UDC 579.22: 582.28 https://doi.org/10.15407/biotech12.04.034

INFLUENCE OF NITROGEN CONCENTRATION ON PHOTOINDUCED GROWTH, ENZYMATIC ACTIVITY AND MELANINE SYNTHESIS BY *Inonotus obliquus* (Ach.:Pers.) Pilát

N. L. Poyedinok[1]
T. I. Tugay[2]
A. V. Tugay[2]
O. B. Mykchaylova[3]
N. N. Sergiichuk[4]
A. M. Negriyko[5]

[1]Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine, Kyiv
[2]Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv
[3]Kholodny Institute of Botany of the National Academy of Sciences of Ukraine, Kyiv
[4]Open International University of Human Development "Ukraine", Kyiv
[5]Institute of Physics of the National Academy of Sciences of Ukraine, Kyiv

E-mail: n.poyedinok@gmail.com

Received 09.06.2019
Revised 17.07.2019
Accepted 30.08.2019

The aim of the work was to study the effect of nitrogen concentrations on photo-induction of growth, enzymatic activity and synthesis of melanin by the medicinal mushroom *Inonotus obliquus* (Ach.: Pers.) Pilát. Irradiated by light of low intensity light, different coherence and in different wavelength ranges, mycelium was cultivated in a dynamic mode on a glucose-peptone medium with different concentrations of total nitrogen. The concentration of the nitrogen source was not shown to significantly affect the photo-induced stimulation of the *I. obliquus* growth. The increase in biomass accumulation of mycelium photoactivated in different modes was almost the same in all variants of the experiment, compared with the biomass of not-irradiated mycelium. A reliable dependence of the photo-stimulation of melanin synthesis on the concentration of nitrogen in the medium was established. Reduced nitrogen concentration more than twice increased the stimulating effect of low-intensity laser radiation with a wavelength of 488 nm. Using substrate with a reduced content of the nitrogen source is advisable to increase the photo-induced stimulating effect in the production of extracellular catalase, tyrosinase and polyphenol oxidase, intracellular peroxidase.

Thus, the cultivation parameters of *I. obliquus* and the light treatment regimes of the inoculum should be adjusted according to the target bioactive components.

Key words: *Inonotus obliquus*, photoinduction, nitrogen, melanin, catalase, tyrosinase, polyphenol oxidase, peroxidase, growth activity.

For several hundreds of years, *Inonotus obliquus* has been used, rather successfully, in traditional medicine to treat cancer and other illnesses. Its chemical composition is known by now. Thus, the mushroom is recognized as a promising producer of not only phenolic compounds, melanins and triterpenoids, but also of other components with anti-oxidant, antitumor, antiviral, hepatoprotective and immunomodulation properties [1–4].

Previously we've established that the low-intensity light of different coherence in the visible part of the spectrum can be effectively utilized to regulate the growth and biosynthesis activity of this mushroom [5, 6]. However, the way the mushroom reacts to light exposure depends on various factors. For many of them, the mechanisms of action are hardly studied at all. The published data mostly touches on the processes of photoreception and photo-regulation of the microfungi metabolism

[7–13]. For *Hypocrea atroviridis* it is shown that the composition of medium and culturing regime determine the photostimulation levels of growth and conidia production. In *Trichoderma reesei*, photo-induced activity of enzymes, involved in plant cell destruction, also depends on the nutrient source [13]. The photoreception and expression of *Aspergillus* enzimes is sometimes linked with carbohydrate metabolism [14]. Other researchers think that there is a relationship between light and the metabolism of nitrogen [15, 16]. We have previously revealed the dependence of photo-activation levels of mycelial growth in five species of fungi on the carbon concentration in substrate [17]. There is no statistically significant evidence of the effect of nitrogen source concentration on the photo-induction of growth stimulation and biomass accumulation in those fungi. However, the nitrogen concentration can affect the photo-induction of secondary metabolites in those species, which does not necessarily correlate with the growth activity [17–20].

To test that hypothesis, we've studied the biosynthetic activity of *I. obliquus* on media with different concentrations of nitrogen (2 and 4 g/l of peptone).

Materials and Methods

Study object was pure culture of *Inonotus obliqus* (Ach.: Pers.) Pilát 1877 from the mushroom culture collection (IBK) of Kholodny Institute of Botany of the National Academy of Sciences of Ukraine.

The medium used to study the effect of nitrogen concentration on photo-induced biosynthetic activity was the glucose-peptone medium (Bacteriological Peptone (laboratorios CONDA), with 30 g/l glucose and different peptone concentrations (2 and 4 g/l, equal to 282 and 564 mg/l of total nitrogen). The mycelium, surface-grown on wort-agar at 25–26 °C, was irradiated. Immediately after that, disks of mycelium were cut out, 5 mm in diameter. Mycelial disks were put in groups of five in Erlenmeyer flasks with 150 ml of nutrient medium, and cultured in dynamic mode (180 rpm) at same temperature for 12 days. The biomass was dried at 60 °C to constant weight. The change in growth indices after irradiation with low-intensity light was calculated in% compared to control.

