J.Natn.Sci.Foundation Sri Lanka 2018 46 (2): 159 - 164
DOI: http://dx.doi.org/10.4038/jnsfsr.v46i2.8416

RESEARCH ARTICLE

Phenotypic variation of cabbage white mold pathogen, *Sclerotinia sclerotiorum* in the upcountry commercial cabbage fields in Sri Lanka

T. Mahalingam[1], B.M.A. Guruge[1], K.P. Somachandra[2], E.A.E.S.S. Jayasekara[2], C.S.K. Rajapakse[3] and R.N. Attanayake[1*]

[1] *Department of Botany, Faculty of Science, University of Kelaniya, Kelaniya.*
[2] *Regional Agricultural Research and Development Centre, Bandarawela.*
[3] *Department of Chemistry, Faculty of Science, University of Kelaniya, Kelaniya.*

Revised: 05 December 2017; Accepted: 21 December 2017

Abstract: *Sclerotinia sclerotiorum* is a cosmopolitan, necrotrophic and soil borne plant pathogen, capable of infecting many economically important crops. Although it is a well-studied pathogen elsewhere, there is no research conducted in Sri Lanka. The objectives of the current research were to study the phenotypic variations among isolates within the pathogen population in the upcountry commercial cabbage fields in Sri Lanka and to determine their sensitivity to a commonly applied fungicide, Mancozeb. Forty-six isolates obtained from infected cabbage heads were identified as *S. sclerotiorum* based on its unique morphological characteristics. Colony diameter was measured on potato dextrose agar (PDA) plates at 23 ºC and mycelial colour was observed after two-week incubation. All the isolates were grown in pH indicator media to determine their acid producing abilities. Sensitivity of the isolates to Mancozeb was assayed *in vitro*. There was a significant difference in colony growth among the isolates and sensitivity to Mancozeb. Two isolates showed abnormal mycelial growth characteristics; a slower growth rate on PDA and reduced pathogenicity on cabbage leaves. Two levels of mycelial pigmentations were observed on PDA. All the isolates were acid producers, inferring that all of them were pathogenic. In summary, the upcountry *S. sclerotiorum* pathogen population was found to be diverse for selected phenotypic traits, especially for mycelial growth, and fungicide sensitivities inferring that the pathogen population is capable of adapting to changing environmental conditions. Therefore, disease management will be challenging if an epidemic occurs, and it is recommended that a proper management system should be identified before further expansion of upcountry cabbage cultivation.

Keywords: Cabbage, phenotypic variation, *Sclerotinia sclerotiorum*, white mold.

INTRODUCTION

Cabbage is an important vegetable with 55 million tons of annual production in 2.6 million ha worldwide (FAO, 2001). In Sri Lanka, cabbage is grown to a large extent throughout the year in the Uva and Central provinces. Wet and cold climatic conditions that prevail in the upcountry, Sri Lanka, provide a conducive environment for many diseases including white mold on cabbage. White mold is a fungal disease caused by an ascomycete, *Sclerotinia sclerotiorum.* It is a necrotrophic plant pathogen infecting more than 400 plant species including cabbage, bean, tomato, potato, canola and lettuce worldwide (Boland & Hall, 1994).

In the mid 2014 and 2016, white mold infections were extensively observed in commercial cabbage fields in Pattipola, Ambewela and Seethaeliya in Nuwara Eliya District in Sri Lanka and the pathogen was identified as *S. sclerotiorum* using morphology and rDNA-ITS sequence data (Mahalingam *et al*., 2017). It was also found that the farmers were unaware of the causative agent and applied improper pesticides and cultural practices (*Personal communication with cabbage*

* Corresponding author (renuka@kln.ac.lk; https://orcid.org/0000-0002-4875-777X)

farmers, April 2014), which led to the persistence of the infection and accumulation of inoculum in soil. No disease free commercial cabbage fields were found in Pattipola and Ambewela, and Mahalingam *et al*. (2017) found that the disease incidence and severity were 2 – 5 % and 90 – 100 %, respectively.

