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Screening Wild Pepper Germplasm for Resistance to *Xanthomonas hortorum* pv. *gardneri*

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Abstract: Bacterial spot disease on peppers is caused by four species of the genus *Xanthomonas*. This disease causes black spot lesions not only on the leaves but also on the fruit, leading to yield and quality loss. *Xanthomonas* species cause major disease outbreaks in tropical, subtropical and humid continental regions worldwide. Bacterial blight caused by xanthomonads occurs on both greenhouse- and field-grown peppers and is particularly important in areas characterized by hot and humid environmental conditions. As pesticides are currently not sufficiently effective in the control of bacterial spot, the development of pepper varieties resistant to *Xanthomonas* species, including X. *hortorum* pv. *gardneri*, is of primary importance for sustainable production. In our research, 119 lines of *Capsicum baccatum* from the USDA ARS gene bank (Griffin, GA) and MATE (Hungarian University of Agriculture and Life Sciences) were tested against strains of *X. hortorum* pv. *gardneri* under greenhouse conditions. Four accessions of the wild pepper species *C. baccatum* appeared to be resistant to seven strains of *X. hortorum* pv. *gardneri* in greenhouse trials. The resistant genotypes of *X. hortorum* pv. *gardneri* identified in this study can be used for the resistance gene pyramidation against different bacterial spotted *Xanthomonas* species in pepper.

Keywords: sustainability; pepper breeding; bacterial spot resistance; *Xanthomonas hortorum* pv. *gardneri*

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1. Introduction

Pepper (*Capsicum* spp.) is one of the most important vegetables and spices worldwide because of its versatility, its high nutritional value, as well as its role in reducing human micronutrient deficiencies [1]. The chili pepper genus is currently known to consist of 43 species [2] that are native to temperate and tropical Central and South America, Mexico and the West Indies, five of which are domesticated: *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* [3]. Despite the crucial agronomic importance of this crop, diseases are still one of the most important damaging factors in pepper production, and only few can be controlled by chemical treatments. However, environmental and consumer concerns, as well as the risk of crop pathogen insensitivity, underline the importance of developing resistant pepper varieties as a more effective way of reducing the impact of disease [4,5]. *Capsicum* germplasm collections represent an immense genetic wealth resource this purpose, but screening for disease resistance is often a lengthy, labor-intensive, expensive and mostly complex process.

Bacterial spot disease of peppers occurs in both processing and fresh market pepper fields, particularly where growing conditions are characterized by high humidity. Known for its aggressiveness, the pathogen *X. hortorum* pv. *gardneri* causes large, star-shaped necrotic lesions on the foliage, stem and fruit. Diseased leaves drop prematurely, resulting in extensive defoliation, thus reducing plant productivity and exposing fruits to potential sunscalding. Stem lesions occur as narrow, light brown, longitudinally raised cankers. Fruit with such large lesions are non-marketable in both the fresh and processed markets.

Bacterial spot is caused by four bacterial species of the genus *Xanthomonas*: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. hortorum* pv. *gardneri* [6]. *X. hortorum* pv. *gardneri* was first described in 1957 [7]. The pathogen *X. hortorum* pv. *gardneri* was recognized as a causative agent of bacterial spot epidemics in the year 2000. Currently, there are few chemicals available that are effective against these pathogens, and even these are ineffective under high disease pressure and have a negative impact on the environment. Since the overuse of copper has led to the development of resistance in the bacterial population, there is a need to find new sustainable and environmentally friendly alternatives [8,9]. The growing number of bacterial spot diseases caused by emerging varieties of xanthomonads makes it increasingly urgent for growers to find practical and sustainable solutions. The use of varieties resistant to bacterial leaf spot offers a potential tool for disease control in field conditions. The spread of new *Xanthomonas* species over the last few years has made the discovery of new sources of resistance a major priority. Thus, host resistance is considered to be a critical element of disease management strategies.

