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DYNAMICS OF THE PHENOLIC CONSTITUENTS AND ANTIOXIDANT ACTIVITY IN SUBMERGED CULTURES OF *Xylaria* SPECIES

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Purpose. This study was conducted to enhance comprehension of the dynamic process of synthesis of phenolic compounds by representatives of the genus *Xylaria*, and the correlation between phenol content and antioxidant properties found in biomass and culture liquid during submerged cultivation.

Methods. Cultivation of *Xylaria polymorpha* and *Xylaria longipes* fungal strains from the IBK Mushroom Culture Collection was carried out on a glucose-yeast-peptone nutrient medium under submerged conditions. Harvesting of both biomass and culture liquid was done on the 3rd, 5th, 7th, and 9th day of cultivation, followed by extraction with ethyl acetate. The total phenol content of extracts was determined using the Folin–Ciocalteu method and the antioxidant potential was evaluated through the DPPH assay.

Results. Findings revealed that the accumulation of phenolic compounds by fungal species of the *Xylaria* genus was specified on a strain level. Notably, *X. longipes* strains exhibited higher production of phenolic constituents compared to *X. polymorpha* and demonstrated superior antioxidant activity at a specific time of cultivation. Furthermore, a strong correlation was established between the dynamics of polyphenol accumulation and antioxidant activity in both mycelial biomass and culture liquid.

Conclusions. Natural phenolic compounds with antioxidant properties were extracted from the biomass and culture liquid of the studied strains. Significantly higher concentrations of phenolic compounds and values of antioxidant activity were found in the biomass compared to the culture liquid. The results indicate that a later day of cultivation is not necessarily equivalent to the production of more phenols, emphasizing the need for a comprehensive assessment of the accumulation of these compounds and the dynamic study of related parameters.

Key words: *Xylaria*; phenolic compounds; antioxidants; dynamics; biomass; culture liquid.

According to many scientists, fungi of the genus *Xylaria* Hill ex Schrank can be attributed to promising producers of a variety of biologically active substances, serving both the final and a side product during cultivation. In previous studies regarding metabolites of *Xylaria* species different groups of natural products such as diterpenoids, sesquiterpenoids, diterpene and triterpene glycosides, steroids, alkaloids and phenolics were discovered [1–3]. Since these compounds proved to possess antibacterial [4], antifungal [5, 6], cytotoxic [7], and other pharmacological activities, the interest in xylariaceous fungi as producers of biologically active substances has been growing. Among them, phenolic constituents are of particular interest because of their remarkable potential as free radical scavengers [8]. However, it is worth noting that most of these compounds were obtained from the fruiting bodies of these fungi, while there are rather limited studies on their cultivation. Nevertheless, the practical use of fungi is closely related to the production of mycelium through different methods of cultivation, which is why many modern studies are focused on the optimization of culture conditions [9, 10]. The method of submerged cultivation applied in this study presents a promising approach for obtaining both mycelium and culture liquid

containing bioactive compounds, facilitating their subsequent analysis.

As a result of a preliminary screening of species of the genus *Xylaria* from the IBK Mushroom Culture Collection for biological activity, *Xylaria polymorpha* (Pers.) Grev. and *Xylaria longipes* Nitschke were selected for the study. Cultural and morphological data on growth rates on different nutrient media were used to select two strains of both species [11]. This study aimed to analyze the growth-associated dynamics of phenol production and antioxidant capacity during submerged cultivation of these fungi. Since phenolic compounds have been registered among the main contributors to the antioxidant activity of fungi [12] a correlation between the accumulation of phenolic compounds and antioxidant activity in both biomass and culture liquid extracts was of particular interest.

Materials and Methods

The basal glucose-yeast-peptone nutrient medium (GYP) composed of (g/l): glucose, 25; peptone, 3; yeast extract, 3; $MgSO_4$, 0.25; KH_2PO_4, 1; K_2HPO_4, was used for the cultivation of the mycelium. Fungal strains IBK 2720, 2736 of *X. polymorpha* and IBK 2718, 2726 of *X. longipes* used in this study are from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany. Strains were initially grown in Petri dishes for 7 days at 25±1 °C on a basal medium with an additional 20 g/l of agar-agar.

The obtained inoculum was homogenized and sterilely inoculated (10% v/v, in 6 duplicates) in 250 ml Erlenmeyer flasks containing 100 ml of GYP medium. Cultivation was carried out for 9 days in darkness on a laboratory shaker under the following conditions: temperature 25±1 °C, agitation speed 120 rpm. The mycelial biomass was harvested by filtration on 3, 5, 7, and 9th day of cultivation and dried at 60 °C until constant weight.