The sources of coherent visible low-intensity laser radiation (LILR) were gas lasers: helium-neon laser LGN-215 with a wavelength of 632.8 nm (red light) ("Poliaron", Lviv, Ukraine), and argon ion laser with a wavelength of 488.0 nm (blue light) (modified LGN-106M1, "Plasma", Russia). The laser ray was defocused with a lens to the size of a Petri dish.

The light-emitting diodes (LED) matrix was the source of low-coherent light. It was produced with 21 high power LED units based on AlGaInN (China Young Sun Led Technology Ltd). Each diode block included two microchips emitting light with wavelengths of 463 nm (blue) and 625 nm (red). The electrical power of each microchip was 1 W. Radiation intensity was regulated from zero to maximum independently for each spectral range, that is separately for the blue, green and red lights by adjusting the current running through the diodes [6].

Radiation power density was measured using a PM-100D digital optical power and energy meter, Thorlabs Inc. with standard photodiode power sensor S120C, operating range of 400–1100 nm. The radiation dose was determined as the product of the power density and the exposure time, and was 230 mJ/cm^2 in all variants of the experiment. We chose the modes of irradiation based on our previous results [5, 6, 18, 20]. Light processing of inoculum was carried out in the complete absence of other light sources.

Methods of melanin extraction from biomass and its quantitative determination in mycelium and culture medium were described in [6], together with methods used for the determination of enzyme activity.

Statistical processing of the results was performed using Excel 2007 software program. All experiments were performed in 4–5 replicates. Statistical processing of the results was calculated in Statistica 6.0 software program. The results of study on the biosynthetic activity of *I. obliquus* on media with different concentrations of nitrogen were statistically significant at $P < 0.05$ according to the *t*-student criterion.

Results and Discussion

Our results did not reveal any significant effect of the nitrogen concentration on the photoinduction of *I. obliquus* growth (Fig. 1). The increase in biomass accumulation by mycelium, photoactivated in different modes, was almost the same in all variants of the experiment compared with control (non-irradiated mycelium). We've obtained similar data for other species of fungi [5]. This allows us to assume that there are common mechanisms of photo-regulation of growth activity in the studied species of fungi.

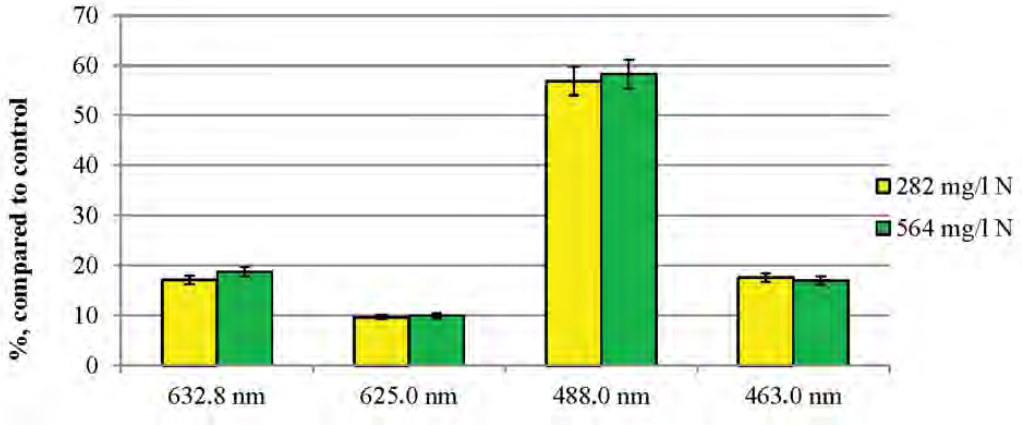

Fig. 1. **Growth of photo-activated mycelium of *I. obliquus* on media with different concentrations of nitrogen** (control = 100%, 0 on the figure)

The results presented on Fig. 2 demonstrate that there is a statistically significant dependence of photo-stimulation of melanin synthesis on the concentration of nitrogen in medium. Reducing the concentration of nitrogen increases the stimulating effect of LILR with a wavelength of 488 nm by more than 2 times.

In this regard, the study of the effect of nitrogen on photo-induced changes under the activity of *I. obliquus* enzymes which were established earlier [6] was of particular interest, specifically catalyzing the synthesis of melanin, extracellular and intracellular tyrosinase, polyphenol oxidase, as well as catalase and peroxidase, which provide antioxidant cell protection and maintaining the concentration of reactive oxygen species at the physiological level.

