ХАРАКТЕРИСТИКА НА ПОЛУЧЕНИ ОТ СЛЪНЧОГЛЕД ИЗОЛАТИ НА *MACROPHOMINA PHASEOLINA* И *FUSARIUM SPP.*
CHARACTERIZATION OF *MACROPHOMINA PHASEOLINA* AND *FUSARIUM SPP.* ISOLATES FROM SUNFLOWER

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Abstract

Macrophomina phaseolina and *Fusarium spp*. are soil borne plant pathogens causing seed-, root-, collar- and stem rot, as well as vascular wilt and discoloration. Several isolates obtained during 2014-2015 from rotten stems and roots of sunflower plants, hybrid *LG 56.63 CL*, from the region of Northeast Bulgaria, were studied for their basic morphological and cultural characteristics, pathogenicity and sensitivity to fungicides. One of the isolates was identified as *Macrophomina phaseolina* (Tassi) Goidanich and two of them were identified as *Fusarium spp.* using colony morphology, density and extent of mycelia growth. On PDA, the colonies of *Macrophomina phaseolina* showed dense growth, initially olive gray, then becoming black. None of the cultures produced pycnidia. Microsclerotia were produced after the 5th day. On PDA the cultures from single-spore isolates of *Fusarium spp.* formed pink to raspberry and purple colored floccuse mycelium, macroconidia, microconidia and chlamydospores. Pathogenicity tests were carried out in a greenhouse by the stem-tape inoculation method. In vitro sensitivity of *Macrophomina phaseolina* and *Fusarium spp.* to five fungicides was determined through the inhibition zone technique (*Thornberry's* method). The antagonistic activity of *Trichoder-ma viride* (Tr 6) against *Macrophomina phaseolina* and *Fusarium spp.* in vitro was evaluated by means of the opposite culture method.

Key words: *Macrophomina phaseolina, Fusarium spp.,* sunflower, fungicides, *Trichoderma viride*.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the major oilseed crop in Bulgaria, 45% of the planted areas being located in the Northeast Black Sea and the Danube river regions. In the recent years, the air temperatures during its active vegetation period have been often high (over 30^{0}C), combined with heavy short rainfalls. Abiotic stress, monoculture farming, increased doses of nitrogen fertilization and the introduction of new hybrids for Bulgaria have a direct impact on the development of pathogenic microorganisms, such as *Macrophomina phaseolina* and the species of *Fusarium* genus.

Macrophomina phaseolina (Tassi) Goid causes charcoal rot in over 500 crop and non-crop plants like cereal crops, maize, sunflower, soybean, cotton, etc. most often in the countries situated in the tropical and subtropical climatic zones. The symptoms are dry rot, wilt, leaf blight and ashy stem blight. Pustovoit warned about yield reduction, decrease of the weight of 1000 seeds, decrease of the protein content in seeds and changes in the fatty acid content of the seed oil (Antonova et al., 2002). Yield reduction can reach up to 50% (Jimenez-Diaz et al., 1983). Other authors think that *Macrophomina phaseolina* does not cause serious damages and it is a benign parasite (Dovet, 1987). In Bulgaria the spread of charcoal rot in the period 1990-1996 was from 16 to 42.8% (Alexandrov, 1999) and in 2002 – 2008 it varied from 3-5% to 80% in some hybrids. The most significant impact on disease manifestation have the water stress (Mayer-Perez et al., 2002) and the soil temperatures of 28-35^{0}C, when pathogenicity of *Macrophomina phaseolina* is the highest (Dhingra and Sinclair, 1978). Disease development is favored by high temperatures and little rainfall during the reproductive period of sunflower and under those conditions seed yield formation could be threatened (Mirza and Beg, 1983). The great genetic, physiological, morphological and cultural

variability of the pathogen is explained by the broad host range (Ijaz et al., 2012). Difficulties in the control of *Macrophomina phaseolina* are determined by the possibilities of microscerotia survival in soil for several years. Disease control includes cultural, regulatory, physical, chemical and biological methods, which should be applied prophylactically. After the appearance of the disease symptoms the methods of control become inefficient and useless. Although the chemical control is good and easily applied, the ecological aspects in agriculture increasingly require the use of biological agents for the control of charcoal rot in sunflower, such as *Trichoderma spp., Trichoderma viride, Aspergillus flavus, Bacillus subtilis* (Gacitua et al., 2009; Ullah et al., 2011; Reetha et al., 2014).

