Ukrainian Journal of Ecology

Ukrainian Journal of Ecology, 2020, 10(5), 166-169, doi: 10.15421/2020_225

ORIGINAL ARTICLE

Accumulation and identification of secondary metabolites from the fungus *Diaporthe* (*Phomopsis*) *helianthi* Munt.–Cvet. et al.

Ye.V. Syvoded*[1], O.V. Kolesnichenko[2], S.M. Hrysiuk[2], R.V. Ivannikov[3]

[1]Kherson State Agrarian University
23 Stretinskaya St, Kherson, 73006, Ukraine
[2]National University of life and Environmental Sciences of Ukraine
15 Heroyiv Oborony St, Kyiv, 03041, Ukraine
[3]M.M. Gryshko National Botanical Garden National of Academy Sciences of Ukraine,
1 Tymiryazivska St, Kyiv, 01014, Ukraine
**Corresponding author E-mail: evgeniyasyvoded@gmail.com*

Received: 08.09.2020. Accepted: 22.10.2020

The study presents variability in the qualitative composition and quantitative indicators of secondary mycelium metabolites of the fungus *Diaporthe* (*Phomopsis*) *helianthi* Munt.-Cvet. et al., the most harmful plant pest of *Helianthus annuus* L. Toxicity of secondary metabolites of *D. helianthi* was analyzed by determining the average length of seedling shoots of *Triticum aestivum* L. test object grown on fungus filtrates of different cultivation dates. The maximum toxic effect of *D. helianthi* was recorded during the germination of *Triticum aestivum* L. seedlings on a 17-day filtrate of pure mycelium culture of the fungus. The mean length of wheat seedlings in this variant of the experiment decreased by 3.5 times compared with the control and 3.3 times compared with the filtrate of the culture medium of 7 days of cultivation. High-performance liquid chromatography (HPLC) identified secondary metabolites of *D. helianthi* as fomosin, fomopsolides, cytosporones, and xanthones. There was a redistribution of secondary metabolites due to increase in number of cytosporones, decrease in content of fomopsolides, and termination of xanthone synthesis with increasing time of cultivation of fomopsis mycelium, under conditions of relatively stable indicators of fomosin content.
Key words: *Diaporthe* (*Phomopsis*) *helianthi* M., secondary metabolites, mycotoxins

Introduction

Diaporthe helianthi (*Phomopsis helianthi*) Munt.-Cvet. et al. – is the causative agent of phomopsis stem canker of *Helianthus annuus* L., the most harmful fungal disease of sunflowers (Kyryk et al., 2015). *Phomopsis* is spread in many European countries (Serbia, Montenegro, Romania, France and Austria), Argentina and Brazil (Mathew et al., 2018). It is believed that the pathogenesis of *D. helianthi* is linked primarily with the secondary metabolites, which regulate the symbiotic, competitive and parasitic spectra of action. Therefore, it is urgent to find the causes of pathogenic variability of secondary metabolites as a heterogeneous group of natural products of metabolism of *D. helianthi*. The metabolism of cells of *Helianthus annuus* L. Is directly affected by the phytotoxic substances of the secondary metabolites (Butler et al., 1977). The aim of this work was to obtain and identify the secondary metabolites produced by *D. helianthi*, to study the properties of their variability during the pathogenesis of phomopsis stem canker. *D. helianthi* can cause 70-100 % death in plants of *Heliantus annuus*, thus the identification and determination of the phytotoxic action of secondary metabolites present an important scientific problem.

Materials and Methods

The fungus samples of *D. helianthi* were collected from the plants of *Helianthus annus* L. in Genichesky district of Kherson region (Ukraine). *D. helianthi* fungus for pure culture was obtained according to the standard techniques on potato glucose agar (PGA) (Dudka et al., 1982) The collected stems of sunflower with signs of phomopsis canker were cut into 5-10 mm pieces, sterilized in 96 % C_2H_5OH, put in Petri dish on the sterile PGA medium (Bötcher, 1987), and kept in thermostat at 23–25 °C. From the obtained colonies of pure culture of *D. helianthi,* disks (d 5 mm) were cut out, transferred into flasks on liquid Czapek nutrient medium (100 ml), and cultured at 25 ºC. The phytotoxicity of *D. helianthi* was determined by bioassay. Wheat (*Triticum aestivum* L.) seedlings were used as a test culture. The culture fluid of *D. helianthi* mycelium of different duration of cultivation (7 days, 14 and 17 days) was collected and filtered for the studies. Seeds of *Tr. aestivum* were kept in filtrates of *D. helianthi* for 24 hr and put into Petri dishes on filter paper moistened with distilled water, and germinated at 25 ºC. The criterion for assessing the toxic effects of filtrates of the culture medium of *D. helianthi* was the variation of the length of the seedlings *Tr. aestivum* on the sixth day of the study. Statistical data processing was performed in Statistica v. 7.0 (Borovikov, 2013). Changes in the qualitative and quantitative composition of secondary metabolites in the filtrate of the culture medium *D. helianthi* of the above