Biomass extraction was conducted with ethyl acetate in a ratio of 1:5 (w/v) for 24 h at room temperature (20±1 °C). Then, the extracts were centrifuged for 15 min at 3000 rpm, after which the supernatant was separated and concentrated using a vacuum rotary evaporator at 40 °C. The culture liquid was initially concentrated using a vacuum rotary evaporator at 40±1 °C and extracted with ethyl acetate in a ratio of 1:2, for 24 h at room temperature (20±1 °C). The upper ethyl acetate fraction was separated using a separatory funnel and then concentrated using a vacuum evaporator at 40±1 °C.

The antioxidant activity of the prepared extracts was determined by 2,2–Diphenyl–1–picrylhydrazyl (DPPH) scavenging assay following Liu et al. [13].

Total phenol content was estimated using a Folin–Ciocalteu (FC) reagent-based assay following Elfahri et al. [14]. The total phenolic value of the samples was obtained from the regression equation $y = 0.0033x+0.0462$ with $R^2 = 0.9904$. The content of total phenolics was estimated as gallic acid equivalents (GAE) and converted into mg/g of dry weight (for biomass extracts) and mg/ml of cultural media (for culture liquid extracts).

Experimental data are indicated as the mean value of at least three independent experiments ± SD (standard deviation). The Student's t-test was applied to express the significance; values at $P < 0.05$ were considered significant. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Results were analyzed in standard statistical packages Microsoft Excel and Statistics 6.

Results and Discussion

Diverse patterns were noted in the accumulation of phenolics for each strain on different days. In the case of *X. polymorpha* IBK 2720 and 2736 strains, the peak total phenolic content (TPC) was observed on days 3 and 7, with recorded amounts of 0.92±0.05 and 1.53±0.09, respectively (Figs. 1, 2). Strain *X. longipes* IBK 2726 demonstrated the highest TPC value of 2.53±0.10 on the 5th day of cultivation, surpassing all other strains studied. Conversely, the strain *X. polymorpha* IBK 2720 exhibited the lowest TPC value among all biomass extracts, also on the 5th day of cultivation (Fig. 1).

The distinct variations in phenolic accumulation across different strains may be attributed to various factors associated with the cultural and morphological characteristics of the strains, as well as their diverse origins. Contrary to the assumption that the decline in phenolic levels is a result of their extraction into the culture medium, the data did not support this claim.

The quantity of phenolics in the biomass exhibited similar fluctuations to those in the culture liquid, as illustrated in Fig. 1–4, but with higher rates. Notably, for the strain *X. longipes* IBK 2726 the phenolic content initially increased on day 5, decreased nearly threefold on day 7, and halved by the 9th day of cultivation. Similarly, but not so pronounced,

the content of phenols in the culture liquid of this strain changed (Fig. 4). A similar trend was observed when comparing the phenolic content in the biomass and culture liquid of *X. polymorpha* IBK 2720. However, in this case, the amount of phenolics first decreased, then increased sharply and fell again on the 9th of cultivation (Fig.1).

In general, the culture liquid extracts contained notably lower quantities of phenols compared to the biomass, with the peak value of 0.45±0.03 recorded on day 9 for strain IBK 2726. Although the method applied here allows estimation of the total phenolic content, it is susceptible to various factors that may affect the values. In fungal cultivation, factors such as extraction methods, solvent choices, and culture media composition can influence bioactive compound concentrations.

Regarding the antioxidant activity, the biomass extracts of all strains studied showed high rates of DPPH scavenging activity. The maximum value was recorded for *X. longipes* IBK 2718 – 87.82±0.19% on the seventh day of cultivation, and a close value was obtained for *X. polymorpha* IBK 2720 on the third day of cultivation at 87.37±0.75%. As compared to other antioxidant assays, the obtained values are high. For instance, in the already mentioned study conducted by Liu et al. [13], the ethyl acetate extracts of the endophytic *Xylaria* sp. had a substantially higher phenol content, than obtained by us. Nevertheless, their antioxidant activity was notably lower compared to the results obtained in our research and amounted to 29.66±0.97%.