Species of *Fusarium* genus are soil pathogens, polyphages and cosmopolites. They infect the plants mainly through wounds and cause rotting of seeds, roots, stems. They often develop in the vascular plant tissues and cause wilting of plants (Tancic et al., 2012). The negative impact of those pathogens on sunflower was reported by a number of authors (Goncharov et al., 2006; Sharfun-Nahar et al., 2007; Mathew et al., 2010).

Sunflower (*Helianthus annuus* L.) is one of the many common hosts of *Macrophomina phaseolina* and *Fusarium spp.* Nematode damages together with abiotic stress associated with high temperatures (25-30-36^{0}C) and soil drought are factors favoring the manifestation of both pathogens (Mathew et al., 2010; Tančić et al., 2012; Ijas et al., 2012).

Climate changes associated with increasing and retaining high temperatures (over 30^{0}C) during the reproductive period of sunflower in the recent years have led to the reappearance of forgotten in Bulgaria pathogens such as *Macrophomina phaseolina*, alone or together with *Fusarium spp*. That was the reason for us to carry out the present study.

MATERIALS AND METHODS

Diversity of symptoms and pathogenic fungal isolate

Symptoms of charcoal rot and *Fusarium* wilt were observed separately or more often together in 2014-2015 in sunflower fields, hybrid LG 56.63 CL, in Northeast Bulgaria. Isolation of the pathogens was performed by the classical method (Zazzerini and Tosi, 1987).

Pathogenicity test

Pathogenicity of the obtained isolates was confirmed by inoculating visually healthy apple fruits of Golden Delicious cultivar by placing pieces of 7-day mycelium colonies in incisions made with a scalpel. The inoculated fruits together with those of the control (injured with a scalpel but not infected) were stored in a wet chamber and after the symptoms developed, re-isolation was performed from the necrotic areas.

Pathogenicity test was fulfilled by the stem-tape inoculation method (Zazzerini and Tosi, 1987). Sunflower plants hybrid LG 56 63, at the stage of 6-8 leaf, were inoculated with two isolates (LG L and LG S), which had shown a positive result in the initial test on the apple fruits. The plants were infected with a 7-day culture of each isolate. The experiments were carried out in a greenhouse at an air temperature varying from 20^{0}C to 25^{0}C and 14-hour photoperiod. The experiment was set in four variants (one variant with each isolate used separately, one variant with an inoculum prepared as a mixture of both isolates and a control), in 3 replications. The results were reported on the 21st day after infecting the plants.

Cultural and morphological characterization of the isolates

Morphological characteristics of the obtained isolates were studied by a microscope analysis (Olympus, CH-2, Japan). The mycelium and conidiospore types and the existence of vegetative forms (chlamydospores and sclerotia) were determined.

The cultural characteristics of the isolates were determined on 5 nutrient media: potato-dextrose agar (PDA), pea agar (PA), corn meal agar (CMA), cherry agar (ChA) and malt agar (MA). Each variant was set in 3 replications. The growth rate was determined when growing the cultures at 24–25^{0}C by measuring the diameter of the colony on the 3rd, 5th and 7th day.

