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FIRST APPEARANCE OF WHITE MOULD ON SUNFLOWER CAUSED BY *Sclerotinia minor* IN THE REPUBLIC OF MACEDONIA

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SUMMARY

Sclerotinia spp. a very destructive fungus causing "white mould" became one of the biggest problems in sunflower breeding in the Republic of Macedonia in 2010. Field monitoring in the region of Bitola show very high infection of around 20-30%. Two types of symptoms where observed during the field monitoring. First symptoms were observed on the leaves of the infected plants in the form of wilting, prior to flowering stage. The most characteristic symptoms were observed, at the lower part of the stem in the form of a stem cancer. Big variable sclerotia in size and shape were observed inside the stem. The appearance of white mycelium on the infected lower parts of the plant was often observed during the wet weather. Other infected plants showed different symptoms. The stem was longer and thinner than in uninfected plants, and the pit was very small, around 9 cm. Sclerotia observed inside the stem were not bigger than 2.5 mm. In vitro investigations confirmed the presence of ascomycetes *Sclerotinia sclerotiorum* (Lib.) de Bary and *Sclerotinia minor* Jagger, for the first time in the Republic of Macedonia.

In vitro investigation of antifungal ability of *Pseudomonas mediterranea* Cattara *et al.*, 2002 against *S. sclerotiorum* and *S. minor* showed that it can be a possible antifungal agent against these ascomycetes.

Key words: ***Sclerotinia sclerotiorum*, *Sclerotinia minor*, sclerotia, symptoms, *in vitro* investigations**

INTRODUCTION

Sunflower (*Helianthus annuus* L.), is an important oilseed plant in food industries for production of vegetable oils. In 2009 sunflower crop in Republic of Macedonia was sown on almost 4211 ha, mostly in the region of Bitola (3170 ha) (Statistical review, 2009; Table 1). Field monitoring showed the presence of white

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mould in the regions of Bitola, Prilep, Lozovo, Kumanovo and St. Nikola. This was the first observation of the presence of this disease in the Republic of Macedonia. Several plant pathogens are identified as the cause of white mould in sunflower crop: *Sclerotinia sclerotiorum* (Lib.) De Bary, *Sclerotinia minor* Jagger and *Sclerotinia trifoliorum* Eriks (Prudy, 1979; Nelson and Lamey, 2000; Gulya *et al.*, 1997). Therefore it was of great interest to identify the specific pathogen that caused the disease in Macedonia. *S. sclerotiorum* is an aggressive fungal agent which attacks and infects nearly 400 plant species (Prudy, 1979; Maširević & Gulya, 1992; Boland & Hall, 1994; Melzer *et al.*, 1997). The fungus cause head and stem rot in sunflower and survive as sclerotia in soil. Sclerotia can germinate carpogenic in apothecia, and myceliogenic in mycelium (Duane, L.T., 1979; Huang & Chi, 2003). Apothecia produce asci that release ascospores which cause primary infections in spring. Both ascospores and mycelia can case infection. *S. minor* and *S. trifoliorum* can cause very similar symptoms, and it is very difficult to distinguish them according to the symptoms in the field. They can be identified by their cultural, biological, macroscopic and microscopic characteristics. In order to find some new methods which would decrease the application of fungicide, an *in vitro* research was conducted using the bacterium *Pseudomonas mediterranea* (Cattara *et al.*, 2002). *Pseudomonas mediterranea* is tomato and pepper plant pathogen that cause pith necrosis. Hence it is closely related to *Pseudomonas corrugata* (Scarlet *et al.*, 1978) a successfully tested biological agent. We investigated the antifungal activity of *P. mediterranea* against *Sclerotinia sclerotiorum* and *S. minor* in *in vitro* conditions. The bacterium does not attack sunflower (*Helianthus annuus* L.), thus we assumed it could be successfully used as a biological control agent against "white mould" in sunflower plants. The aim of this study was to identify the pathogens causing white mould at sunflower in the Republic of Macedonia and to investigate *in vitro* a new, environmentally friendly method to suppress the infection.

