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Evaluation of pepper (*Capsicum annuum* L.) varieties to several methods of inoculation with *Verticillium dahliae* Kleb. in different conditions

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Abstract

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Verticillium wilt caused by *Verticillium dahliae* Kleb. of pepper (*Capsicum* species) is an important soilborn disease. Surveys of ten Bulgarian commercial pepper (*Capsicum annuum* L.) varieties were conducted to gain information on the incidence of diseases. Several methods of inoculation with the pathogen of *Verticillium dahliae* Kleb. have been used to test the resistance of the varieties in different conditions. Field and greenhouse diagnostics along with laboratory assays of wilted plants revealed that the wilting was caused by *Verticillium dahliae* Kleb. In the *Verticillium* field with additional infection at second observation Kurtovska kapia 1 (23.12%) was least affected. At first monitoring of *Verticillium* field without additional infection it was found that the varieties Kapia UV, Kaloyan, Kurtovska kapia 1, Ivaylovska kapia, Sivria 600 and Stryama did not attack by the pathogen but at second – only variety Kaloyan was non infected. Under greenhouse conditions least affected varieties in both observations are Kurtovska kapia 1 (17.35%–19.64%), Zlaten medal 7 (10.00%–10.00%), Milkana F1 (10.31%–27.11%). Explicably, an infestation index is comparatively lower in the conventional field with natural background and Verticillium field than other variants with artificial inoculation. In all investigated variants of testing the varieties Kurtovska kapia 1, Milkana F1 and Stryama were with the highest level of resistance to *Vericillium dahlia*e Kleb.

In pathogenicity tests, isolates of *V. dahliae* Kleb. caused symptoms in inoculated pepper identical to those in field and greenhouse-grown pepper plants. Results indicate that diseases, posing the most serious challenge to pepper cultivars in Bulgaria.

The best hope for control of *Verticillium* wilt in most crops lies in the development of new wilt-resistant varieties, or the incorporation of resistance into commercial varieties.

Key words: Verticillium dahliae Kleb.; pepper; resistance

Introduction

The pepper is one of the main vegetable crops in Bulgaria. Being one of the intensive crops, it is attacked from different diseases, which could cause significant losses depending on the degree of infestation. The Verticillium wilt, caused by the pathogen fungus *Verticillium dahliae* Kleb., is with the greatest economic importance among the whole complex of diseases in pepper. The disease is all over the country and the attack index is on average 46% (Neshev, 1997). It develops in the conducting vessels of the host and causes trache omycosis. There is a monocyclic development.

The control of this fungus is difficult because it is able to survive both in open field and under controlled conditions for several years (Goicoechea, 2006). According to Goldberg (2003) at present there is no effective enough method in the world for control of this pathogen after the disease has been appeared in the sowing. Veloso et al. (2015) reported that fungus *Fusarium oxysporum* Fo47 reduced the severity of wilt caused by the soilborne pathogen *Verticillium dahliae*

Kleb. in pepper and stimulated the biosynthesis of caffeic acid and primed that of chlorogenic acid in pepper roots.

Cultural control methods for *Verticillium* wilt are expensive or have limited feasibility because *V. dahliae* Kleb. microsclerotia can survive in the soil for more than 14 years (Wilhelm, 1955; Pegg and Brady, 2002), and in addition, *V. dahliae* Kleb. from several hosts including pepper are cross-pathogenic on several crops grown in rotation (Tsror et al., 1998; Bhat et al., 2003; Sanogo et al., 2009).

A few sources of peppers with partial resistance against *Verticillium* wilt have been identified either from field screening using natural soil inoculum or greenhouse experiments using conidial and microsclerotial inoculum (Fiume et al., 1982; Pesti et al., 1985; Palloix et al., 1990; González-Salán & Bosland, 1992).