Resistance breeding programs successfully developed commercial pepper lines with hypersensitive and quantitative resistance to various *Xanthomonas* species [10]. Genes regulating resistance have been found in *X. euvesicatoria* [11], *X. perforans* species T3 [12], *X. perforans* species T4 [13], and *X. vesicatoria* [14], and six dominant resistance genes have been identified in pepper to date, namely *Bs1*, *Bs2*, *Bs3*, *Bs4C*, *Bs7* and *BsT* [15]. However, the persistence of resistance can change rapidly due to the changing geographical distribution of the pathogen and the rapid emergence of new pathogenic variants [16]. In the case of *X. hortorum* pv. *gardneri*, only one resistance gene source has been described so far, although in this case the infection was caused by a single *Xantomonas* strain (USVLXG1) and resistance was not tested with other *Xanthomonas* isolates [17]. The introduction of resistance genes with different mechanisms of action could provide a durable and sustainable solution to this ongoing host–pathogen arms race.

Commercial pepper varieties carrying recessive resistance genes, namely *bs5* and *bs6*, have not proven effective against *X. hortorum* pv. *gardneri*. Based on current publications, the recessive resistance gene *bs8* identified in *C. annuum* accession PI 163192 is the only one that was shown to confer resistance to *X. hortorum* pv. *gardneri* [18]. With the increasing frequency of *Xanthomonas* outbreaks around the world, there is a growing rationale to identify new sources of resistance in pepper to emerging bacterial species, including *X. hortorum* pv. *gardneri* and to incorporate these new resistance genes into breeding programs.

Preliminary results are presented below, which are aimed at screening wild pepper cultivars for heritable resistance to *X. hortorum* pv. *gardneri*. The aim of our current work was to identify new sources of resistance to *X. hortorum* pv. *gardneri* in pepper and to identify potential candidate genes involved in resistance to bacterial spot disease. A diverse USDA panel of pepper accessions were screened for resistance to *X. hortorum* pv. *gardneri*, and highly resistant accessions were identified in our work.

2. Materials and Methods

2.1. Plant Materials

The work was carried out on a collection of *C. baccatum* accessions originating from the USDA ARS genebank. We tested 119 accessions including 97 *C. baccatum* var. *pendulum*, 2 *C. baccatum* var. *pratermissum*, 7 *C. baccatum* var. *baccatum*, and 2 *C. baccatum* var. *umbilicatum*, and in 11 cases the variant was not specified (Table S1). In order to improve germination, the seeds were pre-soaked for four hours before they were sterilized with 10% calcium hypochlorite ($Ca(ClO)_2$) solution for twenty minutes with a few drops of Tween20. The seeds were rinsed five times with sterile Milli-Q Water. After that, all of the seeds were placed on MS medium [19] with 20 g sucrose (MS20) in sterilized glass storage jars until the cotyledon stage. Afterwards, the plants were placed into pots filled with Klasmann Traysubstrate soil mixed with 50 g NPK and microelement fertilizer (14% N + 7% P + 21% K + 1% Mg + 1% microelements (B, Cu, Mn, Fe, Zn) to 70 L of soil.

The plants were then grown under controlled greenhouse conditions at 24 ± 2 °C, with 14/10 h photoperiods, and 50–70% relative humidity until they reached the six-mature-leaf stage. A cultivated variety *C. annuum* Fö "Fehérözön" was used as a susceptible control.

2.2. Bacterial Isolates and Plant Infiltration

To find a resistant phenotype, two *Xg* strains were used for plant infiltration—LMG962 from the Belgian Coordinated Collections of Microorganisms (BCCM) and SRB, which is a field isolate from Serbia [7]. Bacteria were cultivated in YDC [20] medium for two days in an incubator at a temperature of 30 °C. After two days of incubation, bacteria were collected and diluted with sterile Milli-Q Water to a final concentration of 10^6 cfu/mL. The infiltration was performed in planta with a needle-free syringe into the intercellular spaces on the abaxial side of the first true leaves. After infiltration, the plants were placed in plant growth chambers at 25 °C with a 16/8 h photoperiod, at 50–70% relative humidity.