Population scale phenotypic diversity of this pathogen is well studied around the world. Atallah *et al*. (2004) reported phenotypic uniformity among the isolates obtained from infected potato plants in the US Pacific Northwest. Attanayake *et al*. (2012; 2013) reported high phenotypic diversity among the isolates obtained in a fine geographic scale of alfalfa field soils, and isolates obtained from infected canola plants from the USA and China. However, no population scale phenotypic variation has been studied in Sri Lanka so far. Studying the phenotypic diversity of the pathogen population gives an indirect estimation of the level of genetic diversity. This is the initial step in devising control measures since an effective control measure should be effective against all the genotypes of the population. The objectives of the present study were to determine the phenotypic variability among isolates of the pathogen population of *S. sclerotiorum* found in upcountry, Sri Lanka, in terms of mycelial growth, acid production ability, pigment production ability as inferred by colony colour, and to determine variation in sensitivity of the isolates for the fungicide, Mancozeb.

METHODOLOGY

Sample collection and establishment of pure cultures

Approximately 30 commercial cabbage fields in Pattipola, Ambewela and Seethaeliya were inspected for the presence of the disease in the years 2014, 2015 and 2016. Diagonal sampling strategy was followed. Sclerotia or infected cabbage leaves were collected from diseased cabbage heads from commercial cabbage fields with a minimum distance of 6 m between sampling sites to avoid clonal sampling. The samples were transported to the laboratory at the University of Kelaniya to generate pure cultures. Sclerotia were surface sterilised with 5 % Clorox (Clorox Company, USA) for 1 – 2 min and 70 % ethanol for 2 min followed by three consecutive washings with sterilised distilled water, and dried on sterilised filter papers. Sclerotia were cut into two pieces and plated on potato dextrose agar (PDA) (Oxoid Thermo Scientific, UK), amended with ampicillin (150 μgmL^{-1}), and incubated at 23 ºC. Pure cultures of the isolates were obtained using the hyphal tip culture method. All the isolates were identified as *S. sclerotiorum* based on their typical mycelial growth pattern, sclerotia production at the periphery of the Petri plate and sclerotial size. Pure cultures were stored at 4 ºC until use.

Characterisation of the population

Mycelial growth and colony colour determination

Mycelial plugs (5 mm diameter) obtained from the actively growing edges of 2 d old fungal colonies were inoculated onto fresh PDA plates and incubated at 23 ºC. Two colony diameter measurements were made at a 90º angle per plate after 36 h of incubation and the mean diameter was obtained. The plates were arranged in complete randomised design (CRD) and each isolate had 3 replicates. Significant differences among the isolates for their growth were determined using one-way ANOVA implemented in Minitab version 16. The isolates were incubated at 23 ºC for 2 wks and colony colour was recorded. The isolates were grouped into 2, based on the colony colour (white and beige) to determine whether there is a significant differnece between the groups.

Acid production

All the isolates were grown on PDA to obtain actively growing mycelia. The actively growing mycelial plug (5 mm diameter) of each isolate was inoculated on PDA plates amended with bromophenol blue (500 μgmL^{-1}) and incubated at 23 ºC. Colour change from blue to yellow indicating acid production was observed for each isolate in 3 replicates. Qualitative data were recorded.

Pathogenicity of selected isolates

Among the 46 isolates, 8 were used to test and confirm their pathogenicity using detached leaf assay as described by Mahalingam *et al*. (2017). The 8 isolates consisted of 6 fast growing isolates with normal mycelial characteristics and 2 isolates (isolates 30 and G3), which had abnormal mycelial characteristics and reduced growth rate. Both white and beige isolates were present among the 8 isolates.

Determination of discriminatory concentration and variation in fungicide sensitivity

Mycelial plugs (5 mm in diameter) from actively growing mycelia of 10 randomly selected isolates of *S. sclerotiorum* were placed at the centre of each PDA plate amended with 8 different concentrations of commercial fungicide, Mancozeb. The tested

concentrations were 5, 10, 25, 50, 75, 100, 250, 500 µgmL^{-1}. The PDA plates with no fungicide amendments were used as controls. The plates were arranged in a complete randomised design (CRD) with 3 replicates per isolate. The inoculated plates were incubated at 25 ºC for 36 h. Two colony diameter measurements were made at a 90º angle and the mean diameter was obtained. Percent inhibition was measured using the following formula,

$$\frac{D_{control} - D_{test\ plate}}{D_{control}} \times 100$$

where, $D_{control}$ is the average colony diameter of the control plate and $D_{test\ plate}$ is the average colony diameter of the fungicide amended plate. The fungicide concentration that showed the highest variance among the 10 isolates for percent inhibition was identified as the 'discriminatory concentration'. Later, 20 isolates from the population were tested for their variation in percent inhibition at this discriminatory concentration as described in Attanayake *et al*. (2013). Significant difference among the isolates for percent inhibition was tested using Minitab 16.

