УДК 577.127

A. V. Chichvarin[1], T. I. Igumenova[2], N. R. Prokopchuk[3], K. V. Vishnevskiy[3]
[1]Starooskol Technological Institute (branch of the "MISIS")
[2]Voronezh State University of Engineering Technologies
[3]Belarusian State Technological University

SYNTHESIS AND RESEARCH OF BIOLOGICAL ACTIVITY OF ADDUCT OF THE FULLERENES AND INDOLEBUTYRIC ACID MIXTURE

The possibility of synthesis of new growth regulators based on carbon fulleren and a 2-indole-3 of oleic acid and research of influence of the received adducts on plant cells (wheat "Kharkovskaja 39") and mushrooms cells (*Kuehneromyces mutabilis* (sheathed woodtuft), *Lentinula edodes* (shiitake), *Ganoderma lucidum* (lingzhi mushroom), *Flammulina velutipes* (enokitake) is considered in the work. The received products, being in general electroneutral, represent the tsvitter-ions possessing unlimited solubility in water and ethyl alcohol that facilitates their use in microdosages for regulation of plants growth. The conducted experiments indicate high biological activity of the received substance that allows us to consider it as the new effective regulator of growth of complex action.

Key words: fullerene growth regulator, indolebutyric acid, vegetation period, inoculation, mycelium, spectrum.

Introduction. Plant hormones, low molecular weight organic substances produced by plants and having regulatory functions are of great practical interest in modern biological research.

Search and purposeful synthesis of the new drugs of these classes are relevant due to the growing demand for high-quality agricultural products. Creation of supramolecular nanostructures with high biological activity makes it possible to obtain substances and form a class of new drugs. Their biological activity manifests itself in very low dosages, which affects the quality of products and their final cost.

The aim of the research was to synthesize new carbon fullerenes adduct with amino acid and to explore the possibility of its use as a biologically active substance.

Main part. In preliminary experiments, it was noted that many plants grown in solution culture and using mixtures of carbon fullerenes and synthetic growth stimulants, such as indoleacetic acid and indolebutyric acid (IBA), had an increased turgor and a higher chlorophyll content in the aerial part. And it was also impossible not to notice a more intensive development of the colonies of mold fungi. An assumption has been made on the basis of a number of observations that fullerene and auxin derivatives should have regulatory properties.

Synthesis of the new adduct of the carbon fullerenes mixture was performed on the basis of the Prato method [2, 3]. 2-indole-3-butyric acid and a mixture of carbon fullerenes of the C_{50}–C_{92} fraction were used as starting reagents. The reaction was carried out under controlled thermocatalytic conditions at the interface of the solvents used in the synthesis – methylbenzene and dimethyl ketone. The reaction medium temperature change indicated the completion of the process. The resulting product was a viscous red-orange liquid with a characteristic odour.

Synthesis of fullerene C_{60} as an example may be represented as a diagram in Fig. 1.

The resultant product in general is electrically neutral and is a zwitterion having unlimited solubility in water and ethanol, which facilitates its use for regulating the growth of plants and fungi.

The method of frustrated total internal reflectance Fourier infrared spectroscopy (FTIR) was used to study the spectral characteristics of the resulting fullerene adduct. The study was conducted in the IR-Fourier spectrometer NICOLET6700. The selected spectral range was from 400 to 4,000 cm^{-1}. The analyzed product samples obtained on the aluminum substrate by recrystallization in ethyl alcohol were used to record the optical characteristics. IR absorption spectrum is shown in Fig. 2.

Reducing the intensity of the characteristic absorption lines in the region 1,150 and 1,250 cm^{-1} and the peaks shift relative to the fullerene spectrum as well as the appearance of new peaks in the region 3,500, 2950, 1,670, 1,460 and 1,730 cm^{-1}, indicate the proceeding chemical reaction and suggest the structure of the stimulant obtained: 2-ethyl-indole-3-*n*-propylene-3.6:1.2[60]fullerene (Fig. 1) [4, 5]. An interesting feature of the synthesized growth stimulant is a globular structure of the sample obtained following the recrystallization in ethyl alcohol, as shown in Fig. 3.