Available literature on the resistance of pepper to this pathogen is not sufficient. Elenkov (1954) reports an increased resistance to Kapia 1005 and 786 as a result of inter-species hybridization. Illenko (1971, 1979) explains the comparative field resistance of the varieties Moldavia 118, Lastochka and Podarok Moldovski with the participation in the Bulgarian variety White pepper. Woolliams et al. (1962) tested 456 pepper varieties to establish partial varieties of resistance and a line with 54% resistance. Symptoms of Verticillium wilting are varied and in pepper crop can be found dwarf, brown and green form caused by *V. dahliae* Kleb. (Harkova, 1964; Aleksis et al., 1966).

At the Maritsa Vegetable Crops Research Institute – Plovdiv (MVCRI), Plovdiv a continuous screening of more than 370 lines, F1 hybrids and varieties of pepper (*C. annuum* L.) is being carried out by artificial inoculation to search for sources of resistance to this important pathogen and some genotypes have been identified that can be successfully used in the breeding process (Masheva et al., 2001; Masheva & Todorova, 2012; Masheva & Todorova, 2013). Selection lines and some varieties are the result of hybridization between Bulgarian or foreign resources, relatively resistant to *V. dahliae* Kleb. As a result of purposeful breeding work at MVCRI the following original varieties Hebar, Kapiya 1300, Kapiya UV (Vertus), Maritsa, Stryama and Buketen 50 have been bred and all of them possess field resistance to Verticillium wilt (Todorov & Todorova, 2002).

The purpose of this research is to evaluate the response of some Bulgarian pepper varieties to *V. dahliae* Kleb. using several methods of inoculation in different conditions of growing.

Materials and Methods

At the MVCRI, Plovdiv, Bulgaria, ten pepper varieties were assessed to several methods of inoculation with fungus *Verticillium dahliae* Kleb in different conditions. The varieties were: 2095 – Kapia UV; 2086 – Kaloyan; 403/15 – Byal Kalinkov; 2078 – Kurtovska kapia 1; 2099 – Ivaylovska kapia; 2058 – Kurtovska kapia 1619; 2054 – Zlaten medal 7; 616/14 – Milkana F1; 2083 – Sivria 600; 2089 – Stryama. 10 to 12 plants from each variety in all tested conditions were used.

Surveys of symptoms on *Verticillium* and commercial field's diagnostics. In *Verticillium* field, which was established more than 20 years, the two root-dip methods were used. The first method was: before planting, the roots of the plants were suspended in spores and mycelia suspension of pure cultures of the *V. dahliae* Kleb. Inoculum was prepared by growing isolates on PDA plates at 25°C for 1 month. Spore suspensions were prepared by adding 15 ml of sterile distilled water to each plate and scraping the cultures with a spatula. The conidial density of each isolate was adjusted to approximately 182 conidia per ml. Under commercial field the same genotypes were used and grown in natural conditions without artificial infection by *V. dahliae* Kleb.

Surveys of symptoms on greenhouse diagnostics. Under greenhouse conditions in pots with soil from the *Verticillium* field and a sterile substrate. Plants of this cultivars were transplanted into plastic pots (with a diameter of 10 on top and 7.5 cm on bottom, and a depth of 9 cm) filled with sterilized soil.

Pepper plants were observed before maturity and at maturity stages of the fruits. During the vegetation, the rate of pathogen attack on a five-degree scale (0–4) was reported twice. An infestation index (%) of McKiney and resistance – invasive plants (%) was calculated. The infected plants were found by walking along through the middle of small fields with area less than 1 ha. Field patterns of wilted plants were observed and recorded. Wilted plants were examined for root rot, vascular necrosis, lesions on aboveground plant parts and defoliation. Infection of root rot accompanied by vascular necrosis in root or stem was used as an indicator of infection by *V. dahliae* Kleb. Plant infection by pathogen was determined based on the presence of root rot and root sloughing, and vascular necrosis in root or stem.

