

Influence of *Xanthomonas euvesicatoria* on quality parameters of pepper seed from Serbia

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SUMMARY

The present study focused on detecting bacteria of the *Xanthomonas* spp. complex (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*) and examining their influence on certain quality parameters of pepper seed collected from the territory of Smederevska Palanka (Serbia). The analysis included 27 non-commercial pepper seed genotypes (including chili and sweet pepper) collected in 2021. Several parameters of the quality of analyzed pepper seed (germination energy, total germination, moisture and seed health) were determined. The results showed that out of a total of 27 analyzed samples of pepper seed, the presence of *X. euvesicatoria* was detected in 13 of them. The presence of *X. vesicatoria*, *X. gardneri* and *X. perforans* was not confirmed. Germination energy of infected seed was 52-84%, and of bacteria-free seed 63-90%; total germination of infected seed was 66-91%, and of bacteria-free seed 80-95%. Seed moisture of infected seed samples was 6.1-12%, and of bacteria-free seed 6.2-8.1%. These parameters did not show significant statistical difference ($p > 0.05$). The presence of seed-borne fungi *Fusarium* sp. accounted for up to 3% in 25 samples, while it was up to 6% in the remaining two; *Alternaria* sp. ranked from 1-4% in 25 samples, and up to 5% in only two samples. The results led to a conclusion that the bacterium *X. euvesicatoria* is the predominant pathogen of the *Xanthomonas* spp. complex, but it did not affect the quality parameters of the tested pepper seed.

Keywords: pepper, bacterial spot, germination, moisture, seed health

INTRODUCTION

Pepper (*Capsicum annuum* L.) as a commercial species is cultivated worldwide. The annual production of pepper (dried chillies and peppers) has reached approximately 3.9 million tons (Li et al., 2018). In Serbia, the area under pepper production was assessed at 10.278 ha in 2021, and overall production at 147.663 tons (Statistical Office of the Republic of Serbia, 2022).

Production of *C. annuum* is greatly hampered by many biotic factors, especially fungal, bacterial and viral diseases. Bacterial spot, caused by four distinct plant pathogenic *Xanthomonas* species (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*) is one of the most destructive diseases affecting sweet and chili pepper (Potnis et al., 2015; Schwartz et al., 2015; Horuz, 2019). Major losses occur under conditions of high humidity, intense rainfall and temperatures between 20-30 °C

(Kurozawa & Pavan, 2005). Rain and wind facilitate *Xanthomonas* spp. spreading from infected to healthy plants (Osdaghi et al., 2021). *Xanthomonas* spp. bacteria have been recognized as a serious disease accompanied by significant damage in pepper production in Serbia (Ignjatov et al., 2010; Vlajić et al., 2017). These pathogens cause lesions on pepper leaves, irregular shape, and haloes of concentric necrotic and surrounding chlorotic tissue. When a severe infection occurs, leaves may fall off. Symptoms on fruit are scab-like, raised, whitish lesions, which leads to their decreased market value (EPPO, 2013).

One of the most important management strategies and preventive control measures for plant pathogens is the testing and certification of seed and its production in areas where pathogens are not present or under unfavorable conditions for their development. In addition, seed is very important for crop production as more than 80% of crops are propagated from seed worldwide. Some studies have reported that even a low level of infection in the seed is enough to cause epidemics in the field (Kolb et al., 2007). According to the ISTA (2020) and the Official Gazette of the Socialist Federal Republic of Yugoslavia (1987), the most common methods for selecting high-quality seed are based on their physical properties, such as weight and germination, biochemical and other physical tests. *Xanthomonas* spp. bacteria are able to survive in seed (externally or internally), and can be spread by infected seed as a primary source of inoculum, leading to infection of subsequent crops (Bashan et al., 1982; Ritchie, 2000; Dutta et al., 2014; Utami et al., 2022). Seed is the major source of long distance dissemination. According to van der Wolf and Duriat (2006), *X. campestris* pv. *vesicatoria* survives in seed over long periods, even more than 16 years. Seeds may also be externally or internally infected with seed-borne pathogenic fungi (Martín et al., 2022). Pepper seed-borne fungi have been reported by several authors, indicating the presence of *Fusarium* sp., *Alternaria* sp., *Colletotrichum capsici*, *Phytophthora capsici*, *Rhizoctonia solani*, *Phoma capsici*, *Macrophomina phaseolina* and *Verticillium* sp. (Mushtaq & Hashmi 1997; Ali, 2007; Agarwal et al., 2007; Chigoziri & Ekefan, 2013). The most common fungi in *C. annuum* are *Fusarium* spp., which alone cause seed rot, seedling rot and root rot leading to significant damage in pepper production (Hasan et al., 2012).

