



## Statistical analysis of resistance variability of pepper accessions to *Xanthomonas euvesicatoria* and *Xanthomonas vesicatoria*

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**Abstract.** The limit values of resistance groups were established to *Xanthomonas euvesicatoria* and *Xanthomonas vesicatoria* by mean score - ms based on conducted statistical analysis of 155 selection lines of Bulgarian, introduced hybrids and pepper varieties: I- immune; R- resistant; MS- medium sensitive; S- sensitive; SS- strongly sensitive. The genetic diversity between the two bacteria suggest the use of separate resistant groups to determine the resistance. Data on the mean score of infestation on 116 pepper accessions to *X. euvesicatoria* and 155 (the previous 116 included) to *X. vesicatoria* were processed and the resistance groups were defined. Breeding pepper accessions with complex resistance requires the application of the established resistance groups to both pathogens. The limit values of the groups by ms based on the statistical analysis of the data on the resistance of pepper accessions to *X. euvesicatoria* and *X. vesicatoria* were specified.

**Keywords:** limit value, pepper accession, *Xanthomonas euvesicatoria*, *Xanthomonas vesicatoria*, statistical analysis

### Introduction

Bacterial spot of pepper was a serious disease worldwide where climatic conditions favourable, the pathogens provoked significant yield losses (OEPP, 2013; Schwartz et al., 2015; Potnis et al., 2015; Dhakal et al., 2019). In Bulgaria, bacterial spot caused by *X. euvesicatoria* and *X. vesicatoria* was a threatening disease and a major limiting factor in the pepper production areas. *X. euvesicatoria* was currently the dominant pathogen of pepper, while *X. vesicatoria* was widespread in tomato. The Bulgarian population of *X. euvesicatoria* was characterized with genetic diversity and private allelic richness (Bogatzevska et al., 2007, 2021; Kizheva et al., 2011; Stoyanova et al., 2014; Vancheva, 2015; Vasileva and Bogatzevska, 2019; Vancheva et al., 2018, 2021).

*X. vesicatoria* and *X. euvesicatoria* were of historical importance and prevalent worldwide. According to the gene-for-gene hypothesis, eleventh pepper races had been identified among *Xanthomonas* strains worldwide and five non-allelic dominant genes (Bs1, Bs2, Bs3, Bs4, and Bs7) were reported to control hypersensitive reaction (HR). Currently available commercial bacterial spot-resistant cultivars contain various combinations of resistance genes. Because of the pathogens' ability to rapidly overcome major genes, breeding for bacterial spot resistance may ultimately dictate incorporation of quantitative resistance and major resistance gene pyramids in commercial cultivars. The predominance of races on the cultivars was to an extent related to the resistance genes present in them (Stall et al., 2009; Potnis et al., 2015).

The use of resistant cultivars was the most appropriate for the development of these plants, it was necessary to know the genetic base that controls the resistance, allowing the selection of the most appropriate breeding method.

The breeding of varieties with durable and complex resistance was a long and composite process that required extensive, diverse immunity researches, predeceasing the selection process. This necessitates to study the species composition of the causes of bacterial spot pepper in Bulgaria, pathotype, race, virulence, aggressiveness, competitiveness of natural populations; the appearance of new races, able to overcome the resistance of established breeding lines and introduce into hybrids and varieties. The cyclical development and spread of the pathogens, the appearing of new species and races in the country requires extensive research on the specific resistance mechanism, genetic research to establish the resistance genes Bs of pepper, differentially interacts with races of bacterial pathogens, and corresponding avrBs genes in their natural populations.

The breeding of resistant lines, hybrids and varieties was associated with the correct choice of resistance components: bacterial pathogens – species; races; host phenophase; infection method; disease ratings on leaves and scale ranging from 0 to 4, classification into groups with proven limit values on the basis of mean score (ms) of infestation (Pesti et al., 1990).

