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Effect of low temperature culture on the biological characteristics and aggressiveness of *Sclerotinia sclerotiorum* and *Sclerotinia minor*

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Abstract – Sunflower White Mold caused by *Sclerotinia sclerotiorum and Sclerotinia minor* is a devastating disease worldwide. To investigate the effect of low temperature (4 °C) on biological characteristics and aggressiveness of isolates of the two species, which were collected from the same field in Baiyinchagan, Inner Mongolia, their mycelial growth rate, oxalic acid secretion level and polygalacturonase activity were compared under normal culture temperature (23 °C) and low temperature (4 °C). Aggressiveness was also evaluated on detached leaves by inoculating the isolates produced in both temperatures. The results suggested that culture of isolates at 4 °C not only promoted mycelial growth, but also enhanced secretion of oxalic acid and polygalacturonase activity of both *S. sclerotiorum* and *S. minor* isolates compared to that cultured at 23 °C. Additionally, the corresponding aggressiveness of tested isolates of the two species also increased after culture at 4 °C. However, *S. sclerotiorum* always showed faster mycelial growth, higher oxalic acid levels and greater polygalacturonase activity than *S. minor* at both 23 °C and 4 °C, indicating that *S. sclerotiorum* is generally the more aggressive species than *S. minor*.

Keywords: *Sclerotinia sclerotiorum* / *Sclerotinia minor* / low temperature culture / biological characteristics / aggressiveness /

Résumé – Effet d'une culture à basse température sur les caractéristiques biologiques et l'agressivité de Sclerotinia sclerotiorum et Sclerotinia minor. La pourriture blanche du tournesol causée par *Sclerotinia sclerotiorum* et *Sclerotinia minor* est une maladie dévastatrice dans le monde entier. Pour étudier l'effet d'une température basse (4 °C) sur les caractéristiques biologiques et l'agressivité d'isolats des deux espèces, collectés dans le même champ à Baiyinchagan, en Mongolie intérieure, leur vitesse de croissance mycélienne, leur niveau de sécrétion d'acide oxalique et leur activité polygalacturonase ont été comparés à une température de culture normale (23 °C) et à basse température (4 °C). L'agressivité a également été évaluée sur des feuilles détachées en inoculant les isolats produits aux deux températures. Les résultats suggèrent que la culture des isolats à 4 °C favorise non seulement la croissance mycélienne, mais augmente également la sécrétion d'acide oxalique et l'activité de la polygalacturonase des souches *S. sclerotiorum* et *S. minor* par rapport à celle de 23 °C. En outre, l'agressivité correspondante des deux espèces a également augmenté après la culture à 4 °C. Cependant, *S. sclerotiorum* a toujours montré une croissance mycélienne plus rapide, des niveaux d'acide oxalique plus élevés et une activité polygalacturonase supérieure à celle de S. minor à la fois à 23 °C et à 4 °C, ce qui indique que la première est généralement l'espèce la plus agressive.

Mots-clés : *Sclerotinia sclerotiorum* / *Sclerotinia minor* / culture à basse température / caractéristiques biologiques / agressivité

1 Introduction

Sunflower White Mold (SWM) caused by both *Sclerotinia sclerotiorum* and *Sclerotinia minor* is one of the most important diseases of sunflower and can cause serious yield losses (Gulya *et al.*, 1997; Van Becelaere and Miller, 2004; Liu *et al.*, 2018). Inner Mongolia, located in northern China is a major confectionery sunflower producing area, with a planting area around 0.86 million hectares. *S. sclerotiorum* and *S. minor* are two necrotrophic pathogens with broad host ranges and

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distributed globally, although *S. sclerotiorum* is more common than *S. minor* in most sunflower planting regions (Wu *et al.*, 2008; Roghayeh *et al.*, 2018). The symptoms of SWM caused by *S. sclerotiorum* and *S. minor* are different. *S. sclerotiorum* causes root, stem, leaf and capitulum rot at different developmental stages of sunflower (Sharma *et al.*, 2016), whereas, *S. minor* causes only basal stem rot (Li *et al.*, 2016b) .