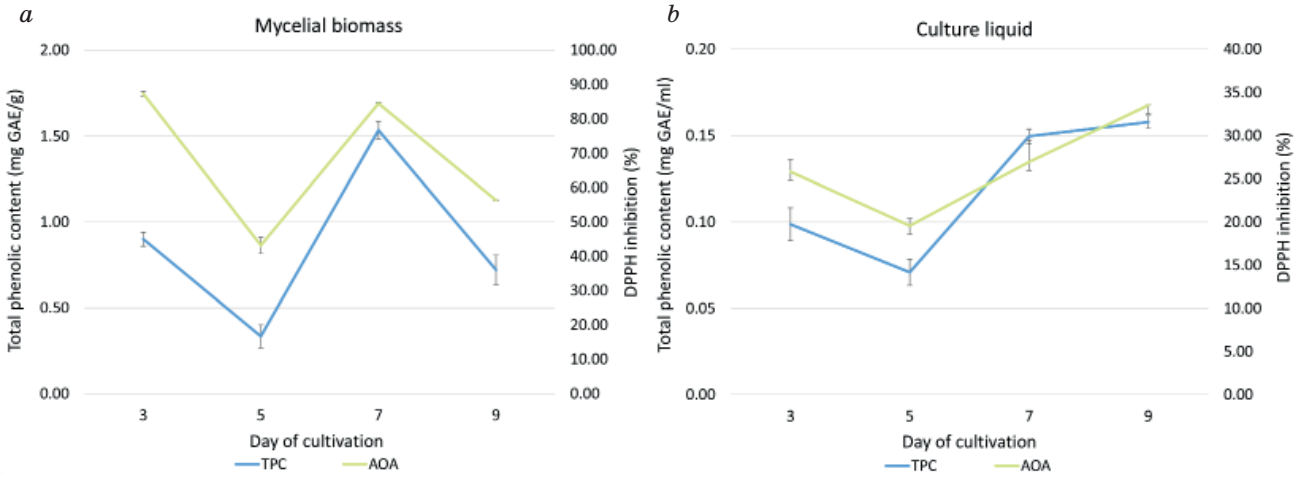

Fig. 1. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. polymorpha* IBK 2720**

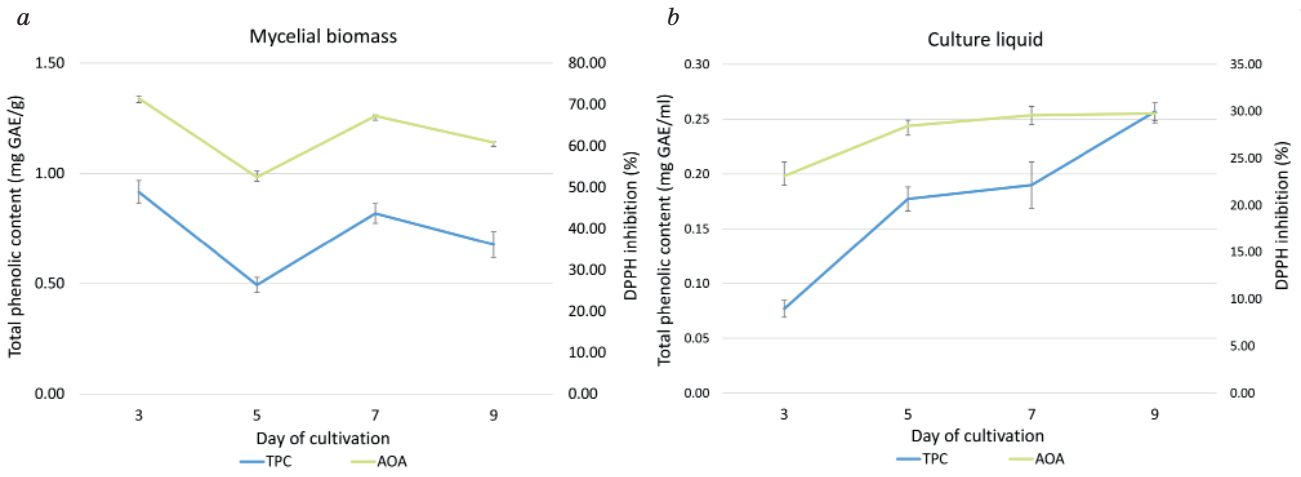

Fig. 2. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. polymorpha* IBK 2736**

Moreover, the strains we studied produced phenols in amounts comparable to other fungi, not only within their taxonomic group but also among fungi that are actively cultivated on the industrial scale. For example, according to Cheung et al. (2003) for the edible mushrooms *Lentinula edodes* (Berk.) Pegler (shiitake) and *Volvariella volvacea* (Bull.) Singer (straw mushroom) amounts of extracted ethyl acetate phenols were 0.03±0.01 and 0.21±0.08 mg of GAE/g of dry weight of fruiting bodies, respectively [15]. These amounts are comparable to our data for mycelium and culture liquid, even though researchers emphasize that fruiting bodies exhibit significantly higher concentrations of phenolic compounds compared to cultivated mycelium.