The aim of this paper was to make statistical analysis of data on resistance variability of pepper accessions to *Xanthomonas euvesicatoria* and *Xanthomonas vesicatoria* (2005-2016) and

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their clustering into groups with proven critical values defined by the mean score (ms) of infestation as follows: I- immune; R- resistant; MS- medium sensitive; S- sensitive; SS- strongly sensitive.

## Material and methods

Data from published articles of Bogatsevskaja et al. (2006), Stoimenova et al. (2005, 2006), Bogatzevska and Pandeva (2009) and Vancheva et al. (2016) were processed by statistical methods to group and define pepper accessions in a mean score (ms) and to define their resistance to the causes of the bacterial spot- *Xanthomonas euvesicatoria* and *Xanthomonas vesicatoria*. The defoliation index was calculated according to Pesti et al. (1990). The mathematical analysis included the results of the evaluation of 155 pepper selection lines (selection of Institute of plant physiology and genetics, Bulgarian Academy of Sciences, Sofia; Maritsa Vegetable crops research institute, Plovdiv), Bulgarian and introduced hybrids and varieties.

Data on mean score (ms) of infestation and defoliation index were processed by variation analysis. The coefficient of variation (CV, %) provided information on the dispersing of the sing, expressed as a percentage. The standard deviation (SD) for resistant grouping was applied. The least significant difference (LSD) used t-tests to present all comparisons in pairs between the group of means. LSD for the multiple comparison test was the equivalent of several separate t tests between all pairs in the groups. This allows direct comparisons between two averages of two separate groups. Any difference greater than LSD was considered significant. The R-4.0.4 with R Studio-1.4 (Mendiburu, 2015) and MS Excel 2020 Descriptive Statistics analysis tool were used to generate reports of univariate statistics for the data, providing information about the central tendency and variability of our data.

## Results and discussion

Obtaining reliable results for the resistance of pepper accessions to bacterial spot agents was determined by the following conditions: pathogenic strains *X. euvesicatoria* and *X. vesicatoria* pathotype, race; the host sensitivity during the phenophase; appropriate method of infection; diagnostic scale with the typical symptoms on the leaves; classification into groups with proven limit values on the basis of the mean score of infestation (ms) as follows: I- immune; R- resistant; MS- medium sensitive; S- sensitive; SS - strongly sensitive and index of defoliation.

Vacuum infiltration with *X. euvesicatoria* and *X. vesicatoria* ( $10^8$  cfu/ml) during the most appropriate phenophase was first true pepper leaves for artificial infection. The method stimulated the natural infiltration of infection and reproduced the typical symptoms of the disease. In this way tissues were not injured and the created vacuum facilitated and accelerated the penetration of bacterial cells through the stomata, pappi and hydathodes. The pathological process in resistant plants

induced HR after 18-24h, and in the susceptible plants, 2-5 days after the infiltration water-soaked, brown spots on the leaves were formed. Bacterial spot of pepper was characterized by small, irregular, water-soaked, greasy-appearing lesions on all above-ground parts of the leaf lamina - typical for *X. euvesicatoria*, and in *X. vesicatoria* large, single, brown, water-soaked lesions were formed, which could envelop the whole surface of the leaves, followed by cell death and tissue necrosis. A necrotic ring formed at the base of the leaf stalk and the leaves fell off (Figure 1). These characteristic symptoms determined the disease score, respectively the diagnostic scale development. The average degree of disease (ms) was scored by (0 to 4) disease rating scale. The number of fallen leaves as a result of the ring shape necrosis of the leaf stalk was reported - defoliation index (Pesti et al., 1990; Bogatzevska et al., 2006; Stoimenova et al., 2005, 2006; Bogatzevska and Pandeva, 2009; Vancheva et al., 2016). The next step was to determine and specify the resistance groups based on statistical analysis of data from resistance variability of pepper accessions to *X. euvesicatoria* and *X. vesicatoria*.

