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Study of the immune response of pepper varieties to infection with the pathogen *Sclerotinia sclerotiorum*

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Abstract

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The immune response of pepper varieties grown in greenhouses to *Sclerotinia sclerotiorum* (Lib.) de Bary was studied. Six varieties of *Capsicum annuum* L. were included in the experiment, of which 5 were Bulgarian (sweet peppers Sivriya, Ivaylovska kapiya, White kapiya, Bulgarian ratund and hot pepper Dzhulyunska shipka) and Pirouette F1 of the Syngenta AG Company. The resistance of pepper varieties to infection with the pathogen *S. sclerotiorum* was studied using the detached leaf assay (DLA) and detached fruit method. The effect of biological preparations (based on antagonists of *Trichoderma viride*, *Bacillus subtilis* and *Enterobacter cloaceae*) on the immune response of plants to infection with *S. sclerotiorum* was studied in greenhouse conditions on the pepper variety Pirouette F1. For this purpose, young plants grown against the background of introduced antagonists were infected with the pathogen *S. sclerotiorum* by decapitation of the stem. The results of the study indicate that the immune response of pepper plants depends on the variety and the biological agents that can cause induced resistance to infection. It has been established that the varieties Ivaylovska kapiya, Sivriya, Dzhulyunska shipka and Pirouette F1 are susceptible to infection by *S. sclerotiorum*. Susceptibility to the pathogen varies among pepper varieties.

Differences in the immune response of pepper varieties to *S. sclerotiorum* infection are expressed in the length of the incubation period, frequency of infection, rate of formation and number of sclerotia. The varieties Pirouette F1 and Dzhulyunska shipka have a weaker immune response (the incubation period of the disease is 4 days, infection rate is 31%). The varieties Sivriya (incubation period of the disease is 5 days, infestation 11%) and Ivaylovska kapiya (incubation period of the disease 4 days, infestation 22%) have a good immune response to infection. The formation of sclerotia in hot pepper fruits occurs two days earlier than in other varieties. It was established on the 8th day in this variety, and in other varieties – on the 10th day of infection. The biological products used influence the immune response of pepper to infection with *S. sclerotiorum*. The least development of stem necrosis was observed in the variant with the biological preparation „Trichodermin", which was used for watering plants in the form of a liquid preparation with a working solution concentration of 1.10^{10} c/ml. The results obtained show the variability of disease resistance among popular pepper varieties grown in greenhouse conditions in Bulgaria.

Keywords: white mold; *Capsicum annuum* L.; varieties; resistance; biological products

Introduction

Sclerotinia stem and fruit mold, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a potentially serious disease of pepper (*Capsicum annuum* L.), affecting both seedlings and mature plants (Kim et al., 2000; Pernezny & Purdi, 2000; Pernezny et al., 2003a; b). The possibilities of controlling the disease are limited and there is no information on the resistance of *Capsicum* spp. to this pathogen (Gonzalez et al., 1998; Heffer Link & Johnson, 2007; Sanogo, 2003; Tsitsi-

giannis et al., 2008; Winton et al., 2006). *Sclerotinia sclerotiorum*, the causative agent of white mold, is a necrotrophic and non-host-specific fungal pathogen that infects > 400 plant species worldwide and is now considered as a serious threat to many economically important crops, including soybean, peanut, and canola (Hegedus & Rimmer, 2005). Controlling the disease through chemical and breeding practices is largely unreliable and the level of host resistance to this pathogen is unstable (Li et al., 2004). Studies of host-pathogen interactions at the cellular level can contribute to the development of more effective disease control measures (Hermosa et al., 2000). The interaction of *S. sclerotiorum* with several different host species was first studied by de Bary (1884). Subsequent studies investigating *S. sclerotiorum infection* processes in compatible interactions (Kora et al., 2008) were conducted on beans (Abawi et al., 1975), soybean (Satton et al., 2001), lettuce, tomato, potato (Purdy, 1979), pea, sunflower (Sedun & Brown, 1987) as well as canola/canola (Huang et al., 2007). These studies confirm that suitable nutrient sources – flower petals, injured or senescent plant tissue – are used by germinating ascospores, both to establish a saprophytic phase and to successfully infect healthy plants (Ahmadi et al., 2012).

The interaction between the pathogen and genotypes of sunflower, bean, rape was studied. However, no such attempts have been made with pepper varieties grown under greenhouse conditions.

The level of resistance in commercial varieties is unknown and the potential sources of resistance to the pathogen in *C. annuum* and other *Capsicum* spp. have not been identified (Andrade et al., 2016).