RESULTS AND DISCUSSION

During field observations, no disease free cabbage fields were observed when the crop was at its maturity. However, no infected plants were observed in immature cabbage fields. Water soaked lesions covered with white fluffy mycelia and dark sclerotia, typical symptoms and signs of white mold infection caused by *S. sclerotiorum* were observed on the infected heads [Figure 1(a)]. It was interesting to note that the infected heads were often covered with healthy surrounding leaves making the early detection challenging for an untrained person [Figure 1(b)]. Out of 50 sclerotia, 46 were viable and used to obtain pure cultures. Each isolate was identified as *S. sclerotiorum* based on their unique mycelial growth pattern, white coloured mycelia and 4 – 10 mm size sclerotia present at the periphery of the PDA plates, and further confirmed with previous rDNA sequence information (Mahalingam *et al.*, 2017).

(a) (b)

Figure 1: Signs and symptoms of the white mold disease on infected cabbage head (a) and infected heads covered with healthy leaves making it challenging to identify infected plants (b)

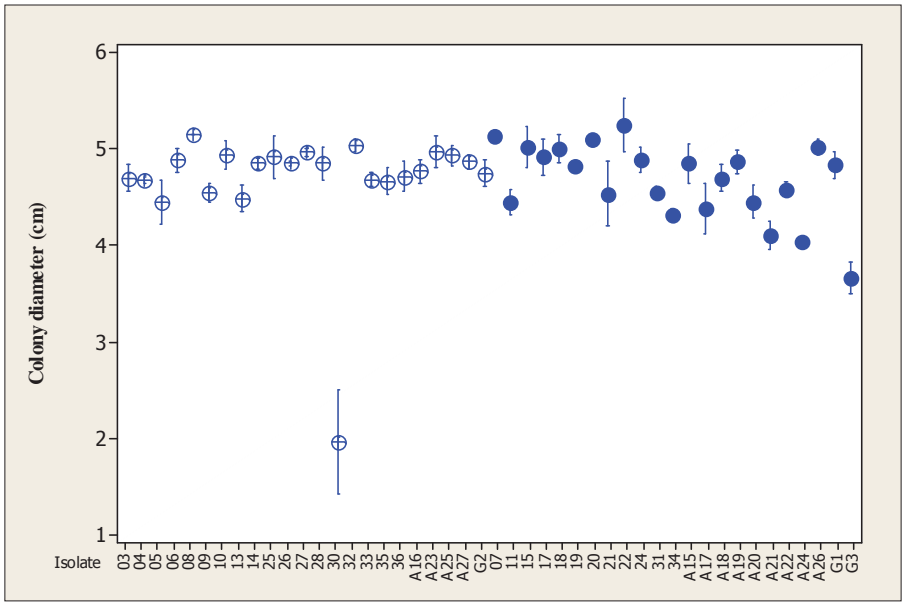

Figure 2: Variation in colony diameters of isolates of *S. sclerotiorum* on PDA plates after 36 hours incubation. Open circles represent white isolates whereas filled circles represent beige isolates

Characterisation of the population

Mycelial growth and colony colour

There was a significant difference ($p < 0.05$) among the isolates in colony diameter, after 36 hours incubation at 23 ºC (Figure 2). Isolates 30 and G3 showed a reduced growth rate and an unusual colony morphology. Several researchers have reported the presence of such *S. sclerotiorum* isolates with atypical, reduced colony growth and reported to be containing a dsRNA element in their cytoplasm (Boland, 1992). The fungal isolates with such cytoplasmic elements have reduced pathogenicity and referred as hypovirulence. Although it is not conclusive that the abnormal isolates found in the current study contain dsRNA elements, they resemble the isolates reported earlier (Boland, 1992). It has been suggested that such isolates can be used as biocontrol agents (Melzer & Boland, 1996) and therefore, worth studying them further.