Isolations from wilted plants

To confirm field diagnostics, three plants with root rot and three plants with vascular necrosis were taken from each variety in each condition visited and further processed in the laboratory. From each plant, four 1-cm segments were cut from the base of each stem and upper part of the taproot. Root and stem parts were washed free of soil, surface-disinfested for 2 min in 90% ethyl alcohol, rinsed in sterile distilled water, and plated onto water agar medium in 9-cm-diameter Petri dishes. Mycelia colonies emerging from the plated segments were transferred to either potato dextrose agar, Czapek-Dox agar medium in 9-cm-diameter Petri dishes for identification based on morphological characteristics and microscopic observations (Talboys, 1960). The number of segments from which *V. dahliae* Kleb. was recorded and the frequency of isolation (%) of the pathogen was computed. The percentage of recovery of mycelia colonies other than those from *V. dahliae* Kleb. was also computed. Average frequency of isolation was calculated for all cultivars.

Pathogenicity

The pathogenicity tests were conducted for each isolation number. The ability of isolates of *V. dahliae* Kleb. to cause wilting was tested on growth chamber on the pepper cultivars. When seedlings were at the six- to eight-leaf growth stage, they were inoculated by 20 ml of conidial suspension (72.3 × 105 conidia per 1000 ml) of each *V. dahliae* Kleb. isolate on top of the soilless medium in each pot. Non-inoculated seedlings were included as controls. The inoculum levels used were within the range of those used by others (Riggs and Graham, 1995). Plants were watered daily starting immediately after inoculation. Isolates of *V. dahliae* Kleb. were tested to produce conidia; a 1-cm mycelial plug of *V. dahliae* Kleb. grown on water agar was added to 100 ml of sterilized Czapek-Dox broth contained in 250 ml Erlenmeyer flasks. The flasks were placed on a rotary shaker and incubated at room temperature (23 to 25°C) in darkness for 7 to 10 days. Then the contents of the flask were passed through three layers of cheesecloth to separate conidia from mycelia plugs and mats. The number of conidia in the filtered suspension was estimated with a chamber of Thoma. The pathogenicity test was repeated once, with 5 plants per isolate per test. Plants were monitored for disease development, and tissue samples were taken from representative diseased plants (5 plants per isolate) and plated onto water agar medium, as described above, to confirm the association of *V. dahliae* Kleb. with the observed symptoms (Riggs and Graham, 1995).

Using multiple range test (Duncan, 1955), the data were processed. Software products using for the investigation were "MS Excel Analysis ToolPak Add-Ins" (https://support.office.com) and "R-3.1.3" in combination with "RStudio-0.98" and install package "agricolae 1.2-2" (Mendiburu, 2015).

Results

The reasons for the sudden increase in incidence of *Verticillium* wilt on different pepper are not known, but may be associated with selection of isolates of *V. dahliae* Kleb. with increased ability to infect pepper plants. In the first observation with proven highest attack index, Byal Kalinkov variety was identified in provocative field with additional infection and under greenhouse conditions – 87.50%–76.67% (Table 1). This is an indication for high intensity of *Verticillium* field with *Verticillium dahliae* Kleb. pathogen.

In the *Verticillium f*ield with additional infection, on the first observation varieties Stryama (11.38%) and Milkana F1 (12.50%) were least affected followed by Kurtovska kapia 1 (20.05%), Kapia UV (23.13%), Kaloyan (29.26%) and Ivaylovska kapia (30.00%). In the second observation conducted at maturity stage in September in this variant it was established higher attack index that ranges from 23.12% for Kurtovska kapia 1 to 90.63% for Byal Kalinkov. Byal Kalinkov was also with the highest index under greenhouse conditions (88.31%).

In the second variant, there is a lesser attack than the first. In *Verticillium* field on first observation the varieties Kapia UV, Kaloyan, Kurtovska kapia 1, Ivaylovska kapia, Sivria 600 and Stryama were non-infected. The range of attack in the rest varieties (Byal Kalinkov, Zlaten medal 7, Kurtovska kapia 1619) varies from 10% to 12.50%. At second observation only the variety Kaloyan was not affected by the disease, and the rest cultivars are under 20% attack (Table 1).