The response to infection was monitored continuously, but the final evaluation was made after 7 days. The plants that showed complete resistance to both isolates were also tested with five further *Xg* strains: Xg51, Xg152, Xg153, Xg156, Xg177 (Table 1), which were donated by Brian Staskawicz (Innovative Genomics Institute University of California, Berkeley).

Table 1. *Xg* strains used in this study and their origin.

Species	Strain Name	Origin	Host Isolated	Year Isolated	Isolation ID	Collector
X. hortorum pv. *gardneri*	LMG962	Republic of Yugoslavia	tomato	1957	-	Sutic D.
	SRB	Republic of Yugoslavia	tomato	1957	-	Sutic D.
	Xg51	- [1]	-	-	-	T. Minsavage
	Xg152	-	-	-	-	-
	Xg153	Gibsonburg, OH	tomato	2010	SM194-10	S. Miller
	Xg156	Blissfield, MI	tomato	2010	SM177-10	S. Miller
	Xg177	Sandusky Co., OH	tomato	2012	SM795-12	S. Miller

[1] means that the exact origin, host, year, isolation ID or collector of the isolate has not been established.

2.3. Bacterial Growth Test

In order to determine how the presence of resistance affected bacterial growth in resistant plants, leaf disc samples were collected from the infiltrated area at different times. For the test of the LMG962 isolate, four resistant and three susceptible plants were used in two replicates at 25 °C and 30 °C. The photoperiod and humidity were the same as for the accession testing. Sampling was performed at 0, 2, 4, 6, 8, 10, 12 days post inoculation (dpi) from the inoculated area. In the case of resistant plants, re-isolation was also performed 30 days after infection to monitor possible changes in bacterial counts. The isolated leaf discs were macerated in 100 µL sterilized water and prepared in dilution lines and were scattered on YDC medium. After two or three days, the colonies were counted. The cfu/cm^2 of leaves was calculated by counting the number of adult colonies.

3. Results

3.1. Evaluation of C. baccatum Accessions for the Susceptibility and Resistance to X. hortorum pv. gardneri

One hundred and nineteen *C. baccatum* accessions were inoculated with two highly virulent strains (LMG962 and SRB) of *X. hortorum* pv. *gardneri* and evaluated for symptoms. Disease evaluation started one week after inoculation and the earliest typical disease symptoms were observed on the leaves 3–6 days after inoculation on the infected pepper plants. The average incubation period varied between 4 and 7 days until the initial abnormal discoloration appeared, while the length of time required to develop complete leaf necrosis in sensitive individuals varied between 6 and 10 days.

Susceptible symptoms typically started with chlorotic lesions, which developed into angular, water-soaked lesions and eventually into brown or black necrotic lesions. Phenotyping of the control line "Fehérözön" (highly susceptible) conformed to the expected disease reactions. In our studies, we found that *C. baccatum* accessions differed in terms of

the earliness and intensity of symptoms. The vast majority of the accessions were susceptible to both strains, showing a similar phenotype to "Fehérözön" used as a control. A small part of the accessions showed partial resistance to one of the *X. hortorum* pv. *gardneri* strain. Based on the evaluation of their reactions, four of the 119 *C. baccatum* genotypes showed strong resistance to both *Xg* isolates used in the first selection round (Figure 1).