Limiting the nitrogen concentration in the nutrient medium lead to increased stimulating effect of both laser and LED irradiation only for the extracellular tyrosinase (Fig. 3). The tyrosinase activity increased by 42.7% and 65.0% on a medium with 284 mg/l of nitrogen after irradiation of the seed mycelium with light with the wavelength of 632.8 nm and 488.0 nm, respectively, compared to cultivation on medium containing 568 mg/l nitrogen. The tyrosinase is known to catalyze the synthesis of melanin, thus a parallel two-fold increase in the synthesis of this pigment in the same variant of the experiment is quite explicable. The results suggest that nitrogen metabolism does not affect the biosynthesis of intracellular tyrosinase.

Similar study was conducted on the changes

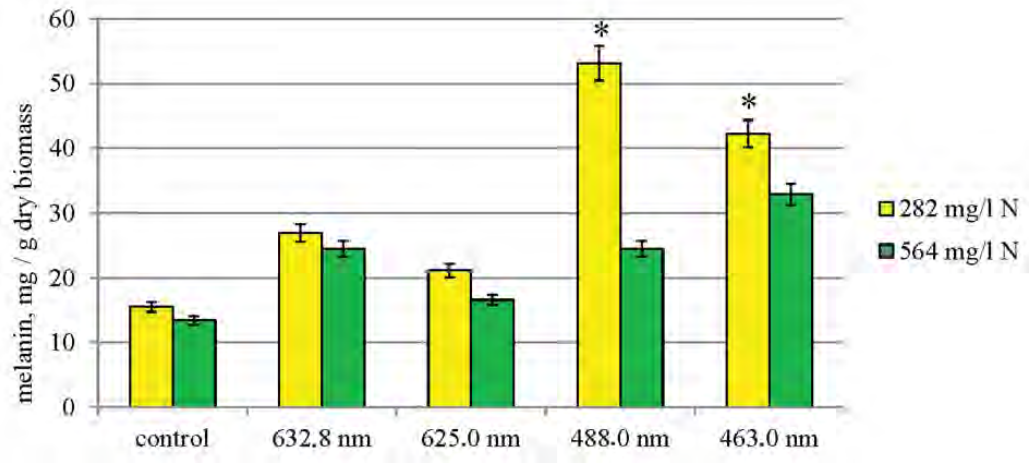

Fig. 2. **Effect of nitrogen concentration (282 and 564 mg/l) on photoinduction of melanin in *I. obliquus*:**
* statistically significant differences of melanin synthesis on media with different nitrogen concentrations ($P < 0.05$), results presented as $M \pm n$, $n = 4$ to 5