Under greenhouse conditions, the results of both observations indicate higher levels of manifestation of the disease than in Verticillium and conventional conditions of testing. This could be explaining with environment conditions – higher temperatures and lower relative humidity of the air. Least affected varieties in both observations are Kurtovska kapia 1 (17.35%–19.64%), Ivaylovska kapia (22.30% – 38.20%), Zlaten medal 7 (10.00%–10.00%), Milkana F1 (10.31%–27.11%) and Stryama (10.60%–30.20%).

In the conventional field, the percentage is comparatively lower than the other variants. The varieties least affected by the pathogen in both observations by less than 10% are Kaloyan, Byal Kalinkov, Kurtovska kapia 1, Ivaylovska kapia and Stryama. In all investigated variants varieties Kurtovska kapia 1, Milkana F1 and Stryama were at the lowest level of attack by *Verticillium* wilt.

There was different level of resistance between varieties in different conditions (Figure 1). As the high resistant stands variety Kaloyan in *Verticillium* field – 100%. More than 80% resistance to pathogen in different conditions is observed in the varieties Kurtovska kapia 1, Ivaylovska kapia, Kurtovska kapia 1619, Zlaten medal 7 and Stryama.

Explicably, resistance is the lowest in the *Verticillium* field with additional infection and under greenhouse conditions. The highest resistance between the different variations develops in the commercial field and *Verticillium* field.

Table 1. Infestation index of pepper varieties by *Verticillium dahliae* Kleb.in different conditions of inoculation

Variety	Observation place	First observation	Second observation
2095	Verticillium field with additional infection	23.13j	45.48f
	Verticillium field	0.00t	16.67n
	Verticillium infected soil in greenhouse	26.67i	42.60g
	Conventional field	12.61m	17.76n
2086	Verticillium field with additional infection	29.26h	62.96c
	Verticillium field	0.00t	0.00u
	Verticillium infected soil in greenhouse	26.18i	58.16d
	Conventional field	4.94pq	9.52rs
403/15	Verticillium field with additional infection	87.50a	90.63a
	Verticillium field	10.00n	10.00qr
	Verticillium infected soil in greenhouse	76.67b	88.31b
	Conventional field	6.62o	8.62rst
2078	Verticillium field with additional infection	20.05k	23.12l
	Verticillium field	0.00t	9.09rst
	Verticillium infected soil in greenhouse	17.35l	19.64m
	Conventional field	3.23rs	7.77t
2099	Verticillium field with additional infection	30.00gh	47.70e
	Verticillium field	0.00t	8.06st
	Verticillium infected soil in greenhouse	22.30j	38.20h
	Conventional field	4.06qrs	8.41st
2058	Verticillium field with additional infection	41.11c	63.89c
	Verticillium field	12.50m	12.50p
	Verticillium infected soil in greenhouse	36.20e	64.10c
	Conventional field	9.90n	14.27o
2054	Verticillium field with additional infection	34.34f	63.33c
	Verticillium field	10.00n	10.00qr
	Verticillium infected soil in greenhouse	38.10d	57.20d
	Conventional field	6.14op	12.14p
616/14	Verticillium field with additional infection	12.50m	36.67i
	Verticillium field	NA	NA
	Verticillium infected soil in greenhouse	10.31n	27.11k
	Conventional field	7.05o	11.80p
2083	Verticillium field with additional infection	30.19gh	45.18f
	Verticillium field	0.00t	15.00o
	Verticillium infected soil in greenhouse	31.10g	44.20f
	Conventional field	4.27qr	11.32pq
2089	Verticillium field with additional infection	11.38mn	38.89h
	Verticillium field	0.00t	12.18p
	Verticillium infected soil in greenhouse	10.60n	30.20j
	Conventional field	2.64s	8.62rst

*2095 – Kapia UV; 2086 – Kaloyan; 403/15 – Byal Kalinkov; 2078 – Kurtovska kapia 1; 2099 – Ivajlovska kapia; 2058 – Kurtovska kapia 1619; 2054 – Zlaten medal 7; 616/14 – Milkana; 2083 – Sivriya 600; 2089 – Stryama. *a,b… Duncan's multiple range test ($P < 0.05$)