Reducing the intensity of the characteristic absorption lines in the region 1,150 and 1,250 cm^{-1} and the peaks shift relative to the fullerene spectrum as well as the appearance of new peaks in the region 3,500, 2,950, 1,670, 1,460 and 1,730 cm^{-1}, indicate the proceeding chemical reaction and suggest the structure of the stimulant obtained: 2-ethyl-indole-3-*n*-propylene-3.6:1.2[60]fullerene (Fig. 1) [4, 5].

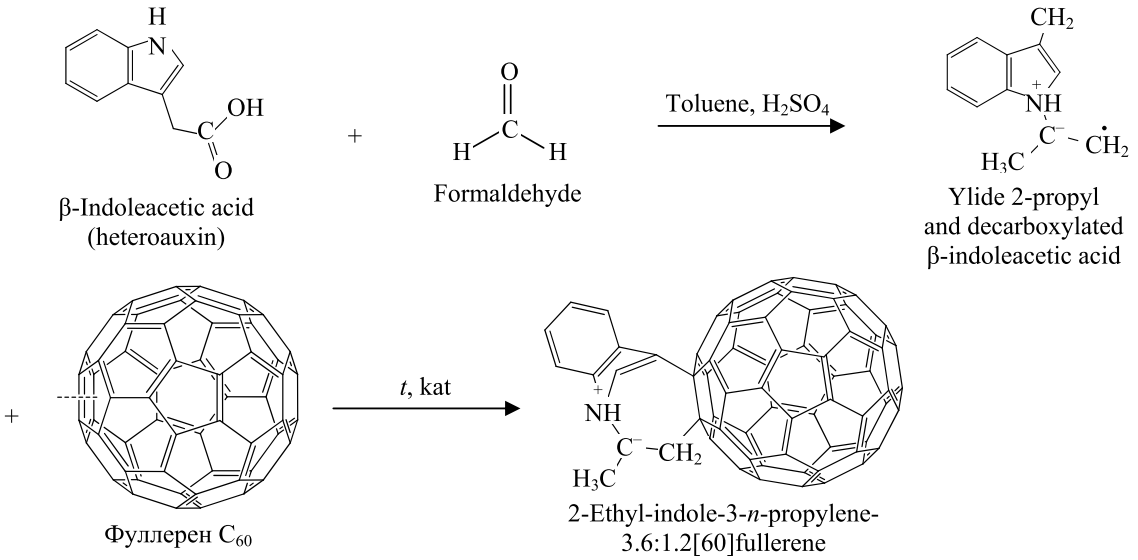


Fig. 1. Adduct synthesis scheme based on the 2-indole-3-butyric acid and fullerenes of the C_{50}–C_{92} fraction

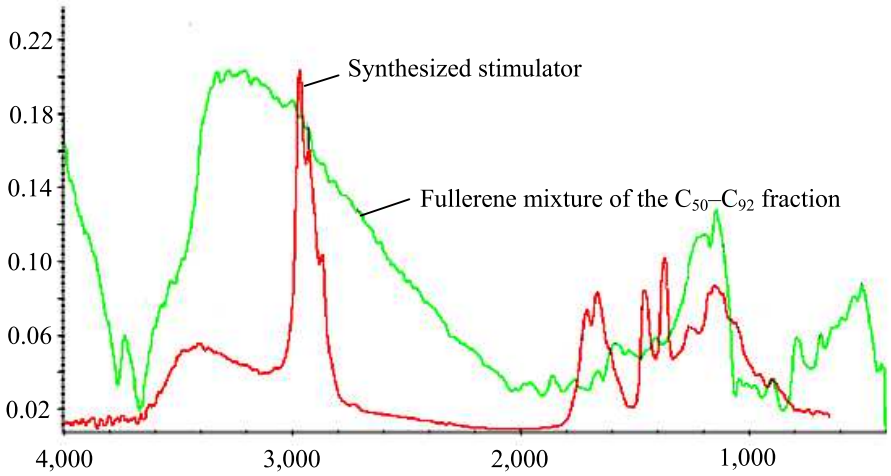


Fig. 2. IR-spectra of the synthesized growth stimulant and fullerene mixture of the C_{50}–C_{92} fraction

An interesting feature of the synthesized growth stimulant is a globular structure of the sample obtained following the recrystallization in ethyl alcohol, as shown in Fig. 3.