The aim of this work was to determine the presence of plant pathogenic bacteria of the genus *Xanthomonas* (*X. euvesicatoria*, *X. vesicatoria*, *X. gardneri* and *X. perforans*) in pepper seed, and their influence on certain parameters (germination energy, total germination, moisture and seed health) of the quality of pepper seed collected in the territory of Smederevska Palanka in Serbia.

MATERIAL AND METHODS

Seed material

A total of 27 pepper seed samples originated from the locality Smederevska Palanka in 2021 (coded as P1-P27). The samples were retrieved from a collection of non-commercial genotypes of pepper seeds (sweet and chili pepper) of the Institute for Vegetable Crops (Smederevska Palanka). All samples for the analysis were stored in paper bags at a temperature of 20-22 °C in the laboratory.

Detection of *Xanthomonas* spp. in pepper seed

Extraction of bacteria from pepper seed

Each pepper seed sample (consisting of 20 g) was soaked in 3 ml g⁻¹ seed of sterile 10 mM phosphate buffered saline - PBS (containing: Na₂HPO₄ x 12H₂O 2.7 g; NaH₂PO₄ x 2H₂O 0.4 g; NaCl 8.0 g; distilled water 1000 ml) for a minimum of 14 h at 4 °C. The samples were then shaken for 2 h at room temperature (24 °C) and 115 rpm, then filtered and centrifuged at 11 000 g for 20 min at 10 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of sterile distilled water.

DNA extraction

Genomic DNA was isolated from the extracts obtained from seed using the DNeasy Plant Mini Kit (QIAGEN, Diagnostics GmbH, Qiagen AG) according to a protocol given by the manufacturer.

Polymerase chain reaction (PCR)

Two conventional duplex-PCR tests were used for detection of 4 species of the *Xanthomonas* complex (*X. euvesicatoria*, *X. vesicatoria*, *X. gardneri* and *X. perforans*). Amplification was performed in two separate reactions, each including two primer combinations: Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR; Bs-XgF/Bs-XgR and Bs-XpF/Bs-XpR, according to a protocol proposed by Koenraadt et al. (2009) (Table 1). PCR was programmed as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min. As a positive control, a strain coded as X22 from pepper isolated in Irig locality in 2016 and identified as *X. euvesicatoria* was used.

Table 1. Primers used in the study

Primer	Primer sequence (5'-3')	Species
Bs-XeF	CATGAAGAACTCGGCGTATCG	<i>Xanthomonas euvesicatoria</i>
Bs-XeR	GTCGGACATAGTGGACACATAC	
Bs-XvF	CCATGTGCCGTTGAAATACTTG	<i>Xanthomonas vesicatoria</i>
Bs-XvR	ACAAGAGATGTTGCTATGATTTGC	
Bs-XgF	TCAGTGCTTAGTTCCTCATTGTC	<i>Xanthomonas gardneri</i>
Bs-XgR	TGACCGATAAAGACTGCGAAAG	
Bs-XpF	GTCGTGTTGATGGAGCGTTC	<i>Xanthomonas perforans</i>
Bs-XpR	GTGCGAGTCAATTATCAGAATGTGG	

Amplified PCR products were visualized by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide under UV light. The expected amplicon sizes in base pairs were as follows: Bs-XeF/R primers 173 bp; Bs-XvF/R primers 138 bp; Bs-XpF/R primers 197 bp and Bs-XgF/R primers 154 bp. The DNA molecular weight marker GeneRuler Low Range DNA Ladder, ready-to-use, was used for fragment size estimation.