The limit values of resistance groups were established by mean score (ms) based on conducted statistical analysis: I -immune; R- resistant; MS- medium sensitive; S- sensitive; SS- strongly sensitive.



**Figure 1.** Symptoms on bacterial spot of pepper caused by *X. euvesicatoria* and *X. vesicatoria* and defoliation

### *Xanthomonas euvesicatoria*

The data for resistance of 116 selection lines from all 155 accessions (Bogatsevskaja et al. (2006), Stoimenova et al. (2005, 2006), Bogatzevska and Pandeva (2009) and Vancheva et al. (2016) of Bulgarian and introduced pepper varieties to *X. euvesicatoria* with mean score of infestation - 0.61 were processed. The variation coefficient (CV, %) was 52%. The defoliation index was in the range from 0 to 52% with an average value of 12%. The clustering by diagnostic scale was respectively: 0- healthy - 53%; 1- single spots of leaves (from 1 to 3) - 35%; 2- 1/3 of the surface of the leaf lamina covered with small necrotic spots - 10%; 3- merged necrotic areas covered with 1/2 of the leaf surface - 2%; 4- covered by necrosis 2/3 of lamina - 0.2%. Variation coefficient had the highest value in the diagnostic scale 4 (Figure 2). The correlation between the scales in the statistical analysis of the pepper accessions was 116: 116: 99: 38: 6 (Table 1).

**Table 1.** Descriptive statistics of averaged percentage distribution diagnostic scale development for *X. euvesicatoria*

| Total for all accessions          | % of the leaves with scale 0 | % of the leaves with scale 1 | % of the leaves with scale 2 | % of the leaves with scale 3 | % of the leaves with scale 4 | ms    | Di, % |
|-----------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-------|-------|
| Maximum                           | 97                           | 62                           | 41                           | 20                           | 10                           | 1.56  | 52    |
| Minimum                           | 8                            | 2                            | 0                            | 0                            | 0                            | 0.03  | 0     |
| Average                           | 53                           | 35                           | 10                           | 2                            | 0.2                          | 0.61  | 12    |
| SD                                | 19                           | 12                           | 9                            | 3                            | 1                            | 0.32  | 11    |
| CV, %                             | 36                           | 36                           | 90                           | 214                          | 605                          | 52.34 | 89    |
| Total number accessions of scales | 116                          | 116                          | 99                           | 38                           | 6                            | NA    | NA    |

\*SD- standard deviation; CV- coefficient of variation; NA- not available; ms- mean score; Di, %- index of defoliation

**Figure 2.** Symptoms on the leaf of pepper caused by *X. euvesicatoria* (diagnostic scale 4)

The mathematical analysis of the mean score classified 116 accessions into *X. euvesicatoria* resistance groups: I- immune 0; R- resistant (0.01-0.44); MS- medium sensitive (0.45-0.79); S – sensitive (0.80-1.12); SS- strongly sensitive (over 1.13). The percentage distribution depending on ms value was respectively: I-immune - 0%; R- resistant - 19%; MS- medium sensitive - 48%; S- sensitive - 4%; SS- strongly sensitive – 4%. The ratio between healthy leaves, leaves with single spots and those evaluated with a diagnostic scale 2 in the resistant group was 81:18:1. The percentage of healthy leaves was the highest in the resistant and reduced in the strongly sensitive group (Table 2).

**Table 2.** Percentage distribution of the number of leaves in the diagnostic scale according to the mean score (ms) of infestation of *X. euvesicatoria*

| MS distribution by groups | Resistance grouping | % of total accessions | % of the leaves with scale 0 | % of the leaves with scale 1 | % of the leaves with scale 2 | % of the leaves with scale 3 | % of the leaves with scale 4 |
|---------------------------|---------------------|-----------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| R                         | 0.01 - 0.44         | 19                    | 81                           | 18                           | 1                            | 0                            | 0                            |
| MS                        | 0.45 - 0.79         | 48                    | 56                           | 37                           | 7                            | 0                            | 0                            |
| S                         | 0.80 - 1.12         | 28                    | 35                           | 43                           | 19                           | 3                            | 0                            |
| SS                        | over 1.13           | 4                     | 23                           | 38                           | 25                           | 12                           | 3                            |