Spring durum wheat "Kharkovskaya 39" was chosen as the object of biological research. Plating was performed on the Petri dishes. Effects on morphological and physiological properties of the selected varieties of wheat were compared using the synthesized growth stimulator relative to the control sample, as well as to the starting synthesis components – hydrated fullerene C_{60} and indolebutyric acid mixture.

Wheat seeds germination experiment was carried out in three biological surfaces. The Knopp solution was added in suitable variants with the analyzed stimulant solution (from 0.0001 to 0.00001 wt %), the indolebutyric acid solution (from 0.001 to 0.0001 wt %) and hydrated fullerene solution (from 0.0001 to 0.00001 wt %). The research results are shown in Table 1.

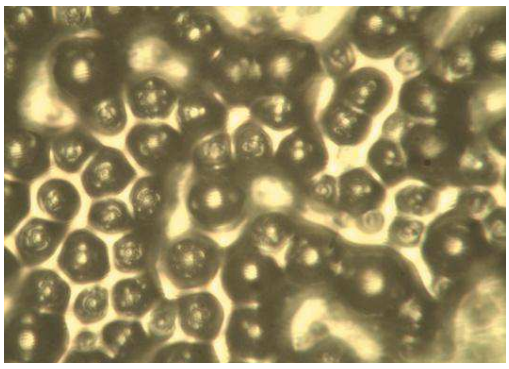


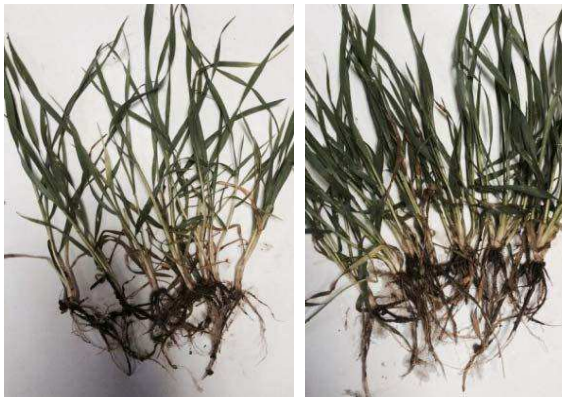
Fig. 3. The structure of the synthesized growth stimulant (increase of 250×)

Table 1

Research results of the synthesized stimulator effect on the morphological properties of wheat "Kharkovskaya 39"

Preparation/concentration, wt %	Wet weight, mg			Dry weight, mg		
	Roots	Sprouts	Total	Roots	Sprouts	Total
Control	333.8	780.7	1114.5	55.0	141.0	196.1
IBA 0.001%	346.0	947.2	1293.2	102.0	248.0	350.1
C60H3 0.0001%	181.0	536.0	717.0	43.2	150.4	193.6
C60H3 0.00001%	237.3	909.6	1146.8	43.8	207.0	250.8
IBA+C60H3 0.0001%	277.3	763.1	1040.4	78.7	169.9	248.5
Stimulator 0.0001%	316.9	529.4	1046.3	87.0	139.8	226.8
Stimulator 0.00001%	381.0	723.6	1104.6	94.3	134.3	228.5

Fig. 4 shows the results of field tests. System analysis of the data obtained shows the effectiveness of the synthesized stimulator in concentrations one order less than indolebutyric acid. A ten-fold increase in the concentration (the standard being 0.00001%) causes organs growth inhibition of the analyzed objects. The obtained data also point to the lack of significant difference between wet and dry weight in percentage terms. A higher tissue water content of the test plants under the influence of the synthesized stimulant and hydrated fullerene was marked. In general, it should be noted that the synthesized product has a greater affect on the length of the aeril part than on the weight and size of the root system. This suggests a heightened osmolytes concentration in plant tissues. Additionally, it should be noted that the root system water content in the presence of the synthesized stimulator is minimum, its length being significantly increased.