Analysis of pepper seed quality parameters

Seed testing of 27 genotypes of pepper was performed using standard methods for assessing seed quality and health (ISTA, 2020). Pepper seed quality was evaluated based on germination parameters (germination energy and total germination), moisture and seed health.

Germination

Germination energy and total seed germination were tested using the standard filter paper method (ISTA, 2020). Samples of different pepper genotypes consisting of a total of 400 seeds (100 per replicate) were placed in Petri dishes with filter paper moistened with 0.2% KNO₃. Quality analyses, especially germination, revealed abnormal seed unable to develop by the end of the test period, which will not eventually develop into healthy seedlings. Seeds were incubated for 7 and 14 days at 23 °C. The final seedling count was made after 14 days for all pepper genotypes.

Moisture

For moisture determination, pepper seed samples were measured thermogravimetrically to their constant weight. To measure moisture content in seed samples, consisting of 5 g, they were kept at a temperature of

105 °C ± 2 °C for 17 h ± 1 h. Seed moisture (SW) is defined as the water in seed, and calculation was performed according to the following formula:

$$SW(\%) = \frac{(m3 - m1)}{(m2 - m1)} \times 100$$

where:

m1 (g) = the mass of a container and lid;

m2 (g) = the mass of a container, lid, and content before drying;

m3 (g) = the mass of a container, lid, and content after drying.

Seed health

The seed health of pepper genotypes was tested for the presence of *Alternaria* sp. and *Fusarium* sp. Seed testing was performed using the standard method on filter paper (ISTA, 2020). According to the Official Gazette of the Socialist Federal Republic of Yugoslavia (1987), the percentage limit for seed infected with either plant pathogenic fungus is under 5%.

After incubation, the results were rated according to the following formula:

$$\text{Seed health (\%)} = \frac{\text{number of infected seeds}}{\text{total number of seeds}} \times 100$$

Statistical analysis

Statistical analysis was performed using the SPSS software (version 23, IBM, USA). The effects of factors were evaluated by ANOVA (F-test) and Tukey's Multiple Range Test ($p \leq 0.05$) to determine the effects of their means. The coefficients of correlation (r) were calculated for the interrelationships between the observed traits. Differences of $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

The presence of a plant pathogenic bacterial population of *Xanthomonas* spp. in seed of different pepper genotypes, and bacterial influence on seed quality parameters (germination energy, total germination, moisture, seed health) were determined in this study. The analysis included 27 genotypes of pepper (sweet and chili pepper) seed obtained in the season of 2021.

Detection of *Xanthomonas* spp. in pepper seed

The results showed that out of 27 analyzed pepper seed samples, 13 samples were confirmed for the presence *X. euvesicatoria* after amplification of product size of 173 bp using the primers Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR in duplex-PCR (Figure 1). *X. vesicatoria*, *X. gardneri* and *X. perforans* were not detected in any of the analyzed pepper seed samples.

In this study *X. euvesicatoria* was identified as the only species present in pepper seed samples collected in 2021. It is not surprising since this species is found as the most common in Serbian pepper production (Ignjatov et al., 2010; Vlajić et al., 2017; Giovanardi et al., 2018). Moreover, the presence of *X. euvesicatoria* has been identified as prevalent on pepper in the American continent (Potnis et al., 2015; Hernández-Huerta et al., 2021; Areas et al., 2015), and in European countries (Bogatzevska et al., 2007; Vancheva et al., 2021). It suggests an expansion of this species as an emerging threat to pepper production (Potnis et al., 2015).

As a *Xanthomonas* sp. can be disseminated via contaminated seed or plant material, its detection in tomato or pepper seed and seedlings is critical for the reduction of potential inoculum sources (Potnis et

al., 2015). The pathogen can be detected using several techniques, but largely via a combination of classical and molecular approaches. Conventional pathogen detection is based on cells and culture characterization, while PCR and Loop-Mediated Isothermal Amplification (LAMP) are available among molecular methods (Koenraad et al., 2009; Araújo et al., 2012; Utami et al., 2022). Considering that *Xanthomonas* species remain inside the seed, the use of disease-free seed and seedlings is one of the key management tools. Diagnostic testing of seed health is typically used to detect the level of seed infection (Utami et al., 2022). In that context, our further efforts will be focused on the use of appropriate seed-pathogen isolation methods in order to detect viable *Xanthomonas* cells, and to demonstrate their role in further development and spreading of bacterial spot disease under field conditions.