In vitro screening of fungicides for control of *Macrophomina phaseolina* and *Fusarium spp.*

The inhibiting effect of the fungicides Boskalid + Dimoxistrobin (Pictor) – 0.05%, trifloxystrobin + cyproconazole (Sphere Max) – 0.05%, zoxystrobin (Amistar Extra) – 0.07% and prochloraz (Mirage) – 0.1% on mycelial growth of the isolates was determined by the modified method of Thornberry (1950). Each variant was set in three replications. Discs with a diameter of 0.5 cm were cut out with a cork borer from the 7-day culture of the isolates and placed in Petri dishes with 9 ml of PDA supplemented with 1 ml of the fungicide at a definite concentration. Pure nutrient medium (without fungicide) – PDA was used as control. The vari-

ants were incubated in a thermostat at 25^{0}C. The inhibiting effect of the fungicides was evaluated by measuring the diameter of the colonies on the 3rd, 5th and 7th day. The coefficient of inhibition was determined by the formula (Batzer et al., 2005)

$$I\% = \frac{C-T}{C} x100$$

Where:

I – coefficient (index) of inhibition;

C – diameter of the mycelium colony in the control (mm);

T - diameter of the mycelium colony in the treated variant (cm).

Antagonistic activity of *Trichoderma viride* (*Tr* 6) against *Macrophomina phaseolina* and *Fusarium spp.* in vitro

The antagonistic activity of *Trichoderma viride* (*Tr* 6) on the mycelial growth of *Macrophomina phaseolina* and *Fusarium spp.* was evaluated by the opposite culture method. Mycelial blocks (5 mm) of 7-day cultures of the pathogens and the antagonist were oppositely placed at the periphery of the Petri dish and incubated at a temperature of 25^{0}C. The antagonistic effect of *Trichoderma viride* was evaluated by measuring the diameter of the colonies on the 3rd, 5th and 7th day.

The percentage of inhibition was obtained using the formula (Khare et al., 2010): PI = (D_1 –D_2)/ D_1 .100 (D_1 – diameter of radial growth of *Macrophomina phaseolina* or *Fusarium spp.* in the control; D_2 – diameter of radial growth of *Macrophomina phaseolina* or *Fusarium spp.* in the treated variants (in the presence of the antagonist).

RESULTS AND DISCUSSION

Symptoms diversity and pathogenic fungal isolate

Sunflower plants with symptoms of wilting were the most common damages typical of both charcoal rot and *Fusarium wilt* – wilting of the leaves, necrosis and corkiness of the stem base, reduction of the root system. Often the stems broke in the necrotic area. Symptoms were most obvious at the flowering stage. A large number of microsclerotia were found in the stem core, imparting gray tinge, which is a typical symptom of *Macrophomina phaseolina*. In the majority of the studied plants microsclerotia and pink coloration of the stem core were observed simultaneously, which is typical of *Fusarium spp*. That also affected the obtained isolates. Isolates of *Macrophomina phaseolina and* of *Fusarium spp*. were obtained from one and the same plants.

Pathogenicity test

Four out of the 15 isolates showed a positive result in the initial screening for pathogenicity to apple fruits. The strongest effect associated with the formation of microsclerotia (LG L) and raspberry pink coating (LG M and LG S) was detected in three isolates. Sunflower plants were inoculated with two of them – LG L and LG S. Obviously expressed symptoms developed 14 days later, first in the variants with LG S isolate (*Fusarium spp.*). Necrotic spots appeared at the stem base. Corkiness and necrosis were detected after cutting the stem longitudinally. Three weeks later the damages caused by the isolate LG L (*Macrophomina phaseolina*) were observed. Necrotic spots appeared on the leaves, enlarging from the margins, and the stem core changed in color and structure, turning brown and loose.

Cultural and morphological characterization of the isolates

Based on a microscopic analysis, it was established that the colony of the isolate LG L consists of branched, septate mycelium, colorless at the beginning, subsequently turning olive green to olive brown and black. Numerous black globular irregular oval, smooth and glossy microsclerotia were formed on the 5th day, which were 86-104 μm in size. Those characteristics show that the isolate belongs to *Macrophomina* species.