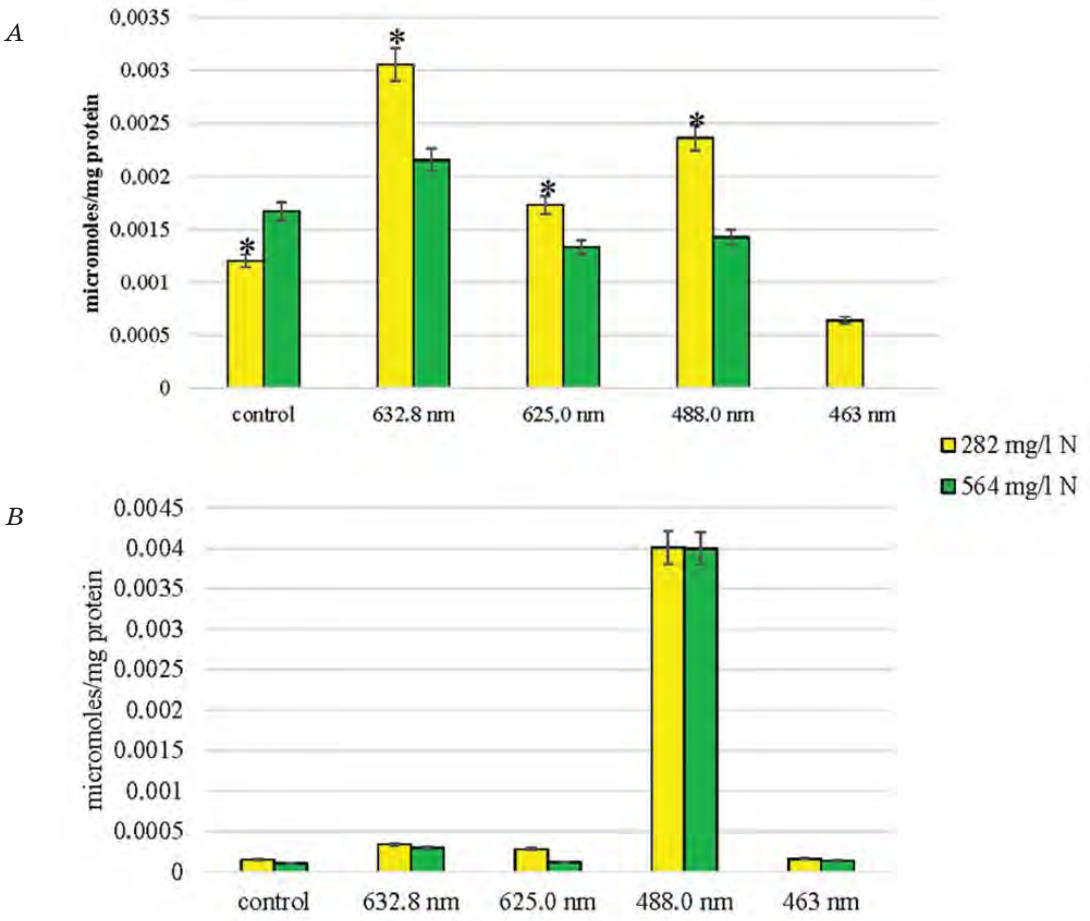

Fig. 3. **Effect of nitrogen concentrations (282 with compared to 564 mg/l) on activity of tyrosinase in *I. obliquus***

Hereinafter (*A*) extracellular, (*B*) intracellular enzyme, results presented as $M \pm n$, $n = 4$ to 5.

* Statistically significant differences of tyrosinase activity on media with different nitrogen concentrations ($P < 0.05$).

in the activity of polyphenol oxidase, another enzyme responsible for the synthesis of melanin in *I. obliquus* (Fig. 4). It was observed that decreasing the concentration of nitrogen in the culture medium lead, on the contrary, to 42.4% decrease in the level of intracellular enzyme activity if mycelium irradiated with blue laser light were used as seed material. A significant decrease in the activity of polyphenol oxidase in mycelium was also noted when using other modes of irradiation, but not as substantial.

In a medium with reduced nitrogen content, the activity of extracellular polyphenol oxidase increased more than twice when the seed mycelium was activated by red low-coherent light (Fig. 4).

Higher extracellular peroxidase activity in *I. obliquus* was observed on a medium containing 284 mg/l of nitrogen for non-irradiated inoculum (control). Irradiation reduced enzymatic activity on media with both concentrations of nitrogen (Fig. 5).

Reduction of nitrogen concentration did not affect the activity of intracellular peroxidase in control and after irradiation of mycelium with non-coherent light. Irradiation with both blue and red coherent light caused an increase in peroxidase activity in the mycelium of *I. obliquus*. However, changes in the concentration of nitrogen in the medium affected the photo-induction of this enzyme differently. For example, cultivating mycelium irradiated with light with a wavelength of 632.8 nm on a medium with 284 mg/l of total nitrogen resulted in lower enzymatic activity than when cultivating it on a medium with 568 mg/l of nitrogen. When those media were inoculated with mycelium activated by blue coherent light, the opposite reaction was observed. We can only assume that light of

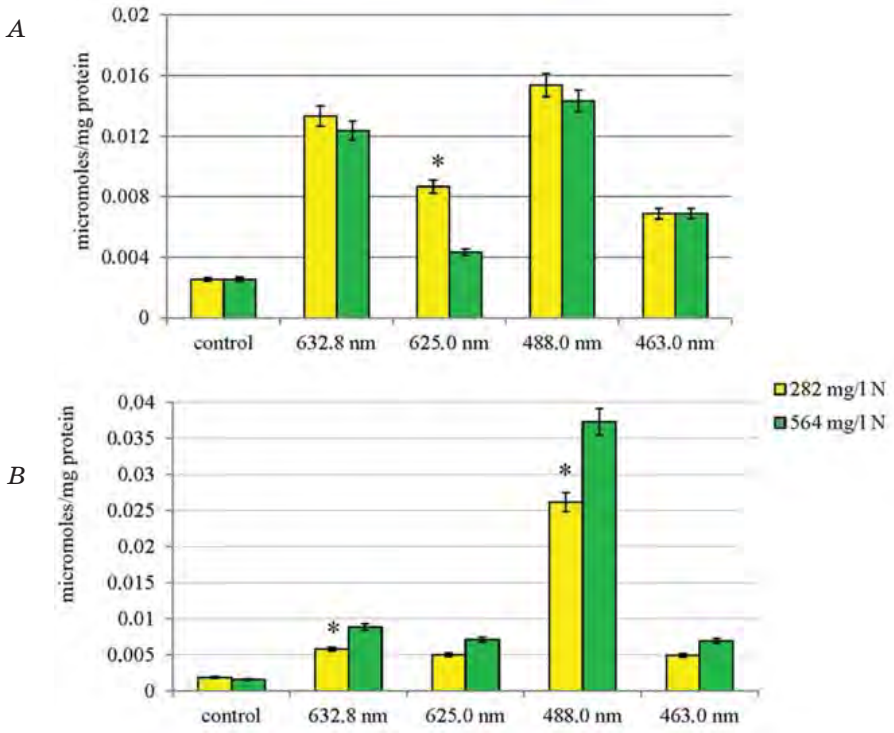

Fig. 4. **Effect of nitrogen concentrations (282 with compared to 564 mg/l) on activity of polyphenol oxidase in *I. obliquus:*** * statistically significant differences of polyphenol oxidase activity on media with different nitrogen concentrations ($P < 0.05$)