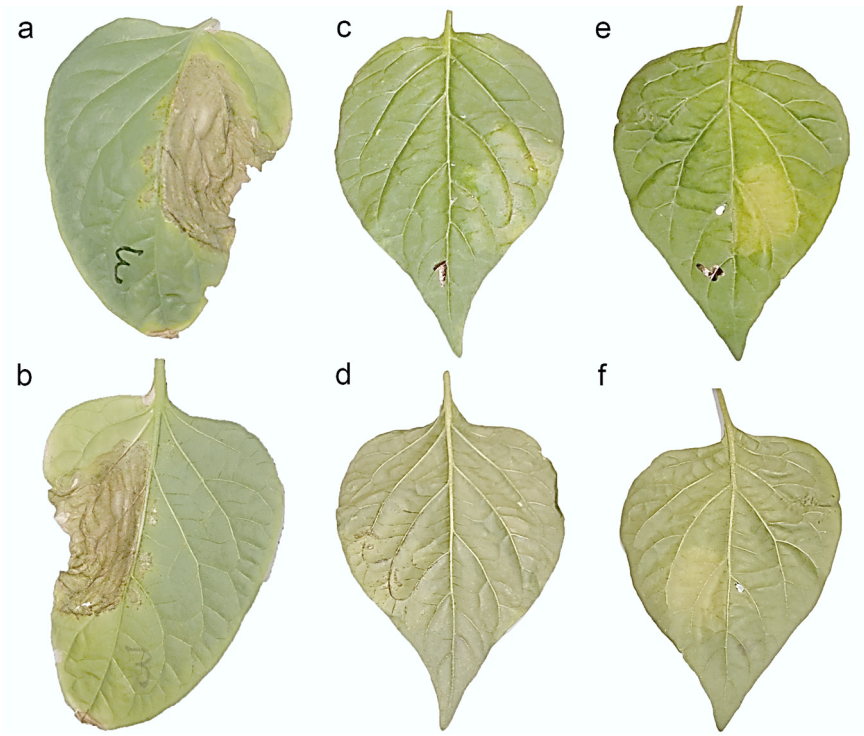

Figure 1. Symptoms of bacterial spot 7 days after inoculation with strain LMG962 in susceptible Fö (**a**,**b**) and the resistant phenotype on Cbp2 plants infected with strain LMG962 (**c**,**d**) and SRB (**e**,**f**). Adaxial and abaxial view.

In the second phase, all four previously resistant phenotypes of *C. baccatum* accessions were challenged with five additional *Xg* isolates. Based on our results, all four accessions also showed strong resistance (Table 2) to all five additional *Xg* isolates (Xg51, Xg152, Xg153, Xg156, Xg 177).

Table 2. *C. baccatum* var. *pendulum* accessions showing partial or complete resistance to the *X. hortorum* pv. *gardneri* strains used.

Species	Variety	Accession	SRB	LMG962	XG51	XG152	XG153	XG156	XG177
C.baccatum	*pendulum*	Cbp2	+	+	+	+	+	+	+
		Cbp3	+	+	+	+	+	+	+
		Cbp4	+	+	+	+	+	+	+
		PI 441541	+	–					
		PI 441542	+	–					
		PI 370010	–	+					
		PI 441543	+	-					
		PI 441578	+	–					
		PI 441552	–	+					
		PI 441533	–	+					
		PI 441520	+	–					
		Cbp1	+	+	+	+	+	+	+

+: resistant response to infection, –: sensitive response to infection.

In the resistant plants, the infected area turned light green, but at 30 dpi, it was no longer possible to distinguish between infiltrated and untreated areas. Small necrotic spots

appeared on the leaves at 10–12 dpi typically along the leaf veins, but these lesions increased very little if at all after 30 days (Figure 2). No difference was observed between the emerging phenotype in resistant plants according to the bacterial strain used for inoculation.

Figure 2. Symptoms of bacterial spot 30 days after inoculation with strain LMG962, SRB and Xg51 in a resistant accession plant.