Among the isolates of Sri Lankan population, two distinct colony colours, white and beige, were observed on PDA (Oxoid Thermo Scientific, UK) plates after two weeks of incubation (Figure 3). Twenty-two isolates were white, whereas the rest of the isolates were beige. Colony diameter and their respective colours have been shown in Figure 2. There was no significant difference ($p = 0.91$) in mean colony diameter between white and beige colour isolates. The variation in mycelial pigmentation of *S. sclerotiorum* has been reported in many studies (Lazarovits *et al.*, 2000; Akem *et al.*, 2006; Sanogo & Puppala, 2007; Woodward *et al.*, 2008; Attanayake *et al*., 2012; 2013). Garg *et al*. (2010) also reported that there was no correlation between mycelial colour and growth rate. It has been reported that mycelial pigmentation aids in viability and persistence of mycelia and sclerotia by protecting cells from adverse biotic and abiotic factors (Butler *et al.*, 2009). Three pigmentation types, normal black, abnormal black and tan sclerotia have been reported in other countries (Huang, 1981; 1982). However, in the current study all the isolates produced only black colour sclerotia.

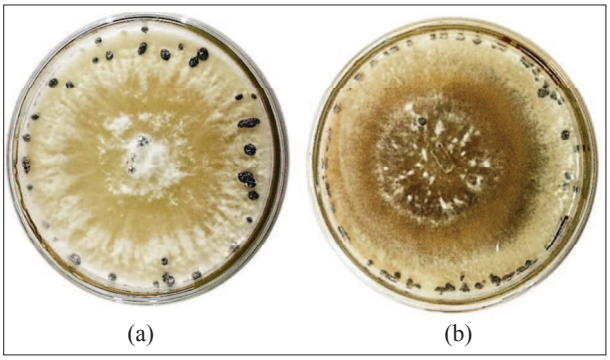

Figure 3: Colony colour variation among isolates on PDA plates; white colour mycelia (a); and beige colour mycelia (b). Note the unique pattern of sclerotia production at the periphery of the PDA plate.

Acid production

All the isolates, including the isolates with abnormal growth patterns were able to change the colour of the PDA plates amended with bromophenol blue from purple to yellow indicating that all the isolates can change pH in their surroundings. Margo *et al*. (1984) showed that the pH change is mainly due to oxalic acid production and has a main role in pathogenicity of the isolates. Oxalic acid has been considered as the main pathogenicity determinant of *S. sclerotiorum* for many years since oxalate-deficient mutants were unable to cause disease (Cessna *et al*., 2000). However, Xu *et al*. (2015) recently reported that it was not oxalic acid but the low pH, which establishes the optimum conditions for growth, reproduction, pathogenicity and virulence expression of the pathogen. Further, they have shown that oxalate-minus mutants were capable of accumulating fumaric acid and retained pathogenicity, but their virulence varied depending on the pH and buffering capacity of the host tissue. In the current study all the isolates including the abnormal isolates were able to change pH in the medium indicating that all of them are pathogenic. No variations in yellow colour intensities were observed among the isolates indicating that all of the isolates were approximately strong acid producers.

Pathogenicity of selected isolates

All six isolates with typical growth patterns produced distinct water soaked necrotic lesions 36 hours after inoculation, and sclerotia were observed after 14 days. Interestingly, slow growing abnormal isolates also produced water soaked lesions and white mycelia, characteristic symptoms and signs of the pathogen infection. However, they failed to produce sclerotia on the inoculated leaves even after 14 days. In addition, the infection rate of these abnormal isolates was slower than that of the typical isolates further suggesting that these are hypovirulent. Several studies have reported the lack of variation in pathogenicity among isolates of *S. sclerotiorum* (Atallah *et al*., 2004; Durman *et al*., 2005). Since all of the isolates used in the current study (except those that had abnormal growth characteristics) were strong acid producers it is unlikely to observe variation among isolates for pathogenicity.

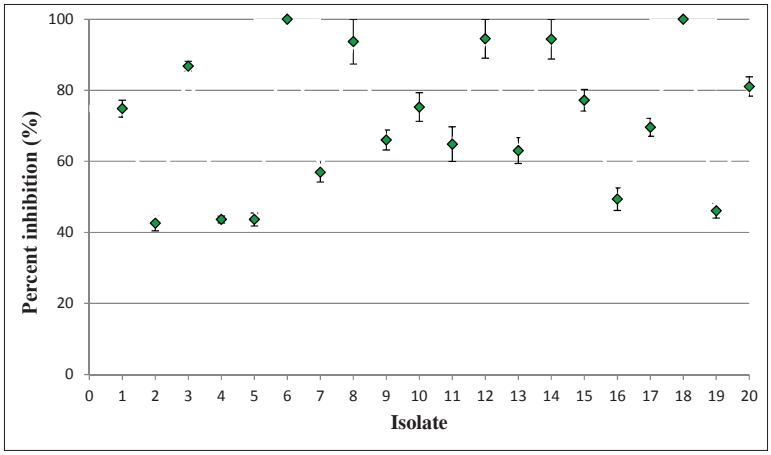

Figure 4: Percent inhibition of *S. sclerotiorum in vitro* at 250 µgmL^{-1} discriminatory concentration of the fungicide Mancozeb, 36 hours after incubation