a *b*

Fig. 4. Winter wheat growth under the influence of distilled water (*a*) and synthesized fullerene adduct (*b*)

In general, the data analysis presented in Table 1 suggests that the synthesized fullerene adduct in concentrations of 0.00001% by weight exhibits the properties of the auxin type growth stimulator. A very low operating concentration of the analyte can be explained by the fact that the biological effect of 2-ethyl-indole-3-*n*-propylene-3.6:1.2[60]-fullerene is caused by the rapid movement of amino acid residue to the receptors due to a high affinity of the fullerene molecule to the biochemical structures.

In the second part of the experiment as the objects of study we examined the cultivated mushrooms mycelium of the following types: *Ganoderma lucidum* (reishi mushroom), *Kuehneromyces mutabilis* (sheathed woodtuft), *Flammulina velutipes* (enokitake), *Lentinula edodes* (shiitake mushroom).

Mycelium inoculation of the analyzed strains was performed in a test agar medium (Czapek agar), and in the medium added with a test growth stimulator in an amount of 0.00001% by weight. For the preparation of 1 liter of solution the following components were used as the basic Czapek agar composition: 20 g agar, 1,000 ml distilled water, 30 g sucrose, 3 g sodium nitrate, 1 g of potassium dihydrogen phosphate, 0.5 g magnesium sulfate 0.5 g potassium chloride, 0.01 g sulfate of iron [4, 5]. Czapek agar preparation was performed in an autoclave with flowing steam at 120°C for 1 hour. The resulting medium was being filtered, poured into Petri dishes and sterilized at 110°C under a pressure of 0.5 kgf/cm^2 for 20 minutes. Inoculation of sterilized medium was performed in a laminar flow box at a temperature of 24°C. The experimental results are presented in Fig. 5.

These data indicate that the mycelium hyphae *Kuehneromyces mutabilis* and *Ganoderma lucidum* develop more intensely in the experimental variation, while *Lentinula edodes* signals its growth only when administered with the fullerene adduct.

A detailed study of the hyphae of the analyzed strains was performed by visual microscopy using a laboratory microscope Polam P312M, the selected zoom range being 250×–550×. The results of research (using *Flammulina velutipes* and *Lentinula edodes* as examples) are shown in Fig. 6 and 7.

The obtained data indicate that the mycelium hyphae *Kuehneromyces mutabilis* and *Ganoderma lucidum* develop more intensely in the experimental variation, while *Lentinula edodes* signals its growth only when administered with the fullerene adduct.

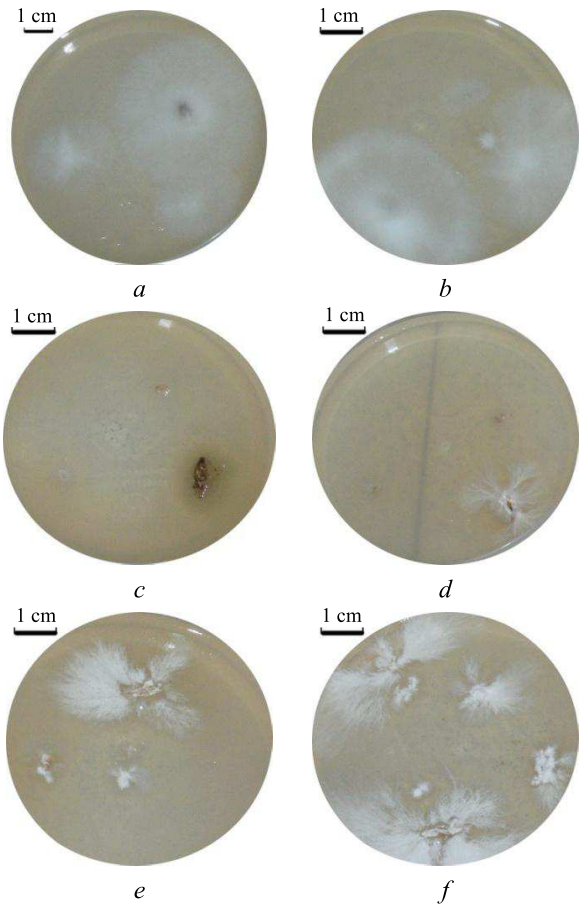


Fig. 5. Cultivated mushrooms mycelium grown in Petri dishes:
a, *b* – *Kuehneromyces mutabilis* (sheathed woodtuft);
c, *d* – *Lentinula edodes* (shiitake mushroom);
e, *f* – *Ganoderma lucidum* (reishi mushroom)