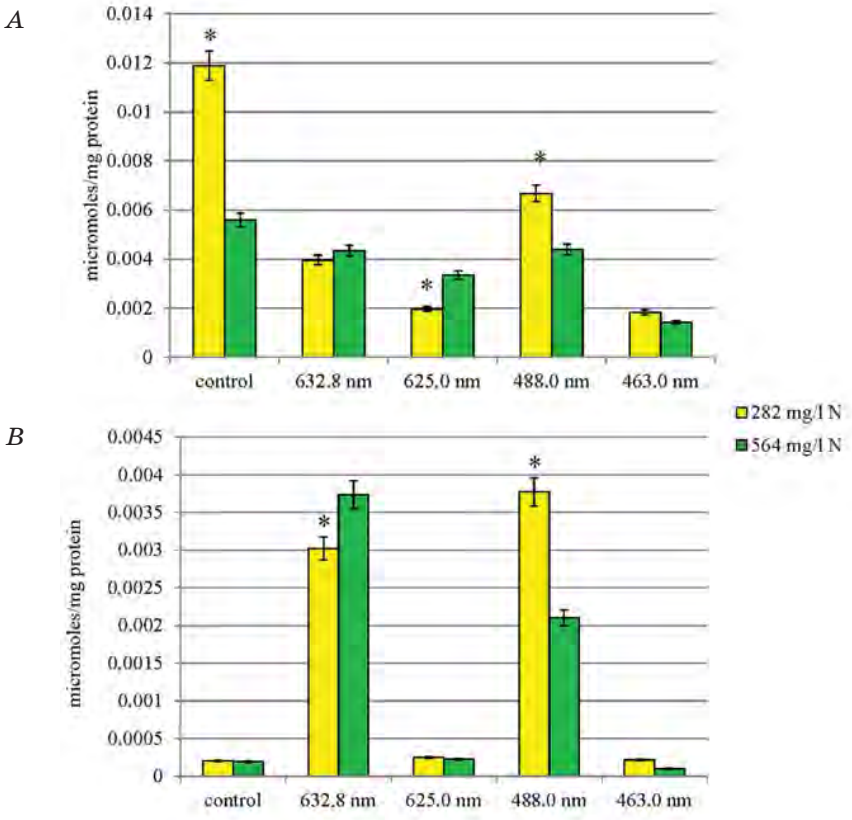

Fig. 5. **Effect of nitrogen concentrations (282 with compared to 564 mg/l) on activity of peroxidase in *I. obliquus:*** * statistically significant differences of peroxidase activity on media with different nitrogen concentrations ($P < 0.05$)