cultivation times were determined by profiling with reversed-phase high performance liquid chromatography (HPLC) on the chromatographic system Agilent 1100. Detection was performed at wavelengths of 206, 254 and 300 nm. In order to detect secondary metabolites, the absorption spectra of the chromatographic peaks were recorded in the ultraviolet and visible ranges.

Results and Discussion

First, we had to obtain *D. helianthi* fungus to make pure culture on PGA (Fig. 1). The effect of the time of cultivation of *D. helianthi* mycelium on changes in the toxicity of its secondary metabolites was studied on the 7th, 14th and 17th days of cultivation.

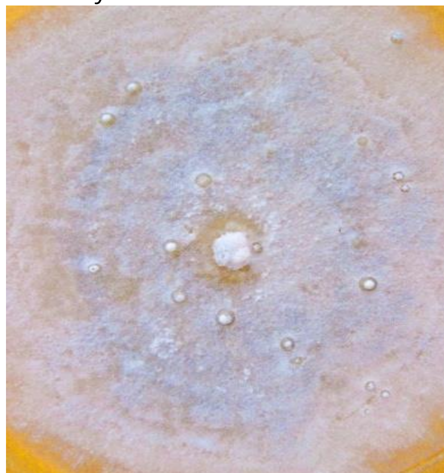

Fig. 1. Pure culture of *D. helianthi*, potato glucose agar (PGA)

These sampling times were based on our model of accumulation of secondary metabolism products in the culture medium, according to which the accumulation of mycotoxins increases and reaches a maximum on the 17th day of cultivation (Syvoded et al., 2018). We observed differences in the processes of formation of *Tr. aestivum* seedlings on filtrates of *D. helianthi* of different cultivation times (Fig. 2).

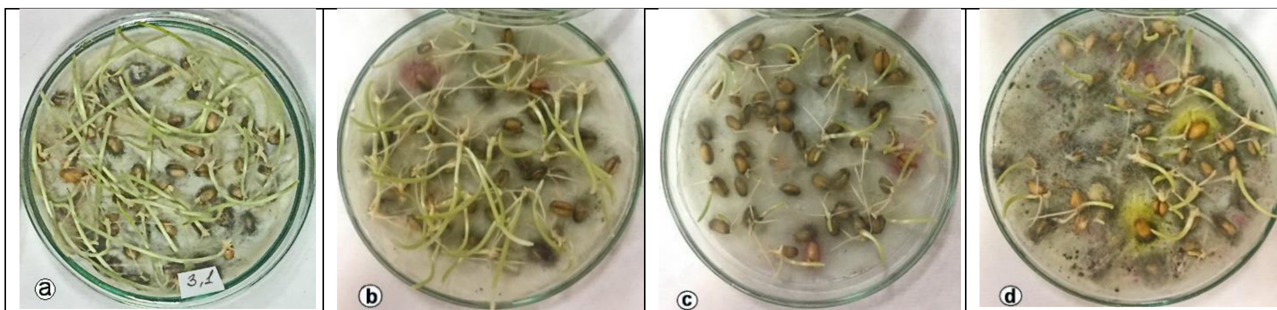

Fig. 2. Seedlings of *Tr. aestivum* grown on filtrates of *D. helianthi* of different times of cultivation:
a – control (liquid culture medium), *b* – 7-day substrate, *c* – 14-day substrate, *d* – 17-day substrate.

The most intensive development of seedlings was observed in the control (Fig. 1, *a*). The mean length of seedlings in this version of the experiment was 4.9 ± 0.26 cm (Table 1).