MATERIALS AND METHODS

Field investigation

The monitoring was performed in the regions of Bitola, Prilep, Kumanovo, Gradsko, Lozovo and St. Nikola in the flowering stage and in the stage of maturing of the plants (Table 1). Samples of diseased material were collected and investigated in *in vitro* conditions.

Table 1: Review of the investigated regions

Region of production		Area / ha	Infection %
1	Bitola	3170	28 - 30
2	St. Nicole	127	18 - 22
3	Lozovo	188	21
4	Kumanovo	305	10 - 13
5	Gradsko	125	-
6	Prilep	26	18 - 20

Isolation of the pathogen

Mycelium from diseased plant parts was transferred to PDA medium containing streptomycin for bacteria suppression. Sclerotia were well washed in sink water, sterilized in 1% NaOCl for 2-3 min and transferred to a Petri dish containing PDA. Ten isolates from sunflower where included for investigation of the pathogen (Table 2). Petri dishes were put in growth chambers at temperature of 22°C and in darkness in order for mycelium growth and sclerotia to form. The identification of the pathogen was made by its macroscopic, cultural, biological and microscopic characteristics (Kohn, 1979).

Table 2: Review of the isolates used in this study

Isolate		Variety	Oregin	Area
1	ss-1	*S. sclerotiorum*	*Helianthus annuus*	Prilep
2	ss-2	*S. sclerotiorum*	*Helianthus annuus*	Prilep
3	ss-3	*S. sclerotiorum*	*Helianthus annuus*	Bitola
4	ss-4	*S. sclerotiorum*	*Helianthus annuus*	Bitola
5	ss-5	*S. sclerotiorum*	*Helianthus annuus*	Bitola
6	sm-1	*S. minor*	*Helianthus annuus*	Kumanovo
7	sm-2	*S. minor*	*Helianthus annuus*	Bitola
8	sm-3	*S. minor*	*Helianthus annuus*	St. Nikole
9	sm-4	*S. minor*	*Helianthus annuus*	St. Nikole
10	sm-5	*S. minor*	*Helianthus annuus*	Lozovo
11	P.m 09/1	*P. mediterranea*	*L. esculentum*	Strumica
12	P.m 09/2	*P. mediterranea*	*L. esculentum*	Strumica
13	Pm S1	*P. mediterranea*	Soil	Strumica
14	Pm S2	*P. mediterranea*	Soil	Strumica

***S. sclerotiorum* inoculum and carpogenically germination of sclerotia**

Sclerotia inside the stem and on the root were washed in water and air dried at room temperature for 7 days so that it could germinate and the apothecia could form. Dry sclerotia were then buried in 1-2 cm deep in autoclaved sand (B.M. Wu *et al.*, 2007). The dishes were then transferred to growth chambers at the temperature of 18-20°C, for 12 hour periods of light and dark and 30% RH (Cobb & Dillard, 2007). The sand was watered daily and the condensation formed on the underside of the Petri dish lid was uncapped with sterile filter paper. The same procedure was implemented with the sclerotia formed on PDA *in vitro*.

Bacterial isolates

Four isolates of *Pseudomonas mediterranea* obtained from tomato plants and soil (Table 2) were investigated for their antifungal efficacy. Bacteria were isolated from tomato plants showing symptoms of tomato pith necrosis and from soil where infected tomato plants were grown. Bacteria were maintained on medium NA and at 27°C.

Antifungal efficacy of *P. mediterranea*

For *in vitro* investigation of antifungal efficacy of *P. mediterranea* one sclerotia, per isolate was put in the centre of the Petri dish containing PDA medium and one day old bacteria were spread by touch onto the some Petri dish in three places. The experiment was conducted in three repetitions for each investigated isolate.