Different symptom manifestations such as root rot, lesions on the upper parts, tissue necrosis and defoliation have been identified in the 10 studied cultivars. The least defoliation was recorded in Kapia UV (14.66%) while in Ivaylovska kapia and Kurtovska kapia 1619 varieties it was most pronounced with 24.00% and 24.66%, respectively (Table 2). In the case of root rot (from 0.94% to 6.36%) and necrosis on the vascular system (0.40%–2.13%), there were no differences between the studied varieties and they were attacked equally in low level. The lesions on the upper parts are the

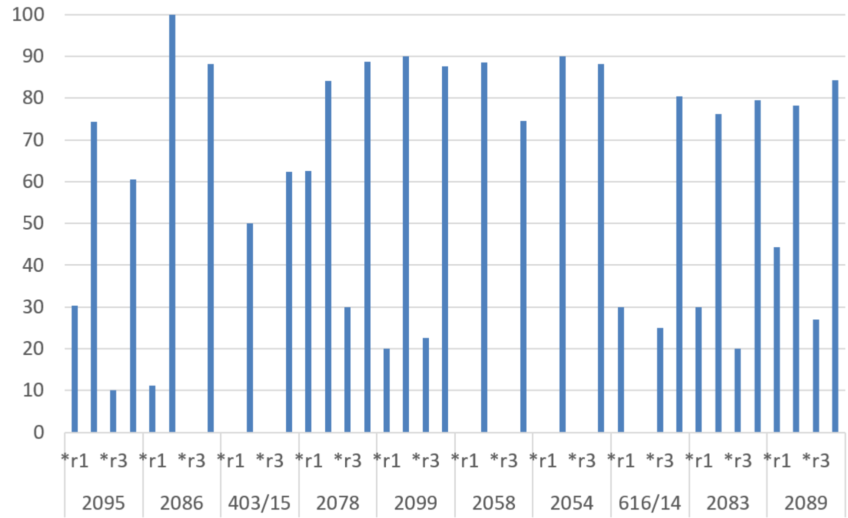

Fig. 1. Reaction of resistance (%) of peppers genotypes to *Verticillium dahliae* Kleb.
*r1 – Reaction of resistance Verticillium field and additional infected plants; r2 – Reaction of resistance of Verticillium field; r3 – Reaction of resistance of Verticillium infected soil in greenhouse; r4 – Reaction of resistance of commercial field. 2095 – Kapia UV; 2086 – Kaloyan; 403/15 – Byal Kalinkov; 2078 – Kurtovska kapia 1; 2099 – Ivaylovska kapia; 2058 – Kurtovska kapia 1619; 2054 – Zlaten medal 7; 616/14 – Milkana; 2083 – Sivria 600; 2089 – Stryama

Table 2. Average incidence of different symptoms of pepper infection by *Verticillium dahliae* Kleb.

	2095	2086	403/15	2078	2099	2058	2054	616/14	2083	2089
I Root rot	4.06 c	2.93 c	4.20 c	5.30 c	6.36 c	2.43 c	2.10 c	0.94 c	1.93 c	3.50 c
II Lesions on the upper parts	10.86 bc	8.76 bc	8.23 bc	11.83 bc	5.63 c	4.46 c	2.76 c	9.16 bc	11.96 bc	11.7 bc
III Tissue necrosis	1.26 c	2.13 c	0.96 c	0.60 c	0.90 c	1.46 c	0.70 c	0.66 c	0.40 c	0.66 c
IV Defoliation	14.66 ab	16.33 ab	16.00 ab	20.00 ab	24.00 a	24.66 a	17.33 ab	16.33 ab	15.66 ab	21.00 a

*2095 – Kapia UV; 2086 – Kaloyan; 403/15 – Byal Kalinkov; 2078 – Kurtovska kapia 1; 2099 – Ivaylovska kapia; 2058 – Kurtovska kapia 1619; 2054 – Zlaten medal 7; 616/14 – Milkana; 2083 – Sivria 600; 2089 – Stryama. *a,b… Duncan's multiple range test ($P < 0.05$)

least developed in the varieties Ivaylovska kapia (5.63%), Kurtovska kapia 1619 (4.43%) and Zlaten medal 7 (2.76%) and the most – in Sivria 600 (11.96%) and Kurtovska kapia 1 (11.83%) and Stryama (11.70%).