The aim of the present work was to study the immune response of pepper varieties to infection with the pathogen *Sclerotinia sclerotiorum*.

Materials and Methods

Seedling production

Seeds of *Capsicum annuum*, sweet peppers varieties Pirouette F1, Ivaylovska Kapiya, Sivriya and hot pepper variety Dzhulyunska shipka were sown in the greenhouse in 28-cell black plastic trays of size 53/34/7 cm, filled with a peat-perlite mixture prepared according to the following recipe: 70% peat "Professional Planting Mix" + 30% perlite.

The plants were watered daily or as needed. The seedling trays are placed on tables in greenhouses, at an air temperature of +28°C ± 2°C. In phase 3–4 true leaves, the seedlings are transplanted into plastic pots with a diameter of 15 cm, containing a sterile peat-perlite mixture for greenhouses, consisting of: peat – with a pH of 5.6–6.4 – 1000 L, perlite – 300 L , labin 10:40:10 ME – 0.3 kg, triple superphosphate ($Ca(H_2PO_4)_2$ $CaHPO_4$) – 0.2 kg, potassium sulfate (K_2SO_4) – 0.2 kg, ammonium nitrate (NH_4NO_3) – 0.1 kg.

Study of the immune response of different pepper varieties to infection with the pathogen* Sclerotinia sclerotiorum *by the detached leaf assay

A method described by Guimaraes et al. (2022) was applied. Leaves from different levels of five plants were used for artificial infection. The tested leaves were wounded with a sterile needle by pricking. On the upper surface of each leaf, 8-mm-diameter blocks of a 7-day-old pure culture of *S. sclerotiorum* grown on standard PDA medium were plated. For the control, plants were used, on the leaves of which sterile blocks of PDA were applied. The inoculated leaves were stored in a humid chamber at +21°C in the dark. The experiment was performed in three replicates, 5 leaves in one replicate of each variety: (5×3). The following data were measured: the incubation period (day of appearance of the infection signs), the dynamics of the development of the fungus daily up to the 10^{th} day after applying the inoculum (cm). The 0–4 scale of Andrade et al. (2016) was used to report the disease, where 0 – means fruits without symptoms; 1 – beginning of formation of small brown spots at the site of injury; 2 – well defined brown spots; 3 – spots with d = 2–3 cm; 4 – with d > 3 cm. Based on the result, a scale for evaluating the disease (Degree of development) was established from 0 to 4: 0 = no symptoms; 1 = symptoms with <10% disease incidence; 2 = symptoms with 11–20%; 3 = symptoms with 21–40%; 4 = symptoms with 41–100% disease frequency. Degree of resistance of the varieties: cultivars with an average scale for the evaluation of the disease < 1 – resistant (R), 1–2 – moderately resistant (MR); 2–3 susceptible (S); and 4 – highly susceptible (HS).

Study of the immune response of different pepper varieties to Sclerotinia sclerotiorum by artificial infestation of detached ripe fruits

Detached healthy pepper fruits at technological and botanical maturity were prepared by surface sterilization with 0.05% NaClO and rinsed three times with sterile distilled water, then allowed to air dry for several minutes. The fruits thus prepared were punctured and inoculated with agar discs (d = 8 mm) containing a mycelium of a *Sclerotinia sclerotiorum* isolate from a pepper plant. For the control sterile discs from PDA were used. All fruits were placed in a theromstat for 14 days at 100% relative humidity and +21°C in the dark. In the experiment, 6 varieties of *C. annuum* were included, of which 5 are Bulgarian (Sivriya, Ivaylovska kapiya, White kapiya, Bulgarian ratund, Dzhulunska shipka) and Pirouette F1 of the Syngenta AG Company. The experiment was carried out twice in 3 replicates, each consisting of 3 fruits.

The pathogen was re-isolated and the resulting cultures were compared with the baseline.

The following data were measured: the incubation period (day of the appearance of the infection signs), the dynamics of the development of the fungus daily up to 10 days after applying the inoculum – the diameter of necrosis (cm), the beginning of the formation of sclerotia (day), the number of sclerotia in one fruit (n).

Study of the immune response of pepper to Sclerotinia sclerotiorum infection in the background of introduced antagonists

The purpose of the research is to determine the influence of biological preparations (based on the studied antagonists *Trichoderma viride*, *Bacillus subtilis* and *Enterobacter cloaceae*) on immune response (increase in stem necrosis) in a comparative experiment with humic acids, microfertilizer SiO_2 and the fungicide methyl thiophanate.