RESULTS

Field investigation

The first symptoms were observed in the flowering stage in the form of leaf wilting. The infected leaves became yellow, turned brown, and died. Characteristic symptoms where observed at the base of the stem in form of the cancer. Numerous sclerotia of different shapes and sizes were noticed on the root and inside the stem and the pith was totally destroyed (Figure 2). The presence of white mycelium was observed in the infected plant parts. Other symptoms observed were: tiny longer than usual stems with black lesions that spread onto the large surface of the steam. A great number of black and light brown sclerotia not bigger than 2.5 mm were present inside the stem and on the lower part of the stem, too (Figure 3). Sclerotia inside the stem were more circular comparing to the sclerotia observed on the stem which were more angular. The pith of the plant was smaller than usual with a diameter of around 9 cm.

Isolation of the pathogen

White cotton like mycelium, latter becoming gray, was observed on medium PDA. Sclerotia formation was observed after 2 weeks. Isolates ss-1, ss-2, ss-3, ss-4 and ss-5 on PDA formed bigger sclerotia, located circularly mostly at the edges of the Petri dishes (Figure 4). Isolates sm-1, sm-2, sm-4 and sm-5, formed smaller and more numerous sclerotia spread on the entire surface of the plate, forming aggregate forms consisting of several sclerotia (Figure 4).

***Sclerotinia* spp. inoculum and carpogenically germination of sclerotia**

Apothecia formation from the sclerotia where observed after 7-8 weeks. The apothecia matured after 2 weeks. Apothecia produced from isolates ss-1, ss-2, ss-3, ss-4 and ss-5 showed the prosenchyma turning out to the apothecial surface. Microscopic observation of the asci showed the presence of 8 ascospores with two nuclei. Apothecia formation from isolates sm-1, sm-2, sm-3, sm-4 and sm-5 showed ectal excipulum at the margin of the apothecium and the presence of globose cells. Ascospores were hyaline, and had four nuclei.

Figure 1: Symptom from S. sclerotiorum on the lower part of the stem and the presence of sclerotia