Symptoms of plant infection by *V. dahliae* Kleb were found in every cultivars surveyed and average incidence of the disease in sampled plants varied from 69 to 85% (Figure 2). From the affected plants rarely was isolate *Rhizoctonia solani* Kühn. Other microorganisms in wilt plants are not almost isolated.

Data from the two-way factor dispersion analysis of variance showed that all systemic factors had proven impact on the variability of the *Verticillium* wilting attack index (Table 3). In the first observation the factor condition of inoculation was with prevailing influence (47.64%) on phenotypic express of attack index followed by variety with 30.22% while in the second – the condition of inoculation had predominant effect with 68.40% followed by interaction variety x condition of inoculation with 16.78% and variety had the lowest influence (14.77%).

Data from the two-way factor dispersion analysis showed again that all systemic factors had proven impact

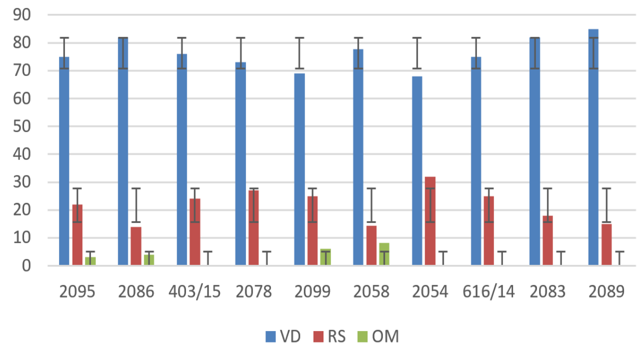

Fig. 2. Average frequency of isolation, %
Average frequency of isolation of *Verticillium dahliae* (Vd), *Rhizoctonia solani* (RS) and other microorganisms (Om) (%) from segments of pepper plants sampled from fields and greenhouse

on the variability of the resistance (Table 4). The factor condition of inoculation was with prevailing influence (82.85%) of resistance while variety (8.62%) and interaction variety x condition of inoculation (8.48%) had almost equal influence.

Table 3. Two-way analysis on variance of index of infestation

First observation			
	Mean Sq	F value	Influence, %
Variety	1449.20***	2247.12	30.21
Condition of inoculation	6854.00***	10627.57	47.63
Variety x Condition of inoculation	366.80***	567.14	22.03
Residuals	0.60		
Second observation			
	Mean Sq	F value	Influence, %
Variety	1081.70***	1785.48	14.76
Condition of inoculation	15018.00***	24810.92	68.40
Variety x Condition of inoculation	424.50***	701.29	16.75
Residuals	0.60		

Table 4. Two-way analysis on variance of resistance

	Mean Sq	F value	Influence, %
Variety	13326 ***	1711.33	8.62
Condition of inoculation	38206 ***	49321.02	82.85
Variety × Condition of inoculation	451***	582.39	8.48
Residuals	1		

Discussion

The study showed widely distributed and *V. dahliae* Kleb. was associated with the symptoms. *V. dahliae* Kleb. has a broad host range, causing vascular discoloration and wilt of many economically important crops (Pegg, 1984). *V. dahliae* Kleb. can infect pepper plants at any growth stage. Symptoms include yellowing and drooping of leaves on a few branches or on the entire plant (Nakayama, 1960). The edges of the leaves roll inward on infected plants, and foliar wilting ensues. The foliage of severely infected plants turns brown. Growth of pepper plants inoculated with aggressive strains of *V. dahliae* Kleb. in the greenhouse, or of pepper plants infected early in the season under field conditions, is severely stunted, with small leaves that turn yellow green. Subsequently, the dried leaves and shrivelled fruits remain attached to plants that die. Brown discoloration of the vascular tissue is visible when the roots and lower stem of a wilted plant is cut longitudinally.