The experiment was carried out in greenhouse conditions with the Pirouette F1 variety, planted according to a scheme (90/60/40 cm) on the plot with mineral fertilization (fertilizer rate N_{22} P_{16} K_{20} kg/ha).

- Control 1 – without introduction of antagonists and organic products.
- Control 2 – one-time watering of the plants with thiophanate methyl (concentration of 0.1%) after planting in a permanent place.
- Spraying with "Optysil" (0.05 ml per 1 L of water) – twice: after transplanting and during the mass flowering.
- Watering with humusil solution (consumption rate of 5 ml/10 L of water per 20 m^2) – twice: after transplanting and during the mass flowering.
- Application of "Trichodermin" (10 ml/10 L of water, for 20 m^2, titer 1.10^{10}) – during transplanting.
- Treatment with "Extrasol" (10 ml/10 L of water, for 20 m^2) – watering twice – after transplanting and during the mass flowering.
- Treatment with a bacterial preparation based on *Enterobacter cloaceae* (10 ml/10 L of water, for 20 m^2) – two waterings: after transplanting and during the mass flowering.

***Content description of used substances**:*

- "Extrasol" ("Bisolbi-Inter" LTD, St. Petersburg, Russian Federation) – Active rhizosphere bacteria *Bacillus subtilis*, dry matter – not less than 19%, organic matter – 58–64% of dry matter, humic acids – 50–85% of the organic content, fulvic and low-molecular organic acids – 15–50% of the organic content, potassium – not less than 9% of the dry content, salts of humic acids – 80–90% of dry matter, trace elements; Titer: 1.10^{10} CFU/ml;
- *Enterobacter cloaceae* (isolate 1B, National Bank for Microorganisms, Sofia, Bulgaria) – genus (Enterobacter), family (Enterobacteriaceae), (Enterobacteriales), class of gamma-proteobacteria (γ proteobacteria), type of proteobacteria (Proteobacteria), kingdom of bacteria. The bacterium was multiplied on a peptone medium during 72 h with continuous shaking in a thermal incubator at a temperature of +28°C. Titer: 1.10^{10} CFU/ml;
- Humic acids ("Agrospace" LTD, Bulgaria): Potassium humates, humic and fulvic acids. Nutrient content not less than: total nitrogen 2.0%; total phosphorus, P_2O_5 in the dry matter 1.0%; Total potassium, as K_2O in dry matter 6.5%; Total calcium, CaO in the dry matter 2.0%; Total magnesium, MgO in dry matter 0.5%; Organic carbon 23.0%; Organic matter 45.0%;
- "Trichodermin" (experimental batch of biopreparation produced in the laboratory of IZK "Maritsa" by deep production technology): *Trichoderma viride*, Trv1, titer 1.10^{10} c/ml;
- "Optysil" ("Intermag", Poland): SiO_2, Fe (16.5–2) – microfertilizer with biostimulating anti-stress action;
- Inoculation of plants was carried out by the decapitation method. The method provides a constant amount of inoculum of *Sclerotinia sclerotiorum* for each experimental plant (Hunter et al., 1978).

For this purpose, the Ss1 isolate of *Sclerotinia sclerotiorum* was grown on (PDA) for 7 days at +20°C; after which blocks of 1 cm diameter were cut from the colony of actively growing mycelium of the fungus to infect the plants. Each block was applied to the decapitated stems of 8-week-old pepper plants and wrapped with moist cotton pieces and foil (10 plants per variant). The decapitated plants, on which sterile (PDA) discs without the pathogen were applied, served as controls. Reading of the percentage of infected plants started 5 days after inoculation. An experiment was carried out in two replicates. The length of the necroses in the decapitated plants was recorded in mm up to and including the 10th day after inoculation. An aggressiveness index of the isolate is calculated according to the formula:

$$X = \sum \frac{(I_i \cdot D_i)}{n},$$

where: I_i – average length of necrosis, mm; D_i – average intensity of mycelium formation, index; n – amount of infected stems (Lavrova et al., 2003).

Results and Discussion

Study of the immune response of different pepper varieties to infection with the pathogen Sclerotinia sclerotiorum by the detached leaf assay

Results of the experiment show that among the tested varieties of pepper there are no completely immune to the pathogen *S. sclerotiorum.* When plants were infected by the foliar method, a difference in the length of the incubation period was found for different pepper varieties (Table 1).