Table 1. Mean length of seedlings of *Tr. aestivum* on filtrates of *D. helianthi* of different time of cultivation, cm

Version of experiment	Time of cultivation, day			Validity of differences, $t_{0,05}$
	2	4	6	
Liquid Czapek nutrient medium	0.6±0.07	3.3±0.11	4.9±0.26	-
Filtrate of *D. helianthi* (7-day culture)	0.4±0.05	2.5±0.15	4.6±0.21	0,91
Filtrate of *D. helianthi* (14-day culture)	0.3±0.02	0.9±0.06	1.9±0.08	11,0
Filtrate of *D. helianthi* (17-day culture)	0.2±0.03	0.6±0.04	1.4±0.06	13,1

The seedlings of *Tr. aestivum* also intensely developed in the version with soaking of seeds in the filtrate of *D. helianthi* of 7-day cultivation of the fungus (Fig. 2, b). The length of test culture seedlings in this version of the experiment did not change significantly compared with the control version of the experiment ($t_{0.05}$=0.91). Using the filtrate of 14-day cultivation of pure culture of the pathogen led to a significant inhibition, 2.6 times, of the germination of test culture seedlings compared with control (Fig. 2, *c*). Germination of *Tr. aestivum* seedlings on the filtrate of a 17-day period of cultivation of pure fungus culture led to the loss of vitality in a significant number of seedlings and was accompanied by intensive development of pathogenic microflora (Fig. 2, d). The mean length of *Tr. aestivum* seedlings was 1.4 ± 0.06 cm, which is 3.5 times less than in control ($t_{0.05}$=13.1) (Table 1). Simultaneously, we collected samples of nutrient medium filtrates on the 7th, 14th, and 17th days of culturing the mycelium to determine changes in the content of secondary metabolites produced by pure culture of *D. helianthi*.

The following secondary metabolites were identified by reversed-phase HPLC: fomosin, fomopsolides, cytosporones, and xanthones (Fig. 3).

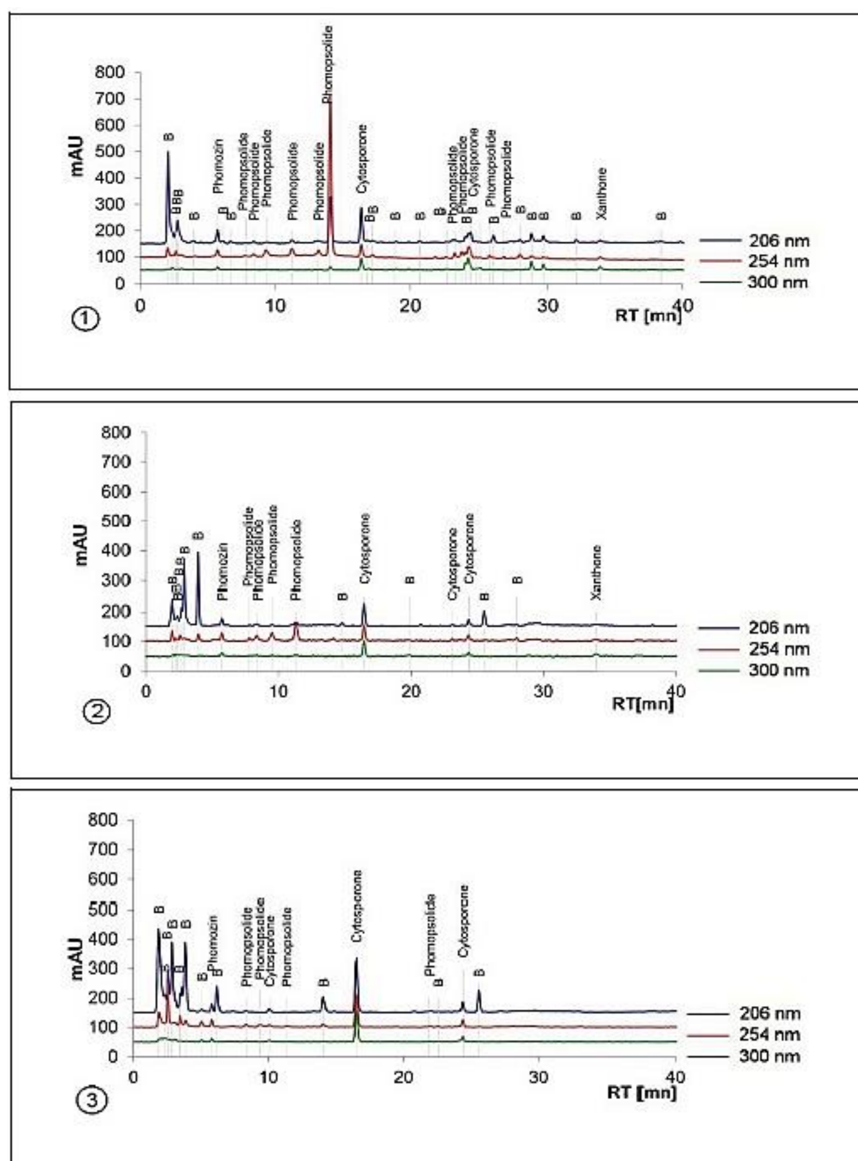

Fig. 3. Chromatogram of the extract of *D. helianthi* at different days of cultivation, at 208 nm, 254 and 300 nm:
1) 7 days; 2) 14 days; 3) 17 days. B – derivatives of benzene, phenol, thiazole and other compounds.