The colony of LG S isolate on PDA developed whitish and in the mature plants – pale to raspberry pink multicellular mycelium. Microconidia, macroconidia and chlamydospores were formed on it. Microconidia are single-celled, elliptical (5-12 x 2.3-3.5 μm). Macroconidia are crescent-shaped, 1- to 3-septate, with a well-defined foot (23-54 x 3-4.5 μm). The pathogen forms globular shaped chlamydospores, 5-13 μm in size.

The best development of *Macrophomina phaseolina* (LG L) was reported on PDA (dense olive-green mycelium), followed by PA (tender substrate olive green mycelium), MA (olive green moderately dense mycelium) and CMA (tender substrate olive green mycelium). The weakest development of the culture was reported on ChA (tender substrate olive green mycelium) (Fig. 1).

The colony of *Fusarium spp.* (LG S) showed the same growth rate on all the nutrient media (Fig. 2). The most rapid development was observed on PDA (dark pink cotton-like mycelium) and CMA (milky pink cotton-like mycelium), followed by PA (raspberry pink substrate mycelium) and MA (pale pink mycelium with dark pink radial stripes). The weakest development was established on ChA (white tender mycelium).

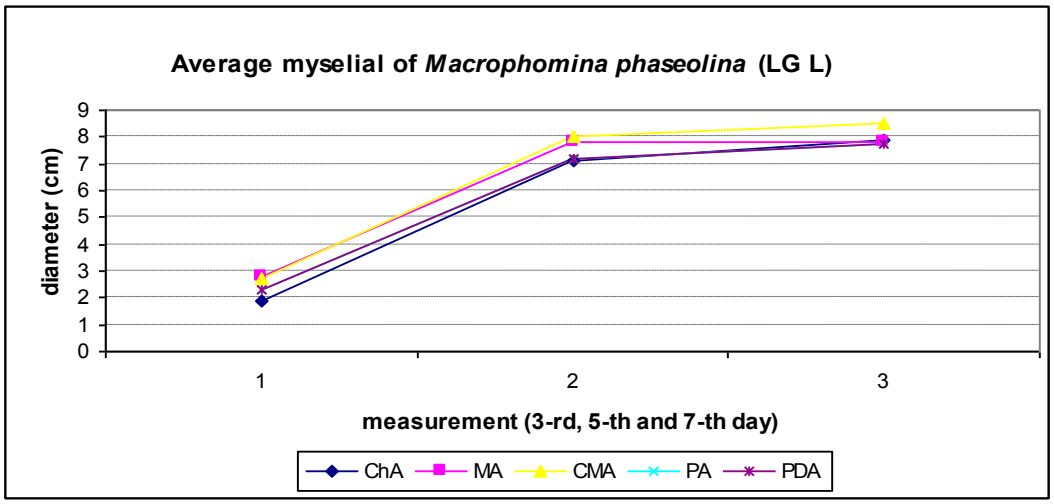

Fig. 1. *Mycelial growth of Macrophomina phaseolina on different artificial culture media*