In the Pirouette F1, Dzhulyunska shipka and Ivaylovska kapiya varieties, the incubation period is 4 days, in the Sivriya variety – 5 days. This does not apply to the indicator „frequency of occurrence" (Figure 1). The percentage of infected sites was calculated to assess disease severity 6 days after inoculation.

In varieties Pirouette F1 and Dzhulyunska shipka the frequency of symptoms (necrotic spots) progressed from 8.88% on the 4th to 31.11% on the 6th day of the total number of infections. In the Ivaylovska kapiya variety, this indicator increased from 2.22% to 22.22% on the 4th and 6th days of inoculation. A relatively low level of susceptibility to infection with the pathogen was reported in the variety Sivriya. The incubation period of the disease in this variety is 5 days, the frequency of infection is 11.11% on the 6th day. According to the research of some authors (McCaghey et al., 2017; Yanar & Miller, 2003; Grau et al., 1982), differences in the varietal response of pepper to infection with the pathogen *S. sclerotiorum* may be due to morphological features of the pepper cultivars and chitin content in the leaves. Compared to them, the infectious process in plants with an increased content of chitin in the leaf mass proceeds more slowly and has a weaker manifestation. The pathogen *S. sclerotiorum,* which refers to necrotrophic fungi, more rapidly decomposes the cell walls of the plant with less chitin content.

As a result of the conducted experiment, the following conclusions can be drawn:

Table 1. Immune response of pepper varieties to infection with the pathogen *Sclerotinia sclerotiorum* (detached leaf assay according to Guimaraes et al. (2022)

No	Variety	IP, day	IR infection rate, %			D, cm	ID,	Degree of resistance
			4	5	6			
1	Pirouette F1	4	8.88	31.11	31.11	1.46	3	S
2	Kurtovska Kapiya	4	2.22	17.79	22.22	2.10	3	S
3	Shipka	4	8.88	26.67	31.11	1.64	3	S
4	Sivriya	5	0.00	8.88	11.11	1.25	2	S

Abbreviation: IP- incubation period; IR – infection rate (%). D – diameter of necrosis, (mean, cm) (ID) – degree of disease development on a 0–4 scale (Andrade et al., 2016). Degree of resistance of the variety: the variety types with an average scale for the evaluation of the disease <1 – resistant (R); 1–2 – moderately resistant (MR); 2–3 susceptible (S); and 4 – highly susceptible (HS)

Fig. 1. Development of necrosis on the leaves of pepper varieties Dzhulyunska shipka, Pirouette F1, Ivaylovska kapiya and Sivriya 6 days after infection with a pure culture of *S. sclerotiorum*. From left to right: Dzhulyunska shipka, Pirouette F1, Ivaylovska kapiya and Sivriya

- The varieties Ivaylovska kapiya, Sivriya, Dzhulyunska shipka and Pirouette F1 are susceptible to infection with the white mold agent *S. sclerotiorum;*
- Differences were found in the immune response of pepper cultivars to infection with *S. sclerotiorum*. The Pirouette F1 and Dzhulyunska shipka varieties have a weaker immune response. The incubation period of the disease is 4 days; the frequency of infection is 31%. With a good immune response to infection are varieties Sivriya (Incubation period of the disease is 5 days, frequency of infections – 11%) and Ivaylovska kapiya (Incubation period of the disease is 4 days, frequency of infections – 22%).

Study of the immune response of different pepper varieties to Sclerotinia sclerotiorum by artificial infestation of detached ripe fruits

The results of the infection of the fruits of different pepper varieties with the pathogen *S. sclerotiorum* confirmed those obtained by the leaf method. The fungus attacks all varieties of pepper. After the introduction of the pathogen into the tissues of the host plant, concentric, slightly sunken, necrotic spots are formed on the surface of the fruit. The fungus develops mainly inside the fruits. For this reason, the diameter of the external necrosis when the fruits are infected with the pathogen *S. sclerotiorum* cannot serve as an indicator of variety resistance. A section of the infected fruits reveals abundant mycelium and numerous sclerotia of the pathogen. Hot peppers, which have a thin pericarp and a high carbohydrate content, are the fastest infected. The formation of sclerotia in hot pepper was reported on the 8th, in the other varieties – on the 10th day of infection (Figure 2).