Pepper seed quality parameters

Germination energy in infected pepper seed ranged from 52 to 84%, and in bacteria-free seed from 63-90%. Total germination of infected seed was found to range from 66 to 91% and of bacteria-free seed between 80 and 95% (Table 2). For all tested pepper genotypes, total germination was above the proposed minimum of 55%; however, some genotypes had total germination of more than 90% (P15, P16, P219 and P23).

The results revealed no statistically significant difference among most of the pepper genotypes ($p > 0.05$) (Table 2) relating to germination energy and total germination; only the genotypes coded as P1, P2, P7, P10, P13, P15, P16, P19, P23 differed at the statistical level of $p < 0.05$, but with no evidence of an influence of *X. euvesicatoria* infection.

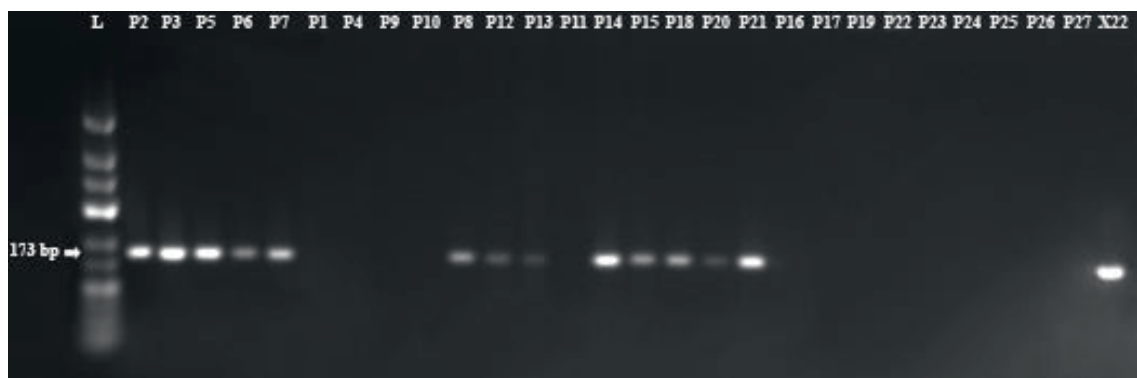


Figure 1. Identification of *X. euvesicatoria* in seed samples using primers Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR in duplex-PCR. Legend: L–Ladder (GeneRuler Low Range DNA Ladder, ready-to- use); P–tested pepper seed samples; X22– reference strain.

Germination is the most important parameter for high classification of seed quality. In a study conducted by Kurtulmus et al. (2016), 101 different pepper varieties had germination rates of c. 85%, so that the authors suggested that such seeds can be selected for producing high-quality pepper plants.

In all analysed pepper genotypes total seed germination was high and *X. euvesicatoria* contamination did not influence the quality of seed ($p > 0.05$). The lowest total germination was observed in the samples P2 and P13, which could be associated with higher moisture percent and detected fungi (*Fusarium* sp. and *Alternaria* sp.). Seed moisture ranged from 6.1-7.9% in the infected and 6.2-8.1% in bacteria-free pepper seed (Table 2). Only in the samples P2 and P13 moisture was higher than 11.5% and 12%, respectively, and with statistical significance compared to the other tested seed ($p > 0.05$).

According to Gebeyehu (2020), moisture content in seeds gradually increases during storage, reducing seed quality depending on the reduction in germination percentage. Low seed moisture and naturally-occurring antifungal substances have been suggested to be the main barriers to their development (Costa et al., 2019).

The presence of *Fusarium* sp. seed-borne fungi was in the range of 2-3% for both infected and bacteria-free tested seed, and *Alternaria* sp. presence ranged from 1-3% in infected seed, while it was estimated at 4% in bacteria-free seed. Only the genotypes P2 and P13 had higher percentages of *Fusarium* sp. (5% and 6%, respectively) and *Alternaria* sp. (4% and 5%, respectively) infection, compared to the other pepper genotypes. In general, the obtained results revealed that the seed-borne fungi were present in all pepper seed samples but they did not significantly affect total germination or reduce overall germination.