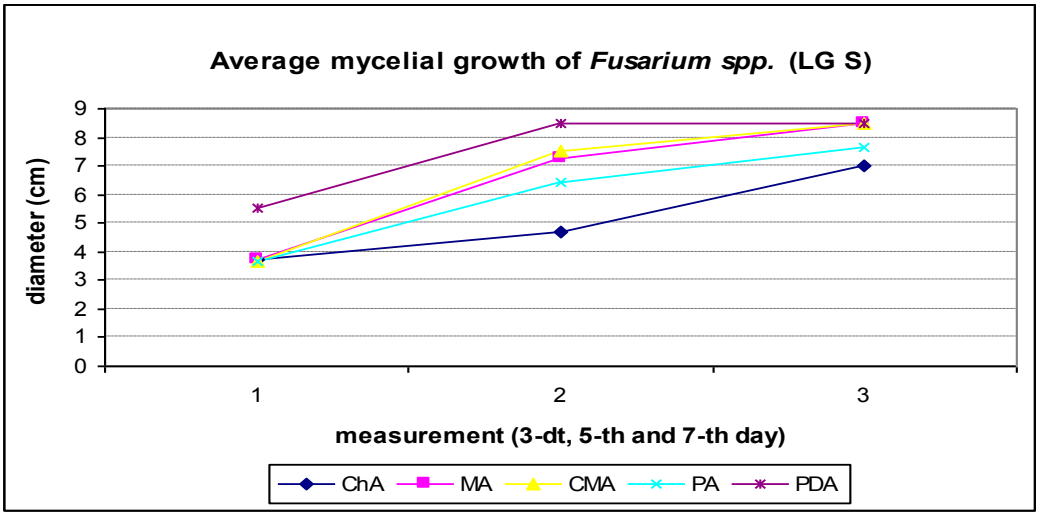

Fig. 2. *Mycelial growth of Fusarium spp. on different artificial culture media*

In vitro screening of fungicides for control of *Macrophomina phaseolina* and *Fusarium spp.*

Out of the tested fungicides, the highest efficiency against *Macrophomina phaseolina* was demonstrated by Pictor – 82%, followed by Amistar Extra – 33%, Sphere Max – 29%, and Mirage – 24% (tabl. 1).

Referring to *Fusarium spp.* (LG S), all the in vitro tested fungicides had a good inhibiting effect – above 50%. Sphere Max (81%), Amistar Extra (77%) and Mirage (74%) demonstrated the strongest inhibiting effect on the pathogen, followed by Pictor -56% (tabl. 2).

All the tested fungicides showed a fungistatic effect.

Antagonistic activity of *Trichoderma viride* (*Tr* 6) against *Macrophomina phaseolina* and *Fusarium spp.* in vitro

The colony of *Trichoderma viride* (*Tr* 6) suppressed the development of *Fusarium spp.* (46%), but did not colonize the culture, while in the variant with *Macrophomina phaseolina* the antagonist almost completely suppressed the development of the pathogen. A narrow zone was detected, differentiating the two colonies, but subsequently, on the 7th day, the colony of *Trichoderma viride* developed on that of *Macrophomina phaseolina* (Fig. 3). That confirms the results of Ullah et al. (2011) and Reetha et al. (2014). According to them the antagonistic effect of *Trichoderma viride* in vitro varied from 51% to above 77%. Treatment of sunflower seeds with *Trichoderma viride* controls successfully *Macrophomina phaseolina* and does not exert a negative effect on their germinability (Singh et al., 2015).

Table 1. Effect of different fungicides on the mycelia growth of Macrophomina phaseolina

№	Fungicides	Mycelium growth (cm)			Percent of inhibition (I %)
		3-rd day	5-th day	7-th day	
1	Pictor (boscalid+dimoxystrobin)	0,5	0,5	1,5	82
2	Sphere Max (trifloxistrobin+cyproconazole)	2,75	4,25	6	29
3	Amistar xtra (azoxystrobin+difeconazole)	2,75	4	5,7	33
4	Mirage (tebuconazole)	1,2	3,5	6,5	24
5	Control	5,87	8,5	8,5	-

Table 2. Effect of different fungicides on the mycelium growth of Fusarium spp

№	Fungicides	Mycelium growth (cm)			Percent of inhibition (I %)
		3-rd day	5-th day	7-th day	
1	Pictor (boscalid+dimoxystrobin)	1,5	2,5	3,75	56
2	Sphere Max (trifloxistrobin+cyproconazole)	0,5	0,5	1,6	81
3	Amistar xtra (azoxystrobin+difeconazole)	0,7	1	2	77
4	Mirage (tebuconazole)	0,8	1,7	2,25	74
5	Control	2,83	8,5	8,5	-