Variation in fungicide sensitivity

Among the eight concentrations of Mancozeb used (5, 10, 25, 50, 75, 100, 250, 500 µgmL^{-1}), the selected 10 isolates showed the highest variance for percent inhibition at 250 µgmL^{-1} concentration. Therefore, 250 µgmL^{-1} concentration was chosen as the discriminatory concentration as described in Attanayake *et al*. (2013). Then from the pathogen population, 20 isolates were assayed for the percent inhibition, and significant difference ($p < 0.05$) among isolates for the percent inhibition was observed. Six isolates had less than 60 % inhibition and seven isolates had 80 – 100 % inhibition (Figure 4). Based on the observations and interviews with farmers, it was found that they do not apply specific control measures for the disease due to unawareness. Therefore, it is too early to predict fungicide resistance development in Sri Lanka. However, the fungicide Mancozeb was chosen since it is often applied to control potato late blight, and both potato and cabbage are commonly grown in the upcountry and serve as hosts for the pathogen. Therefore, the presence of isolates with varying levels of sensitivity to Mancozeb in the population should be taken into account when recommending fungicides.

CONCLUSION

The present study characterised a pathogen population found in Sri Lanka in terms of phenotypic diversity for the first time. It was found that the *S. sclerotiorum* population in the upcountry cabbage fields of Sri Lanka is moderately diverse for the selected traits, namely, mycelial growth, pigment production and fungicide sensitivity. Since phenotypic variation is a result of genetic and environmental variance components, phenotypically diverse populations have a strong ability to adapt to challenging environments such as fungicide applications. Therefore, these findings should be taken into account when control measures are designed. Another interesting finding was that several vegetable crops grown in upcountry were found to be alternative hosts of the pathogen. Some of them were beans and carrots (*Personal communication, K.P. Somachandra, 2016*).

Acknowledgement

The authors would like to thank the National Science Foundation of Sri Lanka (RG/2015/BT/04) for granting financial support to carry out the research project successfully.

REFERENCES

1. Akem C., Bellar M. & Bayaa B. (2006). Comparative growth and pathogenicity of geographical isolates of *Sclerotinia sclerotiorum* on lentil genotypes. *Journal of Plant Pathology* **5**: 67 – 71. DOI: https://doi.org/10.3923/ppj.2006.67.71
2. Atallah Z.K., Larget B., Chen X. & Johnson D.A. (2004). High genetic diversity, phenotypic uniformity, and evidence of out crossing in *Sclerotinia sclerotiorum* in the Columbia basin of Washington State. *Phytopathology* **94**: 737 – 742. DOI: https://doi.org/10.1094/PHYTO.2004.94.7.737
3. Attanayake R.N., Carter P.A., Jiang D., del Río-Mendoza L. & Chen W. (2013). *Sclerotinia sclerotiorum* populations

infecting canola from China and the United States are genetically and phenotypically distinct. *Phytopathology* **103**: 750 – 761.
DOI: https://doi.org/10.1094/PHYTO-07-12-0159-R