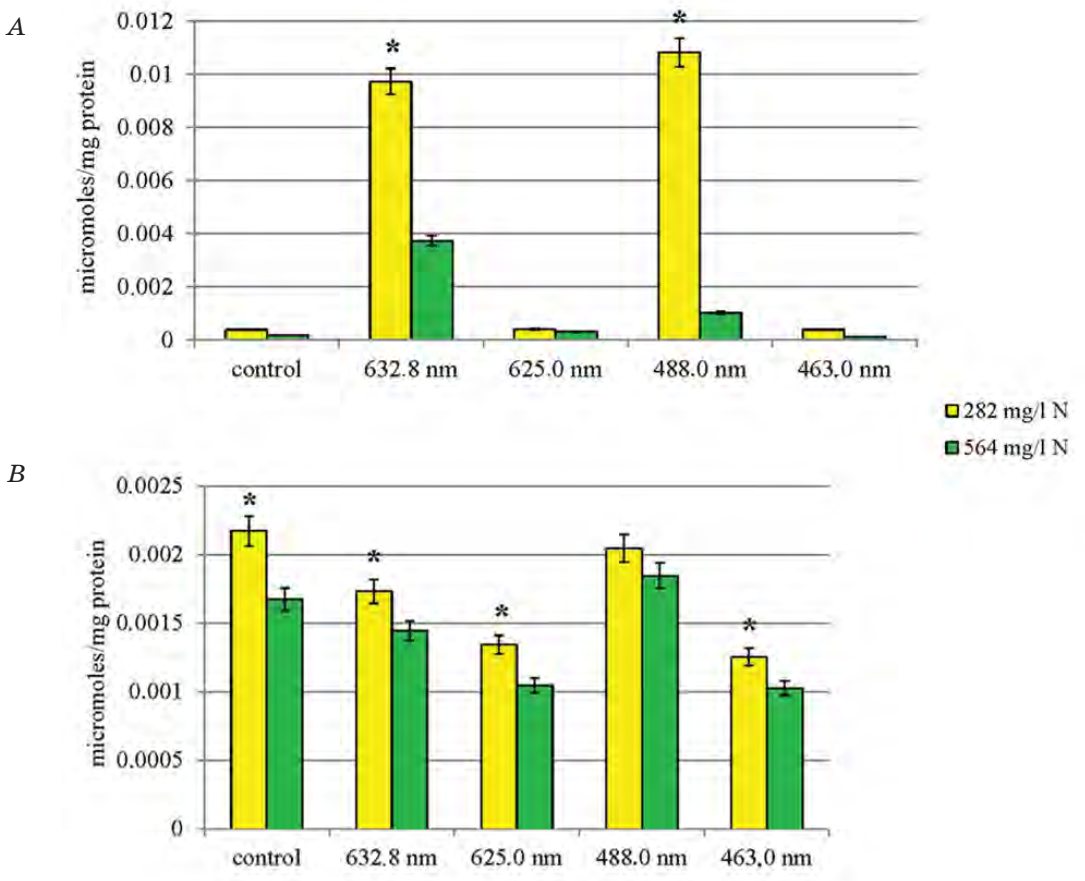

Fig. 6. **Effect of nitrogen concentrations (282 with compared to 564 mg/l) on activity of catalase in *I. obliquus:***
* statistically significant differences of catalase activity on media with different nitrogen concentrations ($P < 0.05$)

different wavelengths and coherence induces various changes in metabolism, changing the peroxidase activity of mycelium. Studying the mechanisms of such changes is undoubtedly of practical and scientific interest and deserves due attention.

Regarding the effect of nitrogen concentrations on the realization of photoinduced changes in catalase activity, our studies unequivocally showed the advantages of using media with a reduced content of nitrogen source to culture the photoactivated inoculum, for an additional stimulating effect (Fig. 6).

The activity of extracellular catalase after irradiation with red and blue coherent light on the medium with 284 mg/l of nitrogen was 2.6 and 10.0 times higher, respectively, than when cultivated on a medium with higher nitrogen content. The activity of intracellular catalase during cultivation on a medium with low nitrogen content was 11.1 to 29.4% higher than with higher nitrogen content in all variants of the experiment, including control. It can be assumed that photo-induction of intracellular catalase does not affect the nitrogen metabolism in *I. obliquus*.

Thus, usinge nutrient media with reduced nitrogen content is advisable to enhance the photo-induced stimulating effect in the production of melanin, intracellular peroxidase, and extracellular catalase, tyrosinase, and polyphenol oxidase.

When cultivating *I. obliquus*, the parameters and modes of light treatment of the seed mycelium should be adjusted in accordance with the target bioactive components. Regulating the mechanisms of metabolic pathways of bioactive components with light of different wavelength ranges and coherence should be subject to systematic control depending on specific biotechnological problems.