The infection process of SWM can be divided into two phases: biotrophic and necrotrophic phase (Kabbage *et al.*, 2013). Following a short biotrophic phase after infection, both *S. sclerotiorum* and *S. minor* can cause tissue maceration and necrosis, leading to rapid cell death and host cell wall degradation. Toxins and cell wall degrading enzymes (CWDEs) such as polygalacturonase (PG) are believed to play critical roles in promoting these processes (Xia *et al.*, 2020). Previously, oxalic acid (OA) was thought to be a necessary virulence factor for *S. sclerotiorum* infection, not only reducing the pH of host cells and enhancing the activity of CWDE, but also inhibiting the oxygen burst and self-phagocytosis, thus facilitating cell apoptosis in host plants (Godoy *et al.*, 1990; Guimarães and Stotz 2004; Williams *et al.*, 2011; Kabbage *et al.*, 2013). This is also called the oxalate-dependent theory. However, some data indicated that OA is not a pivotal pathogenic factor, but only provides an acidic environment for pathogen infection, this is known as the pH-dependent theory (Xu *et al.*, 2018). Apart from the pathogenicity theories mentioned above some effectors have been identified based on genomic sequence analysis, and these have been verified as functional virulence factors if the host does not contain corresponding resistance proteins (Liu *et al.*, 2015).

The two pathogens can form sclerotia as dormant structures at late developmental stages of sunflower, but sclerotia formed by *S. minor* are much smaller than those of *S. sclerotiorum* (Li *et al.*, 2016a). Environmental factors such as soil temperature and moisture can influence sclerotia formation and mycelial growth rate of both *S. min*or and *S. sclerotiorum*. Fernandes *et al.* (2016) reported that the temprature suitable for mycelium growth of both pathogens was possible between 7 °C and 27 °C, but 22 °C is the most suitable cultural temperature and no mycelia growth at 32 °C. The specific temperature and time required for preconditioning of sclerotia to stimulate carpogenic germination vary among different studies (Clarkson *et al.*, 2017; Dillard *et al.*, 1995; Mila *et al.*, 2008).

In this study, low temperature culture (4 °C) was found accidently to affect the development of both *S. sclerotiorum* and *S. minor*. The mycelial growth rate, oxalate secretion ability and PG enzyme activity were compared at 23 °C and 4 °C using isolates of both species, which were collected from diseased sunflower plants. The aggressiveness of the isolates tested was also determined using a detached leaf inoculation method. The aim of this study was to make clear the effects of 4 °C culture on both the biological characteristics and aggressiveness of *S. sclerotiorum* and *S. min*or.

2 Materials and methods

2.1 Fungal and plant material

Sclerotia of both *S. sclerotiorum* and *S. minor* were collected from the same sunflower field in Gaojiadi, Baiyinchagan town, Wulanchabu city, Inner Mongolia (N41°64′20″, E113°13′01″), where located at an altitude of 1419 m with low temperatures in summer. The *S. sclerotiorum* isolates were labeled as S.s-2, S.s-5, S.s-10 and *S. minor* isolates as S.m-1, S.m-3, S.m-8.

Sclerotia collected from individual infected sunflower stems were surface-sterilized in 75% alcohol for 30 s, then in 0.1% NaCIO for 3 min and finally washed 3 times with distilled water. The sterilized sclerotia was then placed on potato dextrose agar (PDA) and incubated for 72 h at 23 °C. Pure cultures were obtained after three hyphal tip multiplications. The sclerotia formed on the edge of the purified colonies were collected and stored at 4 °C for further analysis.

The seeds of confectionery sunflower variety (LD5009) were purchased from Beijing Kaifuri Seed Industry Company.

2.2 Cultural mediums

Potato dextrose agar (PDA) medium was made from 200 g of potato, 20 g of dextrose, and 15 g of agar per liter of distilled water.

Potato dextrose broth (PDB) medium consisted of 200 g potato, 15 g dextrose per liter of distilled water.