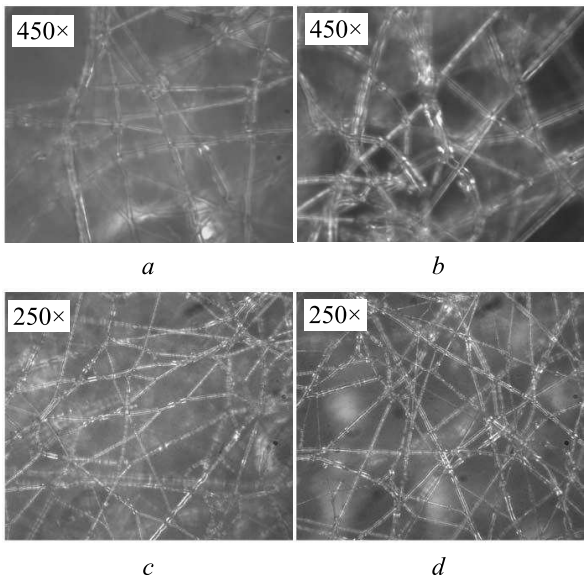


Fig. 6. Mycelium microstructure of *Flammulina velutipes* (enokitake):
a, *b* – control sample;
c, *d* – treated with the fullerene adduct

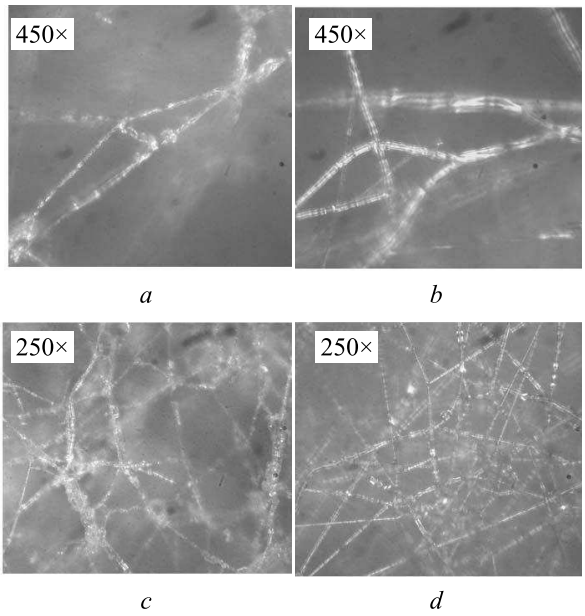


Fig. 7. Mycelium microstructure of *Lentinula edodes* (shiitake mushroom):
a, *b* – control sample;
c, *d* – treated with the fullerene adduct

The research results showed that the density of the *Flammulina velutipes* mycelium hyphae is retained, their thickness increases, the number of branches and the degree of septic treatment increase slightly and there appears gloss. Mycelium surface microstructure of *Lentinula edodes* indicates significantly increased mycelium hyphae density due to the increase of their thickness and the degree of septic treatment. We can also observe the appearance of shine and borders of individual cells. The number of branches and the number of cells per unit length is substantially increased. These trends lead eventually to an increase in the frequency of individual hyphae crossing and formation of diploid cells, which in turn leads to an increase in yield.

Fruiting bodies of oyster mushroom (hiratake) were treated with 0.00001% aqueous solution of the synthesized stimulant. Primary mycelium treatment before plating into substrate blocks results in the mycelium density increase as well as in a significant increase in the number of primordia, which in turn leads to an increase in yield. A general tendency to accelerate the mycelium growth is also marked due to the increase of mycelium temperature and, consequently, to the reduction of the growing season of an oyster mushroom at average of 4 days. The data are presented in Table 2. In addition, 2-ethylindole-3-*n*-propylene-3.6:1.2C[60]fullerene exhibits regulatory nature, increasing resistance to trichoderma.