The accumulation of identified secondary metabolites differed significantly at different times of *D. helianthi* cultivation. The content of fomosin in the culture filtrate of the fungus did not change significantly from 7 to 17 days of cultivation (123–128.5 mAU) (Fig. 4). Other identified secondary metabolites showed multidirectional changes in quantitative and qualitative parameters depending on the timing of fungus cultivation. The content of fomopsolides significantly decreased in the culture filtrate of the 17-day cultivation period of *D. helianthi* M. in comparison with the 7-day cultivation period (16.6 times). In the spectrum of the 17-day fungus mycelium medium, the lines characteristic of xanthones have disappeared, which indicates the complete inhibition of their synthesis. The increase in the period of mycelium cultivation led to a gradual increase in the content of cytosporones (1.09 and 2.41 times, respectively, on the 14th and 17th day of cultivation). Such changes, in our opinion, are due to the fact that the identified substances have different properties. Fomosin ($C_{13}H_{16}O_7 \times H_2O$) is a highly active dimethylglyceric acid (Specian et al., 2012) with species-specific action. It can cause browning of *Helianthus annuus* L. leaves in small concentrations (5 pg in 24 hours), but does not affect plants of *Cucumis melo* L., *Glycine max* l, *Zea mays* l, *Pisum* L. and *Nicotiana* L. (Mazars et al., 1990). Detection of fomopsolides in filtrates of different times of cultivation of *D. helianthi* mycelium testifies to the wide variety of their structure and properties. In studies with *Staphylococcus aureus* found that all fomopsolides have a general biological inhibitory effect, namely antimicrobial properties (Vasudeva et al., 2015). These secondary metabolites are characterized by cytotoxic, fungicidal, allelopathic, bactericidal and antiviral activity (Qing-Wei Tan et al., 2017). Secondary metabolites from the group of cytosporones are bioactive compounds, that are widely distributed in nature and belong to phenolic lipids (Brady et al., 2000).

Xanthones are a product of condensation of γ-pyrone and two benzene rings. These compounds are biogenetically close to such groups of phenolic compounds as flavonoids due to the similarity of their physicochemical properties (Kruglov, 2015). The observed changes are an increase in the number of cytosporones, a decrease in the content of fomopsolides, and inhibition of xantones synthesis. These processes occur against the background of relatively unchanged values of fomosin.

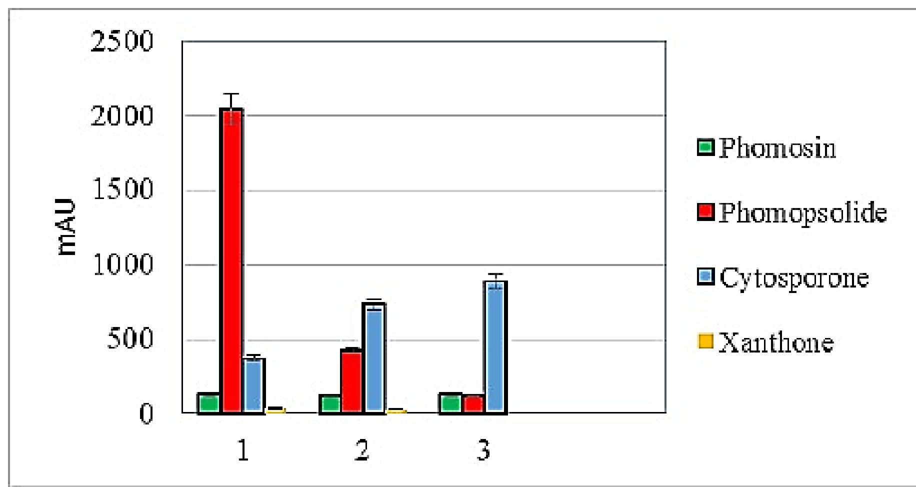

Fig. 4. Changes in content of the secondary metabolites in filtrates of different time of cultivation of *D. helianthi*: 1 – 7-day culture; 2 – 14-day culture; 3 – 17-day culture.

Thus, increasing the time of cultivation of *D. helianthi* mycelium leads not only to the suppression of the germination of *Tr. aestivum* seedlings, but also to the changes in the qualitative and quantitative content of the secondary metabolites. Therefore, in further studies it is important to study the mechanism of action of identified secondary metabolites to understand the processes of pathogenesis of fomopsis canker of *Helianthus annus* L.

