition, it was emphasized that pepper seeds should not

be taken from areas where the presence of the pathogen can be found and plant residues in such areas should be destroyed. In the world, the epidemiology of *X. euvesicatoria* has been study many years ago and there is almost no study on the subject. In Turkey, no study has been conducted on the epidemiology of *X. euvesicatoria*. Our study is the first in this regard. Therefore, our study will contribute greatly to the literature. It will be a reference for the epidemiological studies of *X. euvesicatoria* in different regions, provinces or countries or different bacteria. Thus, this subject has been studied and its publication fills the gap in the literature in this field. It is thought that the study of a seed-borne pathogen will be extremely beneficial in the production of the product groups to be produced. This information will help in the control measures (physical, biological, cultural and chemical) to be taken. It will shed light on the studies to be done on the control, epidemiology and diagnosis of the pathogen. It will be a very important reference to further the work to be done on this subject.

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