4. Attanayake R.N., Porter L., Johnson D.A. & Chen W. (2012). Genetic and phenotypic diversity and random association of DNA markers of the fungal plant pathogen *Sclerotinia sclerotiorum* from soil in a fine geographic scale. *Soil Biology and Biochemistry* **55**: 28 – 36.
DOI: https://doi.org/10.1016/j.soilbio.2012.06.002
5. Boland G.J. (1992). Hypovirulence and double stranded RNA in *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **14**: 10 – 17.
DOI: https://doi.org/10.1080/07060669209500900
6. Boland G.J. & Hall R. (1994). Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**: 94 – 108.
DOI: https://doi.org/10.1080/07060669409500766
7. Butler M.J. & Day A.W. (1998). Fungal melanins: a review. *Canadian Journal of Microbiology* **44**: 1115 – 1136.
DOI: https://doi.org/10.1139/w98-119
8. Butler M.J., Gardiner R.B. & Day A.W. (2009). Melanin synthesis by *Sclerotinia sclerotiorum*. *Mycologia* **101**(3): 296 – 304.
DOI: https://doi.org/10.3852/08-120
9. Cessna S.G., Sears V.E., Dickman M.B. & Low P.S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell* **12**(11): 2191 – 2200.
DOI: https://doi.org/10.1105/tpc.12.11.2191
10. Durman S.B., Menendez A.B. & Godeas A.M. (2005). Variation in oxalic acid production and mycelial compatibility within fields populations of *Sclerotinia sclerotiorum*. *Soil Biology and Biochemistry* **37**: 2180 – 2184.
DOI: https://doi.org/10.1016/j.soilbio.2005.03.017
11. Food and Agriculture Organization of the United Nations (FAO)(2001). *Aggressiveness among isolates of Sclerotinia sclerotiorum from sunflower.* Available at *http://www.fao.org/nr/water/cropinfo_cabbage.html*, Accessed 03 February 2017.
12. Garg H., Kohn L.M., Andrew M., Li H., Sivasithamparam K. & Barbetti M.J. (2010). Pathogenicity of morphologically different isolates of *Sclerotinia sclerotiorum* with *Brassica napus* and *B. juncea* genotypes. *European Journal of Plant Pathology* **126**: 305 – 315.
DOI: https://doi.org/10.1007/s10658-009-9547-7
13. Horticultural Crop Research and Development Institute, Department of Agriculture – Sri Lanka. Available at *https://www.doa.gov.lk/HORDI/index.php/en/crop-2/15-cabbage*, Accessed 23 January 2017.
14. Huang H.C. (1981). Tan sclerotia of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **3**: 136 – 138.
DOI: https://doi.org/10.1080/07060668109501933
15. Huang H.C. (1982). Morphologically abnormal sclerotia of *Sclerotinia sclerotiorum. Canadian Journal of Microbiology* **28**: 87 – 91.
DOI: https://doi.org/10.1139/m82-007
16. Huang H.C. & Hoes J.A. (1980). Importance of plant spacing and sclerotial position to development of Sclerotinia wilt of sunflower. *Plant Disease* **64**: 81 – 84.
DOI: https://doi.org/10.1094/PD-64-81
17. Lazarovits G., Starratt A.N. & Huang H.C. (2000). The effect of tricyclazole and culture medium on production of the melanin precursor 1, 8-dihydroxynaphthalene by *Sclerotinia sclerotiorum* isolate SS7. *Pesticide Biochemistry and Physiology* **67**: 54 – 62.
DOI: https://doi.org/10.1006/pest.2000.2474
18. Magro P., Marciano P. & Di Lenna P. (1984). Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. *FEMS Microbiology Letters* **24**(1): 9 – 12.
DOI: https://doi.org/10.1111/j.1574-6968.1984.tb01234.x
19. Mahalingam T., Guruge B.M.A., Somachandra K.P., Rajapakse C.S.K. & Attanayake R.N. (2017). First report of white mold caused by *Sclerotinia sclerotiorum* on cabbage in Sri Lanka. *Plant Disease* **101**: 249.
DOI: https://doi.org/10.1094/PDIS-05-16-0693-PDN
20. Melzer M.S. & Boland G.J. (1996). Transmissible hypovirulence in *Sclerotinia minor*. *Canadian Journal of Plant Pathology* **18**: 19 – 28.
DOI: https://doi.org/10.1080/07060669609500650
21. Sanogo S. & Puppala N. (2007). Characterization of darkly pigmented mycelial isolates of *Sclerotinia sclerotiorum* on Valencia peanut in New Mexico. *Plant Disease* **91**: 1077 – 1080.
DOI: https://doi.org/10.1094/PDIS-91-9-1077
22. Woodward J.E., Nui C., Wright R.J., Batla M.A. & Baughman T.A. (2008). First report of *Sclerotinia sclerotiorum* infecting peanut in Texas. *Plant Disease* **92**: 1468.
DOI: https://doi.org/10.1094/PDIS-92-10-1468B
23. Xu L., Xiang M., White D. & Chen W. (2015). pH dependency of sclerotial development and pathogenicity revealed by using genetically defined oxalate-minus mutants of *Sclerotinia sclerotiorum. Environmental Microbiology* **17**: 2896 – 2909.
DOI: https://doi.org/10.1111/1462-2920.12818