





Research Article

Bioactivity Study of the C₆₀-L-Threonine Derivative for Potential Application in Agriculture

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The present paper reports data on the biological activity of nanocompositions based on a C₆₀-L-threonine (C₆₀-Thr) derivative. These nanocompositions promote the nonspecific resistance of plants to the action of stress factors (ultraviolet radiation, pesticides, and phytopathogens). Additionally, we determined the perspectives of the C₆₀-Thr adduct application in the cultivation of plants due to the decrease of the pesticide load on the environment. The biological study of C₆₀-Thr revealed the plant growth-stimulating ability due to its influence on the photosynthetic apparatus activity and antioxidant properties.

1. Introduction

The discovery of new carbon allotropes, fullerenes, was made in 1985 and showed new promising prospects in the nanotechnology field. The combination of unique physical and chemical properties of these spherical molecules has been exploited for a wide range of applications. However, the low solubility of fullerenes in polar solvents hindered the use of these promising nanocarbon materials. This problem has been overcome by the synthesis of novel fullerene derivatives. The functionalization of the fullerene core with a variety of chemical groups allowed for the application of these three-dimensional molecules for practical purposes, such as medicine, drug delivery, optoelectronics, electrochemistry, and organic photovoltaics. The present paper is devoted to the biological study of nanocompositions containing the C₆₀-L-threonine derivative. As a result, we developed

C₆₀-L-threonine-containing nanocompositions that promote the nonspecific resistance of plants to the action of stress factors (deficiency of soil moisture and phytopathogens). Additionally, we determined the perspectives of the C₆₀-L-threonine application in the cultivation of plants due to the decrease of the pesticide load on the environment.

Richard Smalley, Robert Curl, and Harold Kroto, who were awarded the 1996 Nobel Prize in chemistry for the buckminsterfullerene (C₆₀) discovery, opened the era of nanoscience and nanotechnology in 1985. Since its discovery, fullerenes have been used in different fields of technology [1–9]. Various fullerene derivatives, their noncovalent interactions, and various physicochemical properties are being actively studied now [8, 10–18]. However, the low solubility of fullerenes in water and water solutions limits their wide application and commercial use. The synthesis of fullerene derivatives has improved miscibility of fullerenes in water,

which showed to have promising results in fields from medicine to engineering [19–28]. The solubility of fullerene derivatives in water allows their application in pharmacology as antioxidants, which protect cells from being attacked by reactive oxygen species (ROS) [29–31].

In particular, the use of fullerenes with amino acids has great relevance for application in the biosciences and medicine. In spite of the importance, there are just a few publications devoted to this subject [32–52]. The existing publications are emphasized for the following research directions:

- (i) Synthesis and biological studies of water-soluble fullerene derivatives [34–39, 45, 46, 52, 53]. Fullerene derivatives with amino acids and peptides could be used for (i) activated transmembrane transport of bivalent metal ions [38], (ii) prevention of oxidative stress-induced cell death without evident toxicity [35], (iii) high DNA cleavage efficiency upon visible light irradiation in the presence of the coenzyme NADH [39], (iv) development of potent inhibitors of cytomegalovirus infection [53], and (v) design of HIV-protease inhibitors [45]. Additionally, they possess anticancer and antioxidant activities [34, 37, 46]
- (ii) Theoretical studies of fullerene derivatives with amino acids. In particular, this group of articles is devoted to the ability of the C_{60} fullerene to interact with amino acids [40], calculation of dissociation constants [41], and molecular structure of hybrid derivatives based on C_{60} and amino acids [33], as well as to the investigation of physicochemical properties of water-soluble fullerene derivatives (investigation of pH, degree of association [33, 47–51], solubility in water, density, specific conductivity, molar conductivity, dissociation constant, activity coefficients, and size distribution) [42–44]. The ability of water-soluble fullerene derivatives to penetrate through the cell membranes (due to lipophilicity and nanosize), to deliver macro- and microelements and to have antioxidant activity, make it interesting to research their influence on the plant cultures. Nowadays, it is essential to find new forms of biologically active nanocompositions for the effective regulation of the plant state and the increase of plant resistance to environmental stress factors [54–56]. Until now, the mechanism of effect of water-soluble fullerene derivatives on plants in agroecosystems remains unclear. Through different research groups, the controversial effects of water-soluble fullerene derivatives on plant growth were established [57–60]. For example, the authors of [61] determined that the polyhydroxylated fullerene $C_{60}(OH)_{20}$ damaged onion cells and, at the same time, promoted the growth of the hypocotyl in *Arabidopsis* and increased the density of the green alga *Pseudokirchnerella subcapitata* [58]. The treatment of bitter melon seeds with a solution of the polyhydroxylated fullerene $C_{60}(OH)_{20}$ led

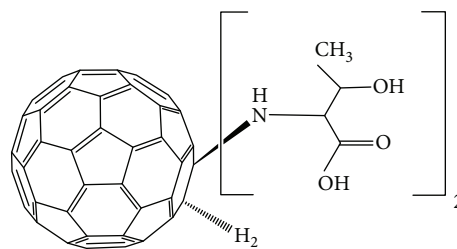


FIGURE 1: Adduct of the C_{60} fullerene with L-threonine.

to an increase of plant biomass by 54%, yield by 128%, and nutrient content by 90% [59]. The authors of [16, 47, 62] concluded that the positive effect of water-soluble fullerene derivatives on plants is presumably associated with the inhibition of reactive oxygen species (ROS) production. Absorption, translocation, and accumulation of carbon nanomaterials (fullerenes and fullerene derivatives and carbon nanotubes) have been evaluated for the following plants: rice, radish, onion, bitter melon, and wheat [9, 57, 59, 61, 63, 64].