\*R- resistant; MS- medium sensitive; S- sensitive; SS- strongly sensitive

### *Xanthomonas vesicatoria*

Various resistance results from 155 selection lines, hybrids, Bulgarian and introduced pepper varieties (Bogatsevska et al. (2006), Stoimenova et al. (2005, 2006), Bogatzevska and Pandeva (2009) and Vancheva et al. (2016) to *X. vesicatoria* were processed. The average mean score (ms) was 0.75, variation coefficient (CV, %) was 49%. The defoliation index (Di,

%) ranged from 0 to 65% with an average value of 13%. The accessions grouping by the degree of infection in accordance to the modified five-point scale demonstrated the following ratio between healthy and damaged leaves 46: 37: 14: 6: 6%. The highest value of CV was in the leaves covered with dark brown lesions with necrosis vein (Table 3, Figure 3).

**Table 3.** Descriptive statistics of averaged percentage distribution diagnostic scale development for *X. vesicatoria*

| Total for all accessions          | % of the leaves with scale 0 | % of the leaves with scale 1 | % of the leaves with scale 2 | % of the leaves with scale 3 | % of the leaves with scale 4 | ms    | Di, % |
|-----------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-------|-------|
| Maximum                           | 99                           | 61                           | 42                           | 26                           | 29                           | 2.41  | 65    |
| Minimum                           | 1                            | 1                            | 1                            | 0                            | 0                            | 0.01  | 0     |
| Average                           | 46                           | 37                           | 14                           | 6                            | 6                            | 0.75  | 13    |
| SD                                | 19                           | 11                           | 9                            | 6                            | 10                           | 0.37  | 12    |
| CV, %                             | 41                           | 30                           | 64                           | 93                           | 162                          | 49.25 | 87    |
| Total number accessions of scales | 155                          | 155                          | 144                          | 79                           | 10                           | NA    | NA    |

\*SD- standard deviation; CV- coefficient of variation; NA- not available; ms - mean score; Di, % - index of defoliation.



**Figure 3.** Symptoms on the leaf of pepper caused by *X. vesicatoria* (diagnostic scale 4)

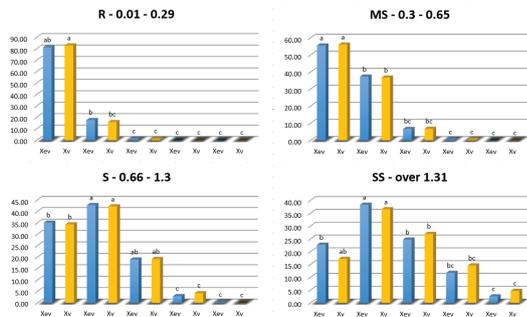
**Table 4.** Percentage distribution of the number of leaves in the diagnostic scale according to the mean score (ms) of infestation of *X. vesicatoria*

| MS distribution by groups | Resistance grouping | % of total accessions | % of the leaves with scale 0 | % of the leaves with scale 1 | % of the leaves with scale 2 | % of the leaves with scale 3 | % of the leaves with scale 4 |
|---------------------------|---------------------|-----------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| R                         | 0.01 - 0.36         | 7                     | 83                           | 16                           | 1                            | 0                            | 0                            |
| MS                        | 0.37 - 0.79         | 46                    | 56                           | 37                           | 7                            | 0                            | 0                            |
| S                         | 0.80 - 1.18         | 39                    | 34                           | 42                           | 19                           | 4                            | 0                            |
| SS                        | over 1.19           | 8                     | 17                           | 37                           | 27                           | 15                           | 5                            |

\*R- resistant; MS- medium sensitive; S- sensitive; SS- strongly sensitive.