3.2. Evaluation of the Bacterial Growth Test

We determined the effect of the identified resistance gene on bacterial growth in the plant and the correlation of visual symptoms with bacterial population levels. The growth in the populations of different bacterial races was monitored for 12 days after inoculation and incubation at 25 °C and 30 °C. "Fehérözön" was used as a negative control. Compared to the almost equal initial values, the bacterial growth curve in planta at 25 °C indicated that resistant plants reached a maximum of 4.75 log at 4 dpi and then showed a minimal decrease with no significant change in the following 6–12 days. In contrast, in the case of the sensitive plants, a value of 6.7 log at 4 dpi was obtained and a steady increase was detected until leaf drop at 8 dpi (Figure 3). In the majority of cases, the infected leaves of the susceptible plants had fallen off by the eighth day and sampling could not be continued. At 30 °C, the resistant plants reached a maximum value of 5.55 log at 6 dpi and then a slight decrease was observed. Susceptible plants showed a value of 7.1 log at 6 dpi until leaf drop (Figure 4). In susceptible plants, the temperature had no significant effect on the growth curve of *Xg* bacteria, but significantly affected the tolerance of plants to infection, because inoculated leaves fell off earlier at higher temperatures. Plants kept at 25 °C were also sampled at 30 dpi and the results show that no significant change in the cfu value in the leaf occurred. In the case of plants kept at 30 °C, it was not possible to collect a sample after one month, due to early aging of the plant and leaf fall.

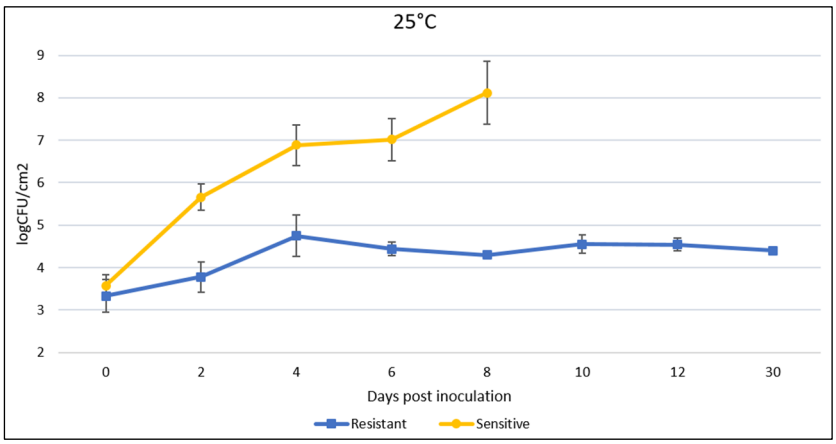

Figure 3. Time course of bacterial population growth at 25 °C after infiltration of leaves of resistant and susceptible plants by LMG962 strains.

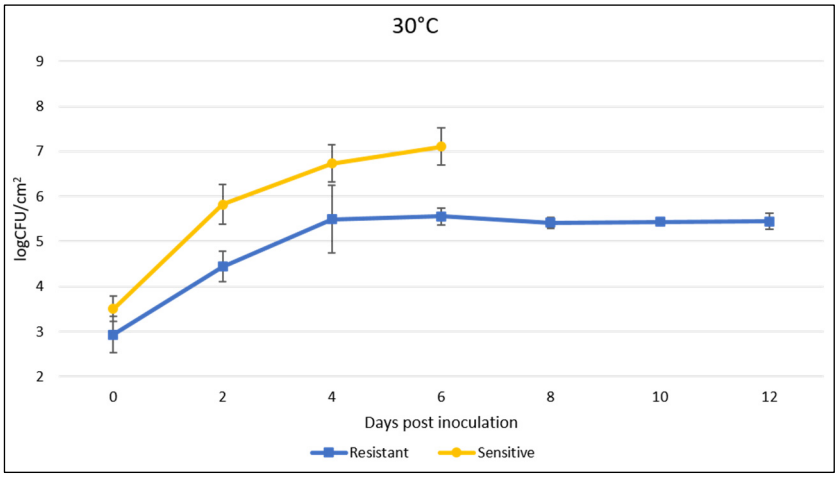

Figure 4. Time course of bacterial population growth at 30 °C after infiltration of leaves of resistant and susceptible plants by LMG962 strains.