Conclusions

The obtained results indicate the presence of secondary metabolites with high biological activity in the liquid culture medium of *D. helianthi.* The main identified classes of secondary metabolites produced by the mycelium of *D. helianthi* are fomosin, fomopsolides, cytosporones and xanthones. In the process of cultivation, the quantitative and qualitative indicators of secondary metabolites were changed, which leads to an increase in the toxicity of the culture filtrate of *D. helianthi.* This redistribution of secondary metabolites occurs due to a decrease in the content of fomopsolides, cessation of xanthone synthesis and an increase in the number of cytosporones against the background of relatively stable levels of fomosin. Redistribution of secondary metabolites of *D. helianthi* under conditions of using the filtrate of the culture medium of the fungus with a cultivation period of 17 days led to a decrease in the length of seedlings of *Tr. aestivum* by 3.5 times compared to control and by 3.3 times compared to the filtrate of the culture medium with a 7-day cultivation period.

References

Borovikov, V.P. (2013). A popular introduction to modern data analysis in the STATISTICA system. Hotline. Telecom Moscow.

Bötcher, I., Wetzel, T., Dreve, F.V., Kegler X., Naumann, K., Fryer, B., Frauenstein, K., Fuchs, E. (1987). Methods for the determination of diseases and pests of agricultural plants. Agropromizdat, Moscow.

Brady, S.F., Wagenaar, M.M., Singh, M.P., Janso, J.E., Clardy, J. (2000). The Cytosporones, New Octaketide Antibiotics Isolated from an Endophytic Fungus. Organic Letters, 2, 4043–4046.

Butler, E.E., Grisan, E.V. (1977). A key to the genera and selected species of mycotoxin-producing fungi. In T.D. Wyllie and L.G. Morehouse (Eds.). Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handboo, Marcel Dekker Inc., N.Y., 2, 432–537.

Dudka, I.A., Vasser, S.P., Ellanskaya, I.A., Koval, E.Z. (1982). Methods of experimental mycology. Chief Editor V.I. Bilay. Naukova dumka. Kiev.

Kyryk, M.M., Pikovskyi, M.Y., Azaiki, S.S. (2015). Diseases of seeds of agricultural crops, Kyiv. ZP Komprint.

Kruglov, D.S. (2015). Classification of biologically active compounds of plant origin in the pharmacognosy course. Basic research, 2(21), 4693–4698.

Mathew, F., Harveson, R., Gulya, T., Thompson, S., Block, C. and Markell, S. (2018). Phomopsis Stem Canker of Sunflower. Plant Health Instructor. Doi: http://doi.org/1094/PHI-I-2018-1103-01/

Mazars, Ch., Rossignol M., Auriol, P., Klaebe, A. (1990). Phomozin, a phytotoxin from *Phomopsis helianthi*, the causal agent of stem canker of sunflower. Phytochemistry, 29(11), 3441–3444. https://doi.org/10.1016/0031-9422(90)85253-C

Reddy, D.V., Sabitha, G., Yadav, J.S. (2015). A cross-metathesis approach to the synthesis of (+) phomopsolide B. Tetrahedron Letters, 56 (27), 4112–4114. Doi: http://doi.org/10.1016/j.tetlet.2015.05.032

Specian, V., Sarragiotto, M., Pamphile, J., Clemente, E. (2012). Chemical characterization of bioactive compounds from the endophytic fungus *Diaporthe helianthi* isolated from Luehea divaricata. Braz. J. Microbiol, 43(3), 1174–1182. https://doi.org/10.1590/S1517-83822012000300045

Syvoded, Ye.V., Kolesnichenko, O.V., Likhanov, A.F. (2018). Cytotoxic and mutagen action of secondary metabolites *Phomopsis helianti* M. Scientific reports of NULIP of Ukraine, 6(76). Available from: www.journals.nubip.edu.ua/index.php/http://dx.doi.org/10.31548/dopovidi2018.06.003

Qing-Wei Tan, Pei-Hua Fang, Jian-Cheng Ni, Fangluan Gao, Qi-Jian Chen (2017). Metabolites, Produced by an Endophytic *Phomopsis* sp. and Their Anti-TMV Activity. Molecules, 22, 2073. Doi: https://doi.org/10.3390/molecules22122073

Citation:
Syvoded, Ye.V., Kolesnichenko, O.V., Hrysiuk, S.M., Ivannikov, R.V. (2020). Accumulation and identification of secondary metabolites from the fungus *Diaporthe* (*Phomopsis*) *helianthi* Munt.–Cvet. et al. *Ukrainian Journal of Ecology, 10*(5), 166-169.

This work is licensed under a Creative Commons Attribution 4.0. License