Minimal medium(MM) media contained 1 g NaOH, 3 g DL-malic acid, 2 g NH_4NO_3,0.1 g $MgSO_4H_2O$, 39 g Bacto-agar in 1000 mL water, pH 4.8 (Li *et al.*, 2016a).

2.3 Low-temperature processing method

Purified *S. sclerotiorum* and *S. minor* isolates were cultured on PDA plates at 23 °C for two days, then 5 mm diameter mycelial plugs were cut from the edge of the colony and transferred on to a new PDA plate. The plates were cultured at either 23 °C (control) or 4 °C (low temperature). When the colony of the control covered 3/4 of the plate, mycelial plugs of both *S. sclerotiorum* and *S. minor* cultured at 4 °C were cut from the edge of colonies to perform the following experiments.

2.4 Determination of mycelial growth rate

The mycelial plugs (5-mm diameter) cut from the actively growing edge of low temperature culture plate were placed on new PDA plates and cultured under normal condition (23 °C). The cross diameter method was used to measure and record the colony diameters of each isolate, with three replications. The average diameter recorded in the table was calculated from the diameter of the three replicates measured after culture for 3 days.

2.5 Oxalic acid (OA) measurement

A 5-mm diameter mycelial plug cut from the actively growing edge of a 2-day-old culture colony was inoculated into 40 mL of PDB and incubated in a static position at 23 °C for 5 days. The filtrate of the cultur was used for analyzing OA concentration following the procedure described by Durman *et al.* (2005). The reaction mixture containing 0.2 mL filtrate (or standard oxalic acid solution), 0.11 mL BPB 0.1 mm,

0.20 mL sulfuric acid 1M, 0.18 mL mL potassium dichromate 100 mm and 4.8 mL mL of distilled water was placed in a 60 °C water bath for 10 min, followed by quenching and addition of sodium hydroxide solution to the final concentration of 0.75 mm. The absorbance was measured at 600 nm using a spectrophotometer (TU-1901, Beijing Purkinje General liability company, China). OA concentration was calculated based on a standard curve. The assay was performed in triplicates and each with three technical replicates.

2.6 Polygalacturonases (PG) enzymatic assay

A 5 mm diameter mycelia plug cut from actively growing edge of colony was inoculated into a flask containing 80 mL PDB. Cultures were shaken at 140 rpm at 23 °C for 10 days. After centrifuging at 5000 rpm for 15 min, the aqueous phase was decanted and assayed for PG enzymatic activity. The 3, 5 dinitrosalicylic acid (DNSA) assay was used to assay PG enzymatic activity (Moyo *et al.*, 2003). A 400 µL aliquot of 0.5% pectin was mixed with culture filtrate and topped up to a final volume of 500 µL by adding 0.1 mol/L sodium acetate. Samples were incubated at 30 °C for 5 h. DNSA reagent (44 mmol/L DNSA, 4 mmol/L sodium sulphite, 75 mmol/L NaOH) was added and the samples were boiled for 10 min to stop the reaction. The volume of samples was adjusted to 2 mL by adding deionized water and the absorbance was measured at 540 nm using a spectrophotometer. Absorbance values were used to determine the enzymatic activity from a standard curve generated with D-(+) galacturonic acid. The assay was performed in triplicate and each with three technical replicates.

2.7 Aggressiveness assessment

When the colony diameter of control isolates covered 3/4 of the petri dish under 23 °C culture condition, the PDA plates cultured under 4 °C were taken out of the incubator. A 5 mm diameter mycelia plug was cut from the edge of colony and transferred into MM medium. Aggressiveness assay was performed on detached leaves of confectionery sunflower variety LD5009. The leaves were collected from sunflower seedlings with 6 true leaves (V6 stage) and placed on a moist filter paper soaked in a covered plastic container (Length 32.5 cm × width 26 cm × height 11.5 cm).

A 5 mm diameter mycelial plug was cut from the actively growing edge of 3-day-old culture on MM media and placed upside down on the abaxial side of the detached leaves. The MM plain plug (control) was inoculated on the other side of same inoculated leaf as a control. The covered plastic container containing the inoculated leaves were kept at room temperature (RT) and the lesion diameters were measured after 48 h post inoculation (hpi). The pathogenicity assay of each isolate was performed on five detached leaves and the average size of the lesion diameters represented the aggressiveness of each isolate.