4. Discussion

C. baccatum is the most popular and widely cultivated chili pepper in South America. There is a wild form called *C. baccatum* var. *baccatum* and a domesticated form (*C. baccatum* var. *pendulum*) [21]. Brazil is considered to be the center of the genetic diversity of *Capsicum* spp., and at least 116 accessions already described are found in Brazil [22,23]. It is expected that new species [2] and new resistance genes for pepper breeding will be found in this important and untapped genetic resource. Pathogens are increasingly threatening the cultivation and yield of most *Capsicum* species worldwide. In this context, the search for new sources of resistance remains a major challenge today and *Capsicum* germplasm collections represent a promising target for this purpose.

Previous searches for resistance to *X. hortorum* pv. *gardneri* concentrated on the *Xg* isolates which are most prevalent in the US. In contrast to previous research, in our present work we also used the isolates most commonly found in Europe and the US. The results of this screening study in which pepper accessions were inoculated with seven *Xg* strains indicated that there were significant differences in resistance between PI lines from the USDA ARS pepper germplasm collection. Screening was carried out in two stages. As a first step, 119 accessions from the *C. baccatum* collection were challenged with two different strains of *X. hortorum* pv. *gardneri* to determine the extent and types of resistance in *C. baccatum*. Sensitive symptoms of bacterial spot were observed in 107 accessions and *Xg* was recovered from all plants, indicating that none of the accessions carried a source of resistance to this pathogen. Eight accessions showed partial resistance to one of the *Xg* isolates and strong resistance was identified in four PI lines (Cbp1, Cbp2, Cbp3 and Cbp4). In the second stage, screening was carried out on Cbp1, Cbp2, Cbp3 and Cbp4 accessions using the five potentially important isolates in Europe and the US. Complete screening was carried out under controlled conditions in growth chambers using a method (leaf screening of young plants with a concentrated bacterial suspension) that clearly indicated the presence of the pathogen and the rapid onset of symptoms.

The screening results showed two main types of resistance (Table 2) in the tested plant population. Species-specific resistance was the most common and was present in 6% of all accessions tested. Resistance to all species used (potential species-nonspecific resistance) was very rare and was present in 3% of accessions tested.

5. Conclusions

Species shifts in bacterial spot pathogen populations are very common [10,24] and pose a serious problem and challenge for pepper breeding programs due to the decline in resistance persistence [25]. Therefore, there is an increasing need to identify new sources of resistance to these pathogens. The resistance germplasm of *X. hortorum* pv. *gardneri*

identified in this study can be of significant importance for both breeders and researchers and is highly relevant for introgression into pepper lines and in terms of gene pyramidation efforts against various *Xanthomonas* species. The resistance genes that we identified in *C. baccatum* might also have significant potential for resistance inhibition in other pepper species such as *C. annuum*. Interspecific crosses provide the opportunity to pass genes from one species to another, but in most cases, this can present several difficulties. A successful wide hybridization among species is highly dependent on the genetic distance between species. According to our current knowledge, two options are available for introgressing *C. baccatum* genes into *C. annuum*. The first option is using *C. chinense* and *C. frutescens* as bridge species [26], while the second option is to carry out direct crosses between *C. annuum* and *C. baccatum* combined with in vitro embryo rescue [27]. Our future goal is to use both methods to introduce our newly identified valuable resistance gene into *C. annuum*. Using the four resistant parental lines, crosses are underway with the aim of creating F1 and F2 progeny to determine the inheritance of disease resistance.

The new sources of resistance identified in this study provide a basis for further work on breeding disease-resistant varieties that are resilient to the changing population structure of pathogens. The current study showed that the different genotypes of *C. baccatum* differed in their response to wilt caused by the pathogen *Xanthomonas*, which also demonstrated that the pepper PI collection offered a valuable public source of resistance for pepper breeders to develop varieties resistant to bacterial spot. In conclusion, the information in this study can be summarized in that the use of a resistant genotype, together with cultural practices and sanitary control measures, is considered the most feasible and sustainable way to control *X. hortorum* pv. *gardneri* wilt.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15020908/s1, Table S1: Screening of *C. baccatum* germplasm to *X. hortorum* pv. *gardneri*.

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