Table 2

Indexes of the oyster mushroom growing season

Vegetation index	Test experiment	Vegetation index
Substrate type	Durum wheat	
Concentration of the administered preparation, % aqueous solution	0	0.00015
Increase in the number of premordia relative to a test experiment, %	–	44.2
Increase in yield relative to a test, %:		
– the first wave	–	21.3
– the second wave	–	11.7
Mycelium temperature, °C:		
– on the 14th day	34	38
– on the 21st day	33	39
Resistance to infection by trichoderma	Average	High
Production cycle, 24-hour period:		
– the first wave	34	31
– the second wave	67	63

Analysis of extracts from the fruiting bodies of oyster mushroom by spectral analysis showed the absence of the stimulant components, such as fullerenes.

It also revealed a slight increase of selenium content, which indicates the environmental safety of the products obtained. On the basis of a laboratory method in Petri dishes and a pot-culture method in the greenhouse, it was noted that Trichoderma has reduced its ability to inhibit the growth and development of the oyster mushroom.

Conclusion. The results obtained allow to recommend a synthesized carbon fullerenes adduct as a new growth promoter to a broader testing for subsequent industrial applications. In particular, the new product can be used to solve the problem of the spring wheat plantings resistance to a spring drought. It can also be used as a component of nutrient media, in particular for poorly cultivated and perspective mushrooms and for the primary mycelium inoculation.

References

1. Shevelukha V. S., Kalashnikova E. A., Kochieva E. Z. *Sel'skohozyaystvennaya biotekhnologiya* [Agricultural biotechnology]. Moscow, Vysshaya shkola Publ., 2008. 710 p.

2. Maggini M., Scorrano G., Prato M. Addition of azomethine ylides to C60: synthesis, characterization, and functionalization of fullerenepyrrolidines. *J. Am. Chem. Soc.*, 1993, vol. 115 (21), pp. 9798–9799.

3. Troshin P. A., Lyubovskaya R. N. Organic chemistry of fullerenes: the basic reaction types of fullerene compounds and perspectives of their practical use. *Uspekhi khimii* [Chemistry success], 2008, no. 4, pp. 323–369 (in Russian).

4. Cherepanova N. P. *Morfologiya i razmnozheniye gribov* [Morphology and reproduction of mushrooms]. Leningrad, Izdatel'stvo Leningradskogo Universiteta Publ., 1981. 120 p.

5. Dudka I. A., Vasser S. P. *Griby. Spravochnik mikologa i gribnika* [Mushrooms. Directory mycologist and mushroom picker]. Kiev, Naukova dumka Publ., 1987. 535 p.

Information about the authors

Chichvarin Aleksandr Valer'evich − Ph. D. Chemistry, associate professor, Department of Chemistry. Starooskol Technological Institute (branch of the National University of Science and Technology "MISIS") (42, microdistrict Makarenko, 309516, Stary Oskol, Russian Federation).

Igumenova Tat'yana Ivanovna – Ph. D. Engineering, associate professor, Department of Chemistry and Chemical Engineering Organic Compound and Polymer Processing. Voronezh State University of Engineering Technologies (19, Revolution Ave., 394036, Voronezh, Russian Federation).

Prokopchuk Nikolaj Romanovich – Corresponding Member of the National Academy of Sciences of Belarus, D. Sc. Chemistry, professor, Head of the Department of Technology of Petrochemical Synthesis and Polymer Materials Processing. Belarusian State Technological University (13a, Sverdlova str., 220006, Minsk, Republic of Belarus). E-mail: tnsippm@belstu.by

Vishnevskiy Konstantin Viktorovich – Ph. D. Engineering, senior lecturer, Department of Technology of Petrochemical Synthesis and Polymer Materials Processing. Belarusian State Technological University (13a, Sverdlova str., 220006, Minsk, Republic of Belarus). E-mail: vik@belstu.by

Received 19.02.2015