As an example, authors of [63–65] showed that the uptake of C_{60} and fullereneol $C_{60}(OH)_{20}$ by wheat germs (*Triticum aestivum* L.) and radish (*Raphanus sativus* L.) depends on their concentration in the root zone, and these compounds were accumulated mainly in the roots. Growth-inhibitory effects of another water-soluble derivative, the carboxyfullerene $C_{70}(C(COOH)_2)_{2-4}$, have been reported in tobacco (*N. tabacum*) cell cultures, showing cell wall deformations and induction of ROS production [66]. The abovementioned observations reveal that the type of fullerene core functionalization is an important determinant of the biological effects of nanocarbon materials on plants. At the present time, there are no data regarding the possible mechanisms of water-soluble derivatives' influence on plant physiology.

For the first time, we have shown that the root zone and foliar treatment of plants (*Hordeum vulgare* L.) by the amino acid derivative of C_{60} with L-threonine (C_{60} -Thr) lead to increase in the net productivity and resistance to oxidative stress. The latter fact is apparently associated with the increase of the photosynthetic apparatus functional activity, as well as with the influence of the C_{60} -Thr derivative on the plant antioxidant systems (lipid peroxidation, the activity of superoxide dismutase, and generation of ROS).

The present paper is devoted to the in situ biological effect of the C_{60} -Thr derivative (Figure 1)— $C_{60}(C_4H_9NO_3)_2$ —on the state of plants.

2. Materials and Methods

2.1. Materials. The amino acid derivative of fullerene $C_{60}(C_4H_9NO_3)_2$ of mass fraction purity 99.8% was produced by ZAO "ILIP" Ltd. (Saint Petersburg, Russia) [67]. The elemental analysis of the commercial sample (C, H, and N) was carried out on a Euro EA3028-HT instrument. The

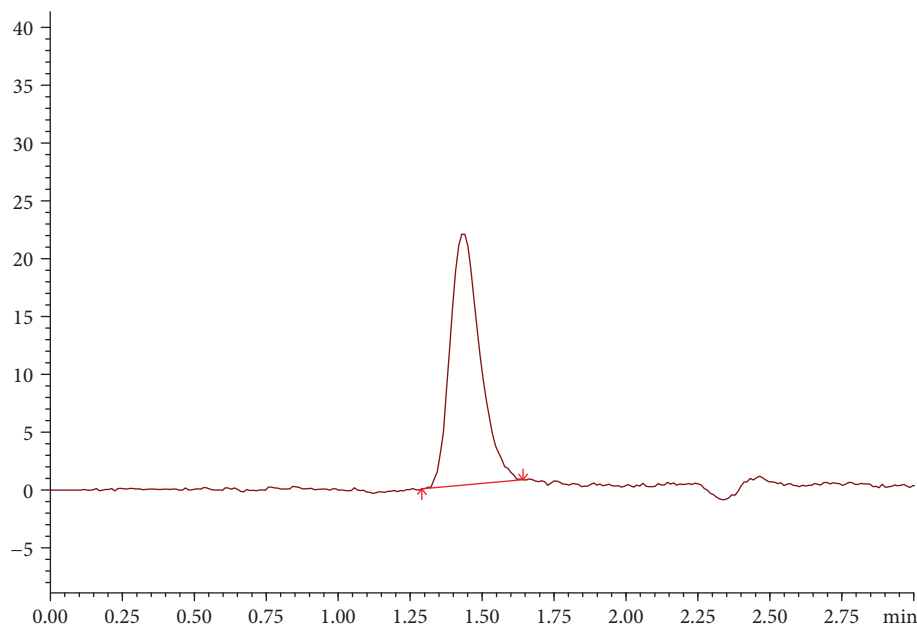


FIGURE 2: HPLC chromatogram of the C_{60} -Thr water solution.

results of the elemental analysis are as follows: C, 78.01; H, 3.84; and N, 5.03%. The obtained experimental data are in good agreement with the calculated values: C, 78.02; H, 3.81; and N, 5.01%.

The HPLC analysis of the C_{60} -Thr derivative was performed using a Shimadzu LC-20 Prominence apparatus with UV detection at 300 nm equipped with a Phenomenex® NH_2 (150 mm \times 2.0 mm, 5 μ m, 100 Å) column; injection volume was equal to 20 μ l, injection speed of 0.2 ml·min⁻¹, and eluent acetonitrile/0.1% water solution of acetic acid (5/95). The chromatogram of the C_{60} -Thr derivative reveals only one peak without any admixture (see Figure 2).

The sample was used without further purification. Additionally, we have not performed separation of positional isomers. Previously, we performed the physicochemical investigation of the C_{60} -Thr aqueous solutions (temperature dependence of solubility and viscosity and concentration dependence of density, specific conductivity, molar conductivity, dissociation constant, activity coefficients, and associate distribution in aqueous solutions) [67]. The relevance of a physicochemical investigation of the water-soluble amino acid derivatives of fullerenes is connected with the development of their application in nanobiomedicine and agriculture.

The absorbance spectrum of C_{60} solutions in *o*-xylene (with a concentration of $C = 10^{-5}$ M) relative to *o*-xylene and C_{60} -Thr in water (with a concentration of $C = 10^{-5}$ M) relative to water in the wavelength range of 200–500 nm was measured on a UV-1800 spectrophotometer under standard conditions in quartz cuvettes with an optical path length of 1 cm. The absorbance spectrum (Figure 3) of individual fullerene C_{60} consists of a maximum at a wavelength of 335 nm and agrees well with the literature data [68]. In turn, the electron spectrum of C_{60} -Thr does not