#### *X. euvesicatoria* and *X. vesicatoria*

The limit values of the groups by mean score of infestation based on the statistical analysis of the data on the resistance of 271 pepper accessions to *X. euvesicatoria* and *X. vesicatoria* were specified. The established resistance clusters were I- immune - 0; R- resistant (0.01-0.29); MS- medium sensitive (0.3-0.65); S- sensitive (0.66-1.3); SS- strongly sensitive (over 1.31). The defoliation index ranged from 0 to 59% with an average value of 12%. The tendency in the accessions resistant to *X. euvesicatoria* and *X. vesicatoria* was preserved, healthy leaves and those with single spots predominated (for Xeu-1-3 single small spots; one large lesion-Xv). Statistical differences were not proven. Sensitive and strongly sensitive accessions were characterized by an increase in the percentage of leaves with symptoms (diagnostic scale 1 to 4), while healthy leaves were strongly reduced (Figure 4).



**Figure 4.** Grouping the mean score (ms) of infestation and percentage distribution of diagnostic scale in the groups to *X. euvesicatoria* and *X. vesicatoria*

**Legend:** R- resistant, MS- medium sensitive, S- sensitive, SS- strongly sensitive; Arrangement by mean score by modified 5<sup>th</sup> scale (Bogatsevska et al., 2006), Xeu- *X. euvesicatoria*, Xv- *X. vesicatoria*; \*a,b... Different letters in the same column indicate least significant difference.

The performed mathematical analysis of ms clustered the selection lines, hybrids, Bulgarian and introduced pepper varieties by resistance to *X. vesicatoria* in the following groups: I- immune 0; R- resistant (0.01-0.36); MS- medium sensitive (0.37-0.79); S- sensitive (0.80-1.18); SS- strongly sensitive (over 1.19). Resistant and sensitive pepper accessions to *X. vesicatoria* were distributed as a percentage of the value of ms, as follows: R- resistant - 7%; MS- medium sensitive - 46%; S- sensitive - 39%; SS- strongly sensitive - 8%. Healthy leaves were 83% of the resistant and only 17% of the strongly sensitive ones (Table 4).

*X. euvesicatoria* was a host specialized pathogen and it caused disease in accessions from the genus *Capsicum* with *avrBs3* and *avrBs4* genes. Strains from *X. euvesicatoria* in pepper plants were more virulent than *X. vesicatoria* (Ignjatov et al., 2010; Vancheva, 2016). Identification of the effectors proteins *avrBs* genes among the *X. euvesicatoria* and *X. vesicatoria* populations in Bulgaria reveals possibility for further investigations and studies of the mechanisms involved in the pathogen-host interaction. Furthermore, those could provide valuable information for further innovative approaches, including induced disease resistance and improved yield characteristics of pepper.

Phylogenetic analysis of all individual genes showed a clear separation of the *X. vesicatoria* from the *X. euvesicatoria* (Dhakar et al., 2019). The genetic diversity between *X. euvesicatoria* and *X. vesicatoria*, and the phylogenetic positions of the two pathogens relative to each other, suggest the use of separate resistant groups to determine the resistance of pepper accessions to bacterial spot.

## Conclusion

The first step in the breeding programs of lines, hybrids and pepper varieties resistant to bacterial spot pathogens was the use of defined groups with mean score of infection with a proven statistical difference, respectively for: a) *X. euvesicatoria* - I- 0; R (0.01-0.44); MS (0.45-0.79); S (0.80-1.12); SS (over 1.13); b) *X. vesicatoria* - I- 0; R (0.01-0.36); MS (0.37-0.79); S (0.80-1.18); SS (over 1.19). Breeding pepper accessions with complex resistance requires the application of the established resistance groups to *X. euvesicatoria* and *X. vesicatoria*: I-

immune - 0; R- resistant (0.0-0.29); MS- medium sensitive (0.3-0.65); S- sensitive (0.66-1.3); SS- strongly sensitive (over 1.31).

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