Figure 2: Sclerotia from S. sclerotiorum different in size and shape

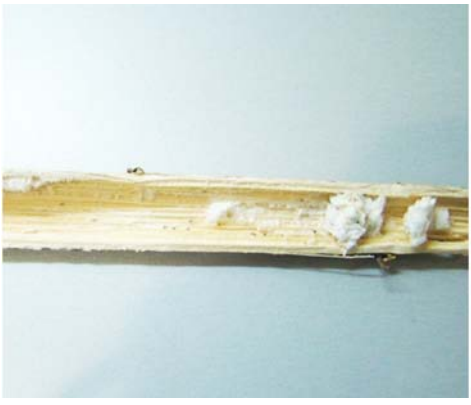

Figure 3: Sclerotia of S. minor inside the sunflower stem

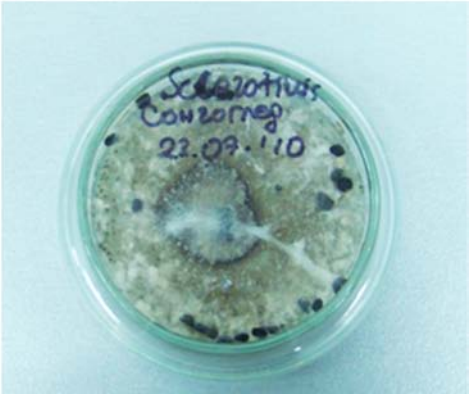

Figure 4: Sclerotia of S. sclerotiorum, isolate ss-1

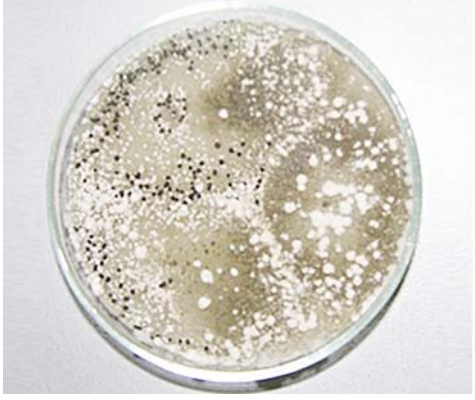

Figure 5: Sclerotia of S. minor, isolate sm-1

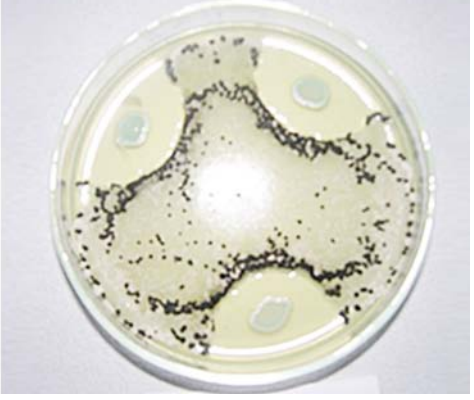

Figure 6: Antifungal efficacy of P. mediterranea against S. minor

Antifungal efficacy of *P. mediterranea*

The result of the antifungal efficacy was observed after two weeks. The mycelia in all isolates were suppressed 1-1.5 cm from bacterial spot where bacterial antifungal metabolites differ in the medium. This area was more yellow than other parts of the medium because of the lipopolypeptide production of the bacteria. Sclerotia at sm-1, sm-2, sm-3, sm-4 and sm-5, were formed and concentrated on the "border" with diffused antifungal metabolites (Figure 6). Isolates ss-1, ss-2, ss-3, ss-4 and ss-5 formed bigger sclerotia located on the edges, in all three repetitions. Only the presence of white mycelium occurred in the Petri dish. Positive controls showed the presence of sclerotia and cotton like white to gray mycelium (Figure 6).

DISCUSSION

The investigation in the field and *in vitro* conditions showed the presence of white mould in the area sown with sunflower plants. Two different types of symptoms were observed in the field and in the laboratory. In the area of Prilep diseased plants showed typical symptoms of white mould: wilting of the leaves, appearance of the cancer in the lower part of the stem, presence of large variable sclerotia and appearance of white mycelium on the infected parts of the plant. Sunflower plants from other investigated areas showed longer and thinner stem than in uninfected plants, a very small pit and small sclerotia not bigger than 2 mm. Thus, we assumed that maybe two different varieties were present causing disease of sunflower crops in R. Macedonia. The laboratory and *in vitro* investigation confirmed the presence of two different varieties of *Sclerotinia* spp.: *Sclerotinia sclerotiorum* (Lib.) de Bary and *Sclerotinia minor* Jagger.

An attempt to suppress the disease was conducted in *in vitro* conditions. In order to decrease the application of fungicide. Four isolates from the plant and soil pathogen *Pseudomonas mediterranea* were used as an antifungal agent. This bacterium is very closely related to the bacteria *P. corrugata* well known antifungal agent against various plant pathogen fungi, among them *Sclerotinia sclerotiorum*. Considering this data, we investigate the antifungal ability of *P. mediterranea* against *S. sclerotiorum* and *S. minor* in *in vitro* condition. The result which was obtained showed that this bacterium, suppressed the growth of mycelium and formation of sclerotia *in vitro* and that there is a possibility for it to be used to control "white mould" in sunflower in the field. In that case, additional tests should be conducted.

We think that *Pseudomonas mediterranea* can be considered as an antifungal agent against *Sclerotinia* spp. because it is soil and water bacterium, pathogen for tomato and pepper, but not for sunflower plants.

CONCLUSION

White mould in the Republic of Macedonia is caused by the ascomycetes *Sclerotinia sclerotiorum* (Lib.) de Bary and *Sclerotinia minor* Jagger, order Helotiales.

Preliminary investigations showed that *Pseudomonas mediterranea* Cattara *et al.*, 2002 can be considered as a biological control agent for suppressing the white mould and the pre laboratory investigation did show positive results.

This is the first report about the presence of *Sclerotinia minor* in the Republic of Macedonia.

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