Microsclerotia produced by *V. dahliae* Kleb. may survive under field conditions for up to 14 years in the absence of a host (Wilhelm, 1995).

Current management strategies to reduce the impact of *Verticillium* wilt in commercial pepper production include the planting resistant cultivars. *Verticillium dahliae* Kleb. penetrates pepper plants through wounded roots and spreads systemically through the xylem, and as the disease symptoms develop acropetally, the vascular tissue turns a characteristic brownish-black. As the disease becomes severe, stunting, epinasty, foliar chlorosis, progressive necrosis, and leaf abscission are also clearly visible (Bhat et al., 2003; Goicoechea, 2006).

Since the absence of wilt symptoms in pepper plants is not necessarily due to the inability of the pathogen to penetrate or colonize plant tissues (Tsror et al., 1998), it is possible that *V. dahliae* Kleb. has always been present in these fields and colonized the roots of pepper plants without noticeable reduction in plant vigour or yield. The intensive production of pepper in this area may have increased selection pressure on strains of *V. dahliae* Kleb. that colonize and reproduce on pepper plants more effectively, resulting in an increase in inoculum levels.

In the *Verticillium* field with additional infection at first observation Stryama (11.38%) and Milkana F1 (12.50%) were least affected, followed by Kurtovska kapia 1 (20.05%), Kapia UV (23.13%), Kaloyan (29.26%) and Ivaylovska kapia (30.00%). At first monitoring of *Verticillium* field without additional infection it was found that the varieties Kapia UV, Kaloyan, Kurtovska kapia 1, Ivaylovska kapia, Sivria 600 and Stryama did not attack by the pathogen but at second only Kaloyan was non-infected. Under greenhouse conditions least affected varieties in both observations were Kurtovska kapia 1 (17.35%–19.64%), Zlaten medal 7 (10.00% – 10.00%), Milkana F1 (10.31%–27.11%) and Stryama (10.60%–30.20%). The higher temperature combined with the early phase of the plant; favor the development of the pathogen, which is the reason for the higher sensitivity of the variety.

Therefore, it is imperative to identify and develop resistant pepper varieties for managing *V. dahliae* Kleb. The very high incidence of wilt and root discoloration in the cv. Byal

Kalinkov and most of the tested varieties demonstrated that the isolates used in this study were highly virulent.

The results of this study about the highest attack index confirmed those in previous study of the resistance of different varieties of pepper in the period 2004–2006 where the highest attack index (46.92%) again was found in the Byal Kalinkov, followed by Kapia 1300 (45.57%) (Masheva & Todorova, 2012). Differences in the response of most of the varieties studied during the years are probably related to weather features. These results support the reported by Palloix et al. (1990) that the expression of sustainability is very variable and depends on the conditions of cultivation. The reaction of native pepper populations to *Verticillium* wild attack, Todorova (2000) also found a high degree of variation in the attack index, both in individual types of samples and in years.

Isolates of *V. dahliae* from pepper plants grew well at 15, 20, and 25°C. The optimum temperature for robust growth of peppers is about 25°C (Nonnecke, 1989). Growth of pepper plants is not only reduced at temperature below 25°C, but pepper plants are also rapidly colonized by *V. dahliae* Kleb. at low temperatures and therefore the severity of *Verticillium* wilt is closely associated with growth of the pathogen (Kendrick & Middleton, 1995).

Jiang (2015) reported that resistance inheritance to *V. dahliae* Kleb. is not well defined due to the limitation of resistant accessions and used molecular markers which would provide promising tools to investigate *Verticillium* wilt resistance, and would improve the efficacy of the breeding program in the chile pepper. Combination classical and molecular approaches to breed for disease resistant hybrid chile peppers (*Capsicum*) will be increase (Gurung et al., 2015).

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