Table 2. Parameters of quality (energy, total germination and moisture) of *Xanthomonas euvesicatoria*-infected (13 genotypes) and bacteria-free (14 genotypes) pepper seed samples

Pepper genotype	Germination energy (%)	Total germination (%)	Moisture (%)	<i>Fusarium</i> sp. (%)	<i>Alternaria</i> sp. (%)	
Infected seed	P2	52±0.5b	66±0.4b	12±0.2b	5±0.5	4±0.5
	P3	67±0.4b	88±0.2a	6.2±0.5ab	1±0.2	1±0.1
	P5	76±0.2a	85±0.1a	7.3±0.0a	2±0.5	1±0.5
	P6	75±0.5a	82±1.0a	7.2±0.1a	3±0.1	1±0.3
	P7	65±0.7b	75±0.5b	6.4±0.0ab	1±0.1	2±0.1
	P8	77±0.2a	87±0.8a	7.5±0.1a	1±0.5	3±0.3
	P12	78±0.8a	86±0.2a	7.5±0.1a	2±0.6	1±0.2
	P13	53±0.4b	63±0.4b	11.5±0.1b	6±0.1	5±0.1
	P14	78±0.8a	87±0.9a	6.9±0.1a	2±0	1±0.4
	P15	84±0b	91±0.7b	6.9±0.2a	1±0.4	2±0.2
	P18	79±0.5ab	88±0.7a	7±0.1a	1±0.3	2±0
	P20	82±1.0ab	89±0.6a	7.9±0.1b	2±0.2	1±0
	P21	77±0.2a	89±0.7a	6.1±0.0ab	2±0.1	1±0.1
Bacteria-free seed	P1	63±0.4b	80±0.6ab	6.9±0a	3±0.2	4±0
	P4	77±0.5a	85±0.6ab	6.8±0.1a	1±0.1	1±0.1
	P9	75±0.2a	84±0.2ab	6.9±0.3a	2±0.1	2±0
	P10	68±0.4b	81±0.2ab	6.2±0.5ab	1±0.4	2±0
	P11	75±0.4a	81±0.2ab	7.1±0.3a	1±0.2	3±0
	P16	79±1.0ab	92±0.2b	7.3±0.5a	2±0.3	1±0.2
	P17	77±0.4a	87±1.0a	7.5±0.1a	3±0.1	1±0.4
	P19	90±0.6b	95±0.6b	6.9±0.1a	2±0.4	1±0.7
	P22	72±0.6b	82±0.7ab	7.1±0.1a	1±0.2	2±0.5
	P23	82±0.5b	95±0.4b	8.1±0.1b	2±0.5	1±0.1
	P24	77±0.5a	85±0.5a	7.2±0.1a	3±0.3	3±0.5
	P25	75±0.8a	86±0.5a	6.8±0.3a	1±0.2	4±0.2
	P26	74±0.7a	80±0.7ab	6.9±0.4a	1±0.1	2±0.1
	P27	75±0.8a	85±0.8a	7.1±0.1a	2±0.1	1±0

Different lowercase letters mean significant effect: a-no statistical significance between genotypes; b- statistical significance between genotypes; ab-difference between genotypes ($p \leq 0.05$); Tukey's Multiple Range test for the column. Values are means ±standard deviation of the mean.

Table 3. The correlation coefficient (r) for the observed traits in 27 pepper genotypes (including seed infected with *Xanthomonas euvesicatoria*)

Traits	Total germination	Germination energy	Moisture	<i>Fusarium</i> spp.	<i>Alternaria</i> spp.	Infected pepper seed
Total germination		0.958***	-0.694	-0.649	-0.418	
Germination energy			-0.422	-0.450	-0.436	
Moisture				0.580	0.582	
<i>Fusarium</i> sp.					0.462	
<i>Alternaria</i> sp.						
Bacteria-free pepper seed						0.341