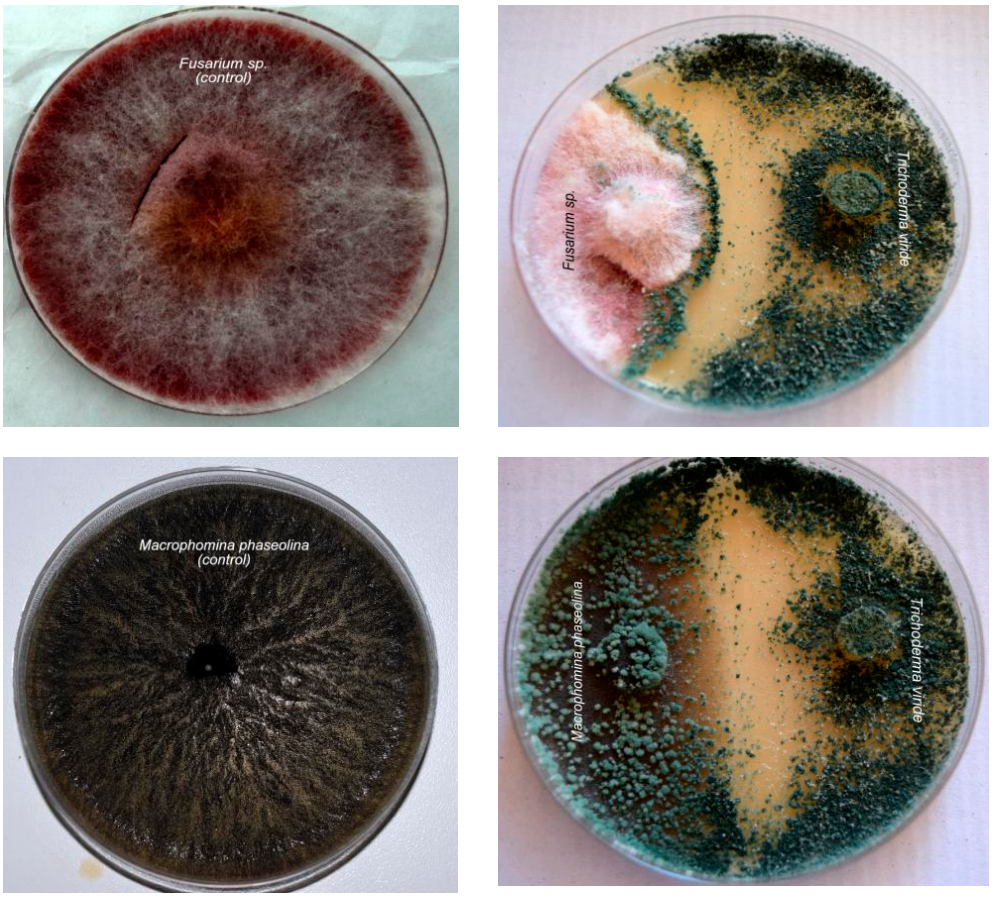

Fig. 3. *Effect of the antagonist Trichoderma viride (Tr 6) on the growth of the cultures of Fusarium sp. and Macrophomina phaseolina, 7 – th day, left – control, right – treatment*

CONCLUSIONS

1. In 2014-2015 the etiology of the damages related to wilting of the plants in the sunflower fields of hybrid LG 56 63 CL in Northeastern Bulgaria was described. *Macrophomina phaseolina* and *Fusarium spp.* were identified as the causative agents of the observed symptoms and a morphological and cultural characteristic of their isolates was made.

2. The results obtained in the screening of the fungicides under laboratory conditions created the prerequisite for their in vitro testing.

3. An initial test was carried out for the antagonistic activity of *Trichoderma viride* (*Tr* 6*)* against *Macrophomina phaseolina* and *Fusarium spp.* The successful impact of *Trichoderma spp*. on the pathogens of *Fusarium* genus is well-known. Physiological and genetic plasticity of *Macrophomina phaseolina* and *Fusarium spp.* creates difficulties in retaining the resistance of sunflower cultivars and hybrids. The lack of specific requirements to the soil type and the many hosts of both pathogens should direct our attention to the search for alternative means of control.

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