REFERENCES

1. *Zied D. C., Pardo-Giménez A.* (Eds). Edible and Medicinal Mushrooms: Technology and Applications. *Wiley & Sons Ltd*. 2017, 562 p. https://doi.org/10.1002/9781119149446
2. *Chang S. T., Wasser S. P*. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. *Int. J. Med. Mushrooms*. 2012, 14 (2), 95–134. https://doi.org/10.1615/IntJMedMushr.v14.i2.10
3. *Zheng W., Miao K., Liu Y., Zhao Y., Zhang M., Pan S., Dai Y*. Chemical diversity of biologically active metabolites in the sclerotia of Inonotus obliquus and submerged culture strategies for up-regulating their production. *Appl. Microbiol. Biotechnol.* 2010, 87 (4), 1237–1254. https://doi.org/10.1007/s00253-010-2682-4
4. *Zheng W., Zhao Y., Zheng X., Liu Y., Pan S., Dai Y., Liu F*. Production of antioxidant and antitumor metabolites by submerged cultures of Inonotus obliquus cocultured with Phellinus punctatus. *Appl. Microbiol. Biotechnol.* 2011, 89 (1), 157–167. https://doi.org/10.1007/s00253-010-2846-2
5. *Poyedinok N. L.* Light regulation of growth and melanin formation in Inonotus obliquus (Pers.) Pilat. *Biotechnol. acta*. 2013, 6 (2), 115–120. (In Russia).
6. *Poyedinok N. L., Mykchaylova O. B., Tugay T. I., Tugay A., Negriyko A., Dudka I. A.* Effect of light wavelengths and coherence on growth, enzymes activity and melanin production of liquid cultured *Inonotus obliquus* (Ach.:Pers.) Pilát. *Appl. Biochem. Biotechnol*. 2015, 176 (2), 333–343. https://doi.org/10.1007/s12010-015-1577-3
7. *Friedl M. A., Kubicek C. P., Druzhinina I. S*. Carbon source dependence and photostimulation of conidiation in Hypocrea atroviridis. *Appl. Environ. Microbiol*. 2008, 74 (1), 245–250. https://doi.org/10.1128/AEM.02068-07
8. *Friedl M. A., Schmoll M., Kubicek C. P., Druzhinina I. S*. Photostimulation of Hypocrea atroviridis growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. *Microbiology*. 2008, 154 (4), 1229–1241. https://doi.org/10.1099/mic.0.2007/014175-0
9. *Schuster A., Kubicek C. P., Friedl M. A., Druzhinina I. S., Schmoll M*. Impact of light on Hypocrea jecorina and the multiple cellular roles of Envoy in this process. *BMC genomics*. 2007, 8 (1), 449. https://doi.org/10.1186/1471-2164-8-449
10. *Tisch D., Kubicek C. P., Schmoll M*. The phosducin-like protein PhLP1 impacts regulation of glycoside hydrolases and light response in Trichoderma reesei. *BMC genomics*. 2011, 12 (1), 613. https://doi.org/10.1186/1471-2164-12-613
11. *Tisch D., Schmoll M*. Targets of light signalling in Trichoderma reesei. BMC genomics. 2013, 14 (1), 657. https://doi.org/10.1186/1471-2164-14-657
12. *Tisch D., Kubicek C. P., Schmoll M*. Crossroads between light response and nutrient signalling: ENV1 and PhLP1 act as mutual regulatory pair in Trichoderma reese. BMC genomics. 2014, 15 (1), 425–438. https://doi.org/10.1186/1471-2164-15-425
13. *Schmoll M*. Light, stress, sex and carbon-the photoreceptor ENVOY as a central checkpoint in the physiology of Trichoderma reesei. *Fungal Biol.* 2017, 122 (6), 479–486. https://doi.org/10.1016/j.funbio.2017.10.007
14. *Zhu J. C., Wang X. J*. Effect of blue light on conidiation development and glucoamylase enhancement in Aspergillus niger. *Acta microbiologica Sinica*. 2005, 45 (2), 275–278.
15. *Ricci M., Krappmann D., Russo V. E. A*. Nitrogen and carbon starvation regulate conidia and protoperithecia formation of *Neurospora crassa* grown on solid media. *Fungal. Genet. Newsl*. 1991, 38 (1), 87–88.
16. *Sommer T., Degli-Innocenti F., Russo V. E. A*. Role of nitrogen in the photoinduction of protoperithecia and carotenoids in *Neurospora crassa. Planta*. 1987, 170 (2), 205–208.
17. *Poyedinok N. L., Mykchaylova O. B., Sergiichuk N. N., Negriyko A. M*. Realization of Macromycete Photoinduced Growth Activity: Influence of Cultivation Ways and the Concentration of Carbon and Nitrogen. *Innov. Biosyst. Bioeng.* 2018, 2 (3), 196–202. https://doi.org/10.20535/ibb.2018.2.3.134629. (In Russia).
18. *Mykchaylova O. B., Poyedinok N. L., Buchalo A. S*. Biotechnological aspects of cultivation of species of the genus *Morchella* on liquid nutrient media. *Immunopathology, Allergology, Infectology*. 2009, 2 (1), 166–167. (In Russia).
19. *Puchkova T. A., Kapich A. N., Osadchaya O. V*. Influence of conditions of cultivation for education biologically active substances by mushrooms of the genus *Cordyceps* and their antioxidant activity. *Trudyi BGU*. 2013, 8 (1), 246–252. (In Russia).
20. *Poyedinok N. L., Mykchaylova O. B., Buchalo A. S., Negriyko A. M*. Light regulation of growth and biosynthetic activity of ling zhi or reishi medicinal mushroom, *Ganoderma lucidum* (W. Curt: Fr.) P. Karst. (*Aphyllophoromycetideae*) in pure culture. *Int. J. Med. Mushr*. 2008, 10 (4), 369–378. https://doi.org/10.1615/IntJMedMushr.v10.i4.100

ВПЛИВ КОНЦЕНТРАЦІЇ АЗОТУ НА ФОТОІНДУКОВАНУ РОСТОВУ, ЕНЗИМАТИЧНУ АКТИВНІСТЬ І СИНТЕЗ МЕЛАНІНУ *Inonotus obliquus* (Ach.:Pers.) Pilát

Н. Л. Поєдинок[1], *Т. І. Тугай*[2], *А. В. Тугай*[2], *О. Б. Михайлова*[3], *Н. М. Сергійчук*[4], *А. М. Негрийко*[5]