Pearson's correlation coefficient: *** $p < 0.001$

The presence of *Fusarium* sp. fungi can result in significant reductions in crop production. Study results on the effects of phytopathogenic fungi on pepper seed germination showed that *F. oxysporum* and *F. solani* reduced seed germination more than the other species. These seed-borne pathogens can reduce pepper seed germination by more than 50% (Liang, 1990; Ali, 2007). Moreover, *Alternaria* spp. is a common pathogen of *Capsicum* spp. worldwide (Nasehi et al., 2014). Soomro et al. (2020) found significant differences in seed germination, number of abnormal seedlings and root length of rapeseed (*Brassica napus*) infected with *Alternaria* spp., compared to fungi-free seed.

In the analysis of parameters of seed quality, the strongest correlation ($r = 0.958$, $p < 0.001$) was found between total germination and germination energy, which indicates a positive correlation between them (Table 3). Pearson's correlation coefficient for these traits indicates that an increase in germination energy will lead to an increase in total germination. Similar results were obtained in a study by Poštić et al. (2020) performed on tomato seeds ($r = 0.8711$, $p < 0.001$). A negative correlation coefficient indicates the possibility of declining energy and total germination, allowing positive growth of *Fusarium* sp. and *Alternaria* sp. at a statistically significant level ($p < 0.05$). The positive correlation coefficient for the traits moisture, *Fusarium* sp. and *Alternaria* sp. indicates that the percentage of seed infected with those fungal species will grow with increasing moisture.

CONCLUSION

The presented research showed that the plant pathogenic bacterium *X. euvesicatoria* is the predominant pathogen of the *Xanthomonas* spp. complex in seed of several pepper genotypes collected in Smederevska

Palanka in Serbia during 2021. The parameters of quality (germination energy, total germination, moisture and seed health) of all 27 tested samples were above the threshold minimum, indicating no influence on the tested quality parameters of pepper seed.

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Uticaj prisustva *Xanthomonas euvesicatoria* na parametre kvaliteta semena paprike u Srbiji

REZIME

U ovom radu izvršena je detekcija bakterija iz kompleksa *Xanthomonas* (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* i *X. gardneri*) i ispitan uticaj prisustva bakterija na određene parametre kvaliteta semena paprike poreklom sa teritorije Smederevske Palanke (Srbija). Analiza je obuhvatila 27 nekomercijalnih genotipova semena paprike (uključujući čili i slatku papriku) prikupljenih u sezoni 2021. godine. U radu su ocenjeni parametri kvaliteta semena paprike i to energija klijanja, ukupna klijavost, vlaga i zdravstvena ispravnost. Rezultati su pokazali da je od ukupno 27 analiziranih uzoraka semena paprike, prisustvo *X. euvesicatoria* detektovano kod ukupno 13 uzoraka. Prisustvo *X. vesicatoria*, *X. gardneri* i *X. perforans* nije utvrđeno ni u jednom uzorku semena paprike. Energija klijanja zaraženog semena je iznosila od 52-84%, a kod semena bez prisustva bakterija od 63-90%; ukupna klijavost u zaraženom semenu je bila od 66-91%, a u semenu bez prisustva bakterija 80-95%. Vlažnost semena u zaraženim uzorcima je iznosila 6,1-12%, a u uzorcima bez prisustva bakterija između 6,2-8,1%. Utvrđivani parametri kvaliteta se nisu značajno razlikovali na statističkom nivou ($p>0,05$). Prisustvo fitopatogenih gljiva koje se prenose semenom je utvrđeno u svim uzorcima semena paprike, i to *Fusarium* sp. do 3% kod ukupno 25 uzoraka, dok je kod dva uzorka zaraza bila i do 6%; prisustvo *Alternaria* sp. je bilo od 1-4% kod ukupno 25, a kod dva uzorka više, do 5%. Dobijeni rezultati ukazuju da je bakterija *X. euvesicatoria* dominantan patogen iz kompleksa *Xanthomonas* vrsta, ali da utvrđeno prisustvo nije značajno uticalo na parametre kvaliteta semena paprike.

Ključne reči: paprika, bakteriozna pegavost, klijavost, vlaga, zdravstvena ispravnost