It has been observed by numerous authors that phenolic content correlates with antioxidant activity. This is attributed to the structural chemistry of phenolics, which facilitates hydrogen or electron donation from hydroxyl groups located along the aromatic ring. Such mechanisms contribute to effective free radical scavenging activities and demonstrate metal-chelating potential [16]. The correlation of total phenolic content with DPPH scavenging activities of extracts in our study is shown in Figs. 1–4 and calculated correlation coefficients are presented in Table 1. Notably, the biomass of *X. polymorpha* IBK 2736 displayed the strongest correlation between the total phenolic content and DPPH activity, with a Pearson's coefficient of 1.00.

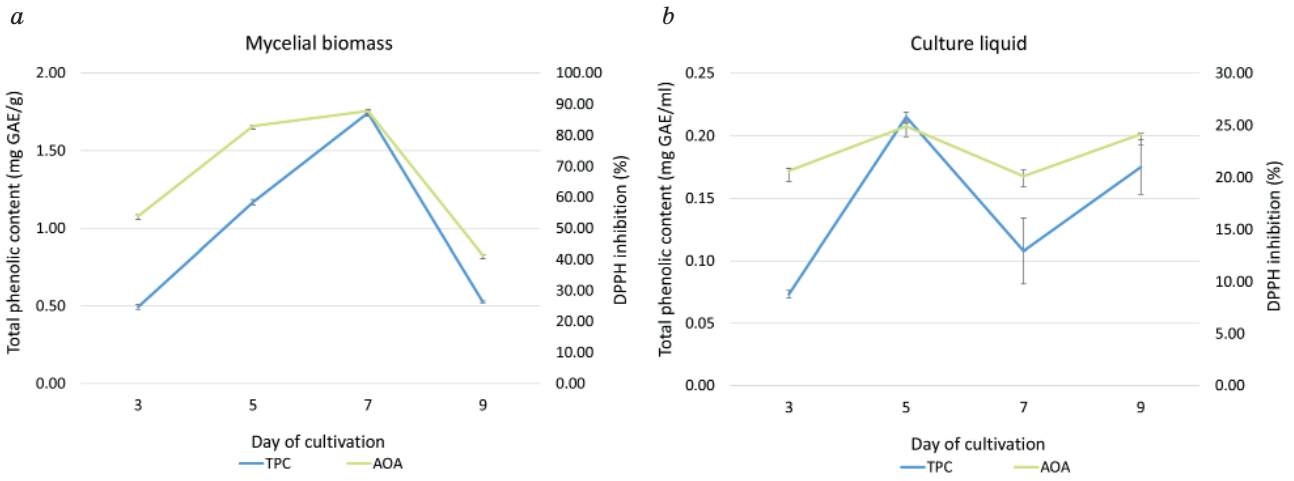

Fig. 3. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. longipes* IBK 2718**

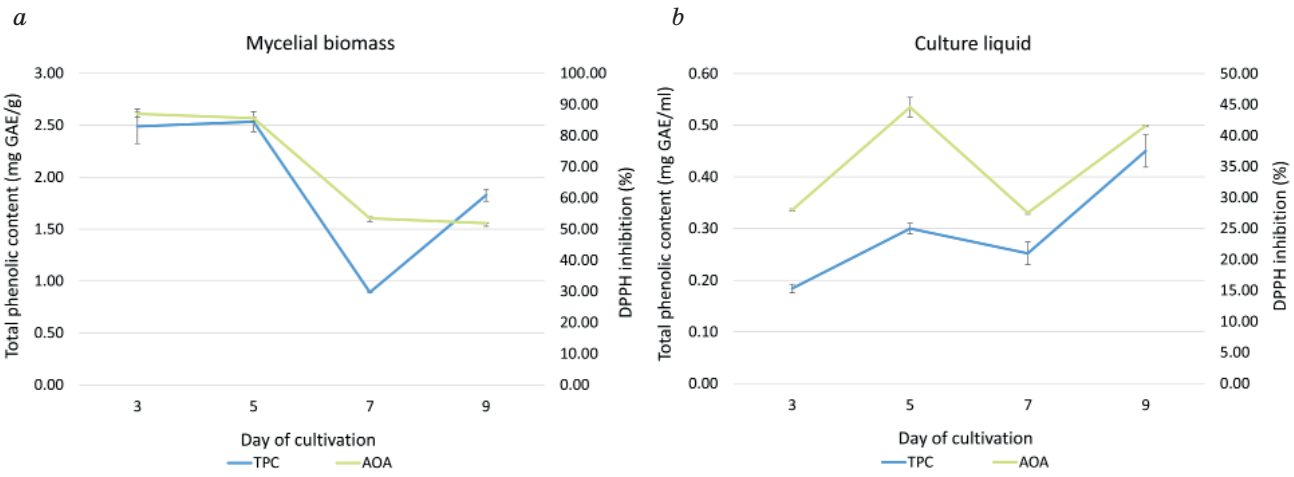

Fig. 4. **Dynamics of the total phenolic content and antioxidant activity in mycelial biomass (*a*) and culture liquid (*b*) of *X. longipes* IBK 2726**