[1]ДУ «Інститут харчової біотехнології та геноміки НАН України», Київ
[2]Інститут мікробіології і вірусології ім. Д. К. Заболотного НАН України, Київ
[3]Інститут ботаніки ім. М. Г. Холодного НАН України, Київ
[4]Відкритий міжнародний університет розвитку людини «Україна», Київ
[5]Інститут фізики НАН України, Київ

E-mail: n.poyedinok@gmail.com

Метою роботи було вивчити вплив концентрації азоту на фотоіндуцію ростової, ензиматичної активності та синтез меланіну лікувального гриба *Inonotus obliquus* (Ach.: Pers.) Pilát. з Колекції культур шапинкових грибів Інституту ботаніки ім. М. Г. Холодного НАН України. Опромінений світлом низької інтенсивності різної когерентності та в різних діапазонах довжин хвиль міцелій культивували в динамічному режимі на глюкозо-пептонному середовищі з різними концентраціями загального азоту.

Отримані результати дослідження не виявили достовірного впливу концентрації азоту на фотоіндуковану стимуляцію росту *I. obliquus*. Збільшення накопичення біомаси фотоактивованим в різних режимах міцелієм, порівняно з неопроміненим, було практично однаковим у всіх варіантах досліду. Встановлено достовірну залежність фотостимуляції синтезу меланіну від концентрації азоту в середовищі. Зниження концентрації азоту збільшує стимулювальний ефект низькоінтенсивного лазерного випромінювання з довжиною хвилі 488 нм більш ніж у 2 рази. Використання живильних середовищ зі зниженим вмістом джерела азоту є доцільним для підвищення фотоіндукованого стимулювального ефекту за отримання позаклітинної каталази, тирозинази і поліфенолоксидази, внутрішньоклітинної пероксидази.

Таким чином, параметри культивування *I. obliquus* і режими світлового оброблення посівного міцелію слід коригувати відповідно до цільових біологічно активних компонентів.

Ключові слова: *Inonotus obliquus*, фотоіндукція, азот, меланін, каталаза, тирозиназа, поліфенолоксидаза, пероксидаза, ростова активність.

ВЛИЯНИЕ КОНЦЕНТРАЦИИ АЗОТА НА ФОТОИНДУЦИРОВАННУЮ РОСТОВУЮ, ЭНЗИМАТИЧЕСКУЮ АКТИВНОСТЬ И СИНТЕЗ МЕЛАНИНА *Inonotus obliquus* (Ach.:Pers.) Pilát

Н. Л. Поединок[1], *Т. И. Тугай*[2], *А. В. Тугай*[2], *О. Б. Михайлова*[3], *Н. Н. Сергийчук*[4], *А. М. Негрийко*[5]

[1]ГУ «Институт пищевой биотехнологии и геномики НАН Украины», Киев
[2]Институт микробиологии и вирусологии им. Д. К. Заболотного НАН Украины, Киев
[3]Институт ботаники им. Н. Г. Холодного НАН Украины, Киев
[4]Открытый международный университет развития человека «Украина», Киев
[5]Институт физики НАН Украины, Киев

E-mail: n.poyedinok@gmail.com

Целью работы было изучение влияния концентрации азота на фотоиндуцию ростовой, энзиматической активности и синтез меланина лекарственного гриба *Inonotus obliquus* (Ach.: Pers.) Pilát. из Коллекции культур шляпочных грибов Института ботаники им. Н. Г. Холодного НАН Украины. Облученный светом низкой интенсивности разной когерентности и в разных диапазонах длин волн мицелий культивировали в динамическом режиме на глюкозо-пептонной среде с разными концентрациями общего азота.

Полученные результаты исследования не выявили достоверного влияния концентрации азота на фотоиндуцированную стимуляцию роста *I. obliquus*. Увеличение накопления биомассы фотоактивированным в разных режимах мицелием, по сравнению с необлученным, было практически одинаковым во всех вариантах опыта. Установлена достоверная зависимость фотостимуляции синтеза меланина от концентрации азота в среде. Снижение концентрации азота увеличивает стимулирующий эффект низкоинтенсивного лазерного излучения с длиной волны 488 нм более чем в 2 раза. Использование питательных сред с пониженным содержанием азота целесообразно для повышения фотоиндуцированного стимулирующего эффекта при получении внеклеточной каталазы, тирозиназы и полифенолоксидазы, внутриклеточной пероксидазы.

Таким образом, параметры культивирования *I. obliquus* и режимы световой обработки посевного мицелия следует корректировать в соответствии целевыми биологически активными компонентами.

Ключевые слова: *Inonotus obliquus*, фотоиндукция, азот, меланин, каталаза, тирозиназа, полифенолоксидаза, пероксидаза, ростовая активность.

The study was supported by a project funded by the National Academy of Sciences of Ukraine (2019-2021).