The amount of sclerotia formed depends not only on the thickness of the pericarp, but also, above all, on the carbohydrate content of the fruit. The carbohydrate-rich fruits of the Bulgarian ratund and Ivaylovska kapiya varieties are a good nutritional substrate for the development of the fungus. Numerous large sclerotia are formed in the fruits of these varieties. In the White капiya variety, the amount of formed sclerotia is the smallest (Table 2).

Table 2. Response of different pepper varieties to infection with *Sclerotinia sclerotiorum* (detached fruits method)

Variety	Content of carbohydrates, mg/g	Pericarp thickness, mm	Formation of sclerotia, days	The number of sclerotia in a fruit		D, cm.
				10th day	13th day	
Bulgarian Ratund	9.0	5–7	10	48	56	3.73
Ivaylovska Kapiya	11.5	6–8	10	36	44	11.83
Sivriya	4.6	5–6	10	17	31	6.42
Pirouette F1	5.6	4–6	10	5	19	3.30
White Kapiya	6.1	6–8	10	2	17	3.33
Hot pepper	6.9	2–3	8	12	17	6.17

Abbreviation: D – necrosis diameter, cm (average for the variety); carbohydrate content is based on literature data (25)

Fig. 2. Infection of pepper varieties with the pathogen *S. sclerotiorum*: top row – 8th, bottom row – 10th day of inoculation. Varieties: Bulgarian ratund, Ivaylovska kapiya, Sivriya, Pirouette F1, White kapiya, Dzhulyunska shipka

Study of the immune response of pepper to Sclerotinia sclerotiorum infection in the background of introduced antagonists

The stem decapitation method allowed to establish differences in the immune response of plants treated with biological and organic products to infection with the pathogen *S. sclerotiorum* (Figure 3).

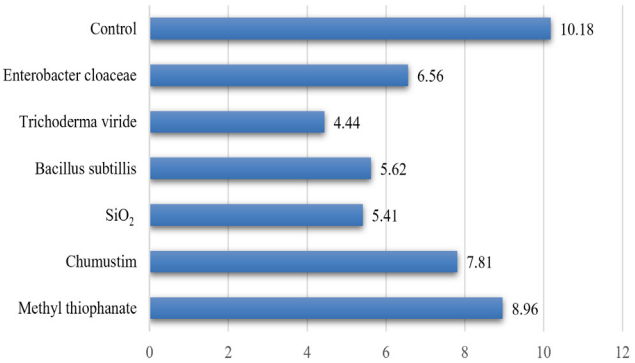

Fig. 3. Increase in necrosis after infection of pepper plants with the pathogen *S. sclerotiorum* by the stem decapitation method, cm

The "necrosis length" indicator in the experiment varied from 2.00 mm to 20.00 mm, depending on the imported bioproducts. The plants tested showed varying degrees of susceptibility to the pathogen. The results of the artificial infection of pepper with the pathogen *S. sclerotiorum* by the decapitation method show that the immune response of the infected plants differs, depending on the applied biological agents. The strongest development of necrosis was reported in the variant without use of biological preparations. Trichodermin, applied for watering plants as a liquid preparation with the concentration of a working solution of 1.10^{10} c/ml, has growth regulator properties and enhances the immune response of plants to white mold infection. Growth of necrosis in this variant is two times weaker than the average for the experiment. The results of the experiment confirm results obtained by other researchers, who propose antagonistic fungi for combating white mold in vegetable crops (including pepper) as an effective method with a multidirectional effects on the development, immune response and yield of plants (Rocha-Ramirez et al., 2002; Zhao et al., 2020). Rhizosphere fungi of the genus Trichoderma can provide long-lasting protection even with a single application at the beginning of the season. Remaining on the roots, they can reproduce along with the growing root system and remain viable during the entire growing season of the crop.

Conclusion

The varieties Ivaylovska kapia, Sivriya, Dzhulyunska shipka and Pirouette F1 are susceptible to infection with the white mold pathogen *S. sclerotiorum.* Susceptibility to *S. sclerotiorum* varies among pepper varieties. Differences in the immune response of pepper varieties to *S. sclerotiorum* infection are expressed in the length of the incubation period, the frequency of infections, the rate of formation and the number of sclerotia. Hot peppers and varieties with a high carbohydrate content are the fastest infected. Use of biological preparations based on *Trichoderma viride*, *Enterobacter cloaceae*, *Bacillus subtilis* in pepper crops increases the resistance of plants to the pathogen *S. sclerotiorum*. The least development of stem necrosis was observed in the variant with the biological preparation „Trichodermin", which was used for watering plants in the form of a liquid preparation with a working solution concentration of 1.10^{10} c/ml.

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