Table 1

Pearson's correlation coefficients of antioxidant activity and phenolic contents

Species	IBK strain	Mycelial biomass	Culture liquid
X. polymorpha	2736	1.00	0.93
	2720	0.82	0.89
X. longipes	2718	0.92	0.94
	2726	0.85	0.71

These correlations do not account for distinguishing characteristics of phenolic profiles, which can vary both qualitatively and quantitatively, depending on the types of phenolics present in the samples. It is important to note that phenolic compounds possess different donor-proton capacities, which determine their antioxidant activity. Therefore, phenolic compounds and their characteristics remain to be investigated in representatives of the genus *Xylaria*.

Conclusions

The results demonstrate that *X. polymorpha* and *X. longipes* accumulate phenolic compounds strain-specifically, which should be considered when selecting strains that produce biologically active substances. Most noticeable concentrations of phenolic compounds were accumulated in biomass compared to culture liquid. Studied trains of *X. longipes* accumulated more phenolic compounds, reaching the maximum of 2.53±0.10 mg GAE/g for the strain IBK 2726 on the 5th day of cultivation. In comparison, the highest amount of TPC was 1.53±0.09 mg GAE/g *for X. polymorpha* IBK 2720 on the 7th day of cultivation. These results support the belief that extended cultivation time does not result in increased metabolite accumulation, and thus it is effective to study the variation of phenols through dynamico17s. A strong correlation between the total phenol content and DPPH radical scavenging activity was observed for all biomass and culture liquid extracts.

The authors declare no conflicts of interest. No funders had any influence on the study design, data collection, analysis, or publication of the results.

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ДИНАМІКА ВМІСТУ ФЕНОЛІВ ТА АНТИОКСИДАНТНОЇ АКТИВНОСТІ ПРИ ГЛИБИННОМУ КУЛЬТИВУВАННІ ВИДІВ РОДУ *Xylaria*

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Мета. Дослідження динаміки накопичення фенольних сполук представниками роду *Xylaria*, а також кореляції між вмістом фенолів та антиоксидантними властивостями, виявленими у біомасі та культуральній рідині при їх глибинному культивуванні.

Методи. Штами грибів *Xylaria polymorpha* та *Xylaria longipes* з Колекції культур шапинкових грибів Інституту ботаніки імені М.Г. Холодного НАН України (ІВК) вирощували на глюкозо-дріжджово-пептонному живильному середовищі за умов глибинного культивування. Біомасу та культуральну рідину відбирали на 3, 5, 7 та 9-ту добу культивування із подальшою екстракцією етилацетатом. Загальний вміст фенолів у всіх екстрактах визначали за допомогою методу Фоліна-Чокалтеу. Антиоксидантний потенціал оцінювали за допомогою спектрофотометричного аналізу поглинання вільних радикалів 2,2–дифеніл–1–пікрилгідразилу.

Результати. Встановлено, що накопичення фенольних сполук було штамоспецифічною характеристикою. Зокрема, штами *X. longipes* продукували більше фенольних сполук упродовж усього часу культивування, порівняно зі штамами *X. polymorpha*, та проявляли вищу антиоксидантну активність на певну добу. Крім того, було встановлено високу кореляцію між динамікою накопичення фенольних сполук та антиоксидантною активністю як у біомасі, так і в культуральній рідині.

Висновки. Фенольні сполуки з антиоксидантними властивостями було екстраговано із біомаси та культуральної рідини досліджених штамів. Значно вищі концентрації фенольних сполук та значення антиоксидантної активності було виявлено у біомасі порівняно з культуральною рідиною. Показано, що продовження процесу культивування не завжди призводить до збільшення концентрації фенольних сполук, що підкреслює необхідність подальшого комплексного вивчення накопичення цих речовин та взаємозв'язків із супутніми параметрами.

Ключові слова: *Xylaria*; фенольні сполуки; антиоксиданти; динаміка; біомаса, культуральна рідина.