2.8 Data analysis

Differences in biological characteristics and aggressiveness of cultured isolates under normal and low temperature were analyzed using SPSS 17.0 software. Duncan's new complex range method was used to test the significance of differences under 0.05 level.

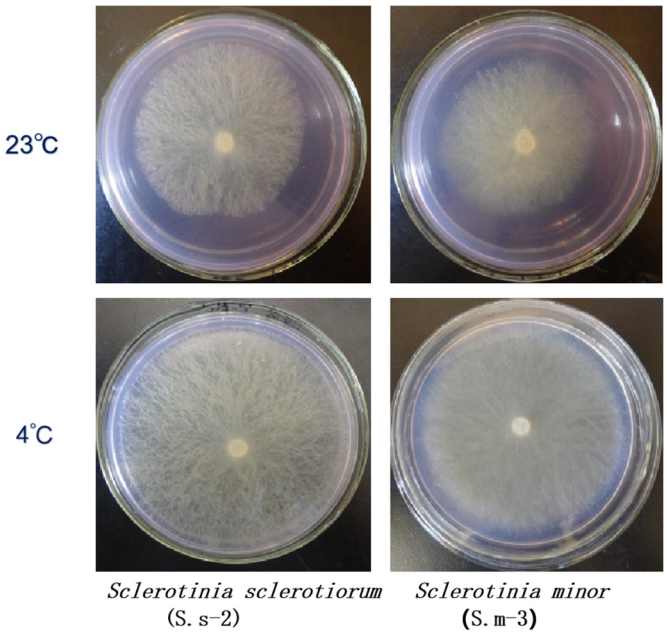

Fig. 1. The comparison on mycelial growth rate of both *S. sclerotiorum* and *S. minor* after culture under 4 °C (S.s-2 and S.m-3 representative isolates of *S. sclerotiorum* and *S. minor*.

3 Results

3.1 Promoting mycelial growth of *S. sclerotiorum and S. minor* after culture at 4 °C

The mycelia growth rate of all tested isolates cultured at 4 °C was faster than those cultured at 23 °C (Fig. 1). The average diameter of *S. sclerotiorum* and *S. minor* cultured under 23 °C for two days was 6.95 ± 0.57 cm and 5.02 ± 0.34 cm respectively, whereas, under same culture period at 4 °C was 8.07 ± 0.09 cm and 6.69 ± 0.23 cm respectively, indicating that the low temperature accelerated the mycelia growth of isolates of both species used in this study (Tab. 1). However, the average mycelia growth rate of *S. sclerotiorum* was significantly faster than that of *S. minor* under both cultural temperature conditions (Tab. 1).

3.2 OA secretion and PG enzymes activity of *S. sclerotiorum* and *S. minor* after culture at 4 °C

The OA secretion ability of both *S. sclerotorum* and *S. minor* isolated was observed boosted after culture at 4 °C. The average OA value of *S. sclerotiorum* and *S. minor* after 4 °C culture was 32.73 ± 2.89 µg/mL and 27.32 ± 0.21 µg/mL, compared with 27.69 ± 1.42 µg/mL and 20.63 ± 2.25 µg/mL respectively at 23 °C (Tab. 1). The average OA values showed the same pattern as mycelial growth rate, higher for *S. sclerotorum* than for *S. minor* at both temperatures.

Table 1. The average value of biological characteristics and aggressiveness of *S. sclerotiorum* and *S. minor* after culture at different temperature.

	Growth rate / (cm ± SE)		Oxalic acid concentration / (μg·mg-1 ± SE)		Polygalacturonases enzymatic / (U·mg-1 ± SE)		Lesion diameter / (cm ± SE)	
temperature treatment	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C
S. sclerotiorum	6.95 ± 0.57b	8.07 ± 0.09a	27.69 ± 1.42b	32.73 ± 2.89a	22.56 ± 1.37b	26.37 ± 2.62a	2.57 ± 0.27b	3.34 ± 0.38a
S. minor	5.02 ± 0.34b	6.69 ± 0.23a	20.63 ± 2.25b	27.32 ± 0.21a	21.57 ± 0.32b	24.54 ± 0.90a	2.08 ± 0.11b	2.43 ± 0.14a

Table 2. The average value of biological characteristics and aggressiveness of tested isolates after culture at different temperature.

	Growth rate / (cm ± SE)		Oxalic acid secretion / (μg·mg-1 ± SE)		Polygalacturonases enzymatic / (U·mg-1 ± SE)		Lesion diameter / (cm ± SE)	
temperature treatment	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C
S.s-2	6.40 ± 0.10b	8.10 ± 0.00a	26.69 ± 0.68b	35.84 ± 0.96a	21.46 ± 0.54b	27.94 ± 0.27a	2.26 ± 0.09b	3.24 ± 0.05a
S.s-5	6.93 ± 0.47a	7.97 ± 0.12a	27.06 ± 1.02b	32.24 ± 0.62a	22.12 ± 1.18b	27.82 ± 0.91a	2.72 ± 0.08b	3.76 ± 0.05a
S.s-10	7.53 ± 0.25a	8.13 ± 0.25a	29.31 ± 1.04a	30.13 ± 0.53a	24.09 ± 0.61b	23.35 ± 0.55a	2.72 ± 0.04b	3.02 ± 0.11a
S.m-1	4.73 ± 0.15b	6.43 ± 0.15a	20.35 ± 1.55b	27.29 ± 0.77a	21.48 ± 0.95b	23.61 ± 0.55a	2.18 ± 0.04b	2.56 ± 0.05a
S.m-3	4.97 ± 0.21b	6.87 ± 0.31a	23.01 ± 0.56b	27.13 ± 0.43a	21.30 ± 0.52b	24.62 ± 0.77a	2.10 ± 0.07b	2.46 ± 0.11a
S.m-8	5.40 ± 0.20a	6.77 ± 0.77a	18.54 ± 0.84b	27.55 ± 1.11a	21.92 ± 0.80a	25.40 ± 1.34a	1.96 ± 0.05b	2.28 ± 0.08a

OA levels of *S. sclerotiorum* isolates S.s-2 and S.s-5 increased from 26.69 ± 0.68 μg/mL and 27.06 ± 1.02 μg/mL at 23 °C to 35.84 ± 0.96 μg/mL and 32.24 ± 0.62 μg/mL respectively at 4 °C. For *S. minor* isolates, the same trend was detected, with S.m-8 showing the greatest increase (9.01 μg/mL), followed by S.m-1 (6.94 μg/mL) and S.m-3 (4.12 μg/mL).

The average PG enzymatic activities of *S. sclerotiorum* and *S. minor* were 26.37 U/mg and 24.54 U/mg respectively after culture under 4 °C, compared with 22.56 U/mg and 21.57 U/mg respectively at 23 °C (Tab. 1), with no significant difference between species.

PG enzymatic activity level of S.sclerotiorum isolates S.s-2 and S.s-5 increased from 21.46 ± 0.54 U/mg and 22.12 ± 1.18 U/mg at 23 °C to 27.94 ± 0.27 U/mg and 27.82 ± 0.91 U/mg at 4 °C (Tab. 2).

For *S. minor*, PG enzymatic activity of all three isolates increased significantly, especially for S.m-8, the increased value listed at the top, it is 3.48 U/mg, whereas, for S.m-3 and S.m-1, the increase value is 3.32 U/mg and 2.13 U/mg respectively.

3.3 Enhanced aggressiveness of *S. sclerotiorum* and *S. minor* post 4 °C culture

To assay the aggressiveness of *S. sclerotiorum* and *S. minor* after culturing at 4 °C, lesion diameter on detached leaves was measured under lab condition. After 2 dpi, S.s-10 and S.s-5 cultured at 23 °C appeared more aggressive than S.s-2 with lesion diameters of 2.72 ± 0.08 cm, 2.72 ± 0.04 cm and 2.26 ± 0.09 cm respectively. However, after culturing at 4 °C, the aggressiveness of S.s-5 was enhanced with a lesion diameter of 3.76 cm, followed by S.s-2 (3.24 cm) and S.s-10 (3.02 cm). The aggressiveness of three isolates of *S. minor* showed a minor difference after being cultured at 23 °C, the leison diameter of S.m-1 is 2.18 cm,, followed by S.m-3 (2.10 cm) and S.m-8 (1.96 cm). Especially for S.m-1 (2.18 cm), compared with S.m-3 (2.10 cm) and S.m-8 (1.96 cm). Their aggressiveness increased accordingly after being cultured at 4 °C.

The average lesion diameter of *S. sclerotiorum* and *S. minor* cultured at 4 °C was 3.34 ± 0.38 cm and 2.43 ± 0.14 cm respectively, while it was 2.57 ± 0.27 cm and 2.08 ± 0.11 cm at 23 °C (Tab. 1). The enhanced aggressiveness of *S. sclerotiorum* at 4 °C was more significant than that of *S. minor*., the two species showed a significant difference.

4 Conclusion and discussion

In Inner Mongolia, both *S. sclerotiorum* and *S. minor* were isolated from diseased plants with SWM symptoms, but, *S. minor* isolates were only collected from sunflower fields which were located in the northern piedmont region of Yin mountain with high altitudes and low temperatures in summer. The lowest temperature in August is about 6 °C. Sometimes, SWM infected plants caused by *S. sclerotiorum* were also observed in fields in which SWM was dominantly caused by *S. minor*, but co-infection by both *S. sclerotiorum* and *S. minor* was never encountered. Previous studies showed that mycelial growth of both *S. sclerotiorum* and *S. minor* occurred at

temperatures ranging from 12 °C to 27 °C, and sclerotia of *S. minor* germinated and exhibited mycelial growth at temperatures ranging from 6 °C to 30 °C, but *S. minor* shows much more sensitivity to low temperature (Domingues *et al.*, 2016). Based on the low temperatures in summer in the region where we observed SWM caused by *S. minor*, it was speculated that *S. minor* is favorised by such low temperature. Isolates of *S. sclerotiorum* collected from regions with mild temperatures do need cold "conditioning" treatment in winter to promote sclerotia germination and producing apothecium (Phillips, 1987), although germination at different temperatures varies between isolates collected from different geographic origins. This result, and the extensive distribution of *S. sclerotiorum*, suggests that this species is adapted to a much wider range of environmental conditions than *S. minor* (Young *et al.*, 2004; Clarkson *et al.*, 2017).

Since the northern piedmont region of Yin Mountain is the only region where *S. minor* was collected from diseased sunflower plants, this study was to determinel if low temperature could affect the biological characteristics of both *S. sclerotiorum* and *S. minor* isolated from this region and thus affect the isolate aggressiveness. Our results showed that not only mycelium growth rate increased, but also OA secretion ability and PG enzyme activity were boosted after culture at 4 °C, suggesting that aggressiveness was also enhanced accordingly. Additionally, the average value of the key pathogenic factors of *S. sclerotiorum* such as OA value and PG enzyme activity was much higher than that of *S. minor*, indicating that genetic differences between *S. sclerotiorum* and *S. minor* may cause the response differently to temperature variations. The result also provided an exaplanation why *S. sclerotiorum* is the dominant causal agent for SWM worldwide, whereas, *S. minor* is only observed in certain sunflower planting regions which have typical environmental condition (Najafzadeh *et al.*, 2018).

In this study we also showed that low temperature treatment can significantly enhance the aggressiveness of both *S. sclerotiorum* and *S. minor*, but increased value for *S. sclerotiorum* was greater than that of *S. minor*. This agrees with earlier observation that the pathogenicity of *S. minor* isolates collected from rapeseed in the central region of China showed mild pathogenicity compared to *S. sclerotiorum* (Yang *et al.*, 2016). However, the molecular mechanism underlying such kind of phenomena is still poorly understood and needs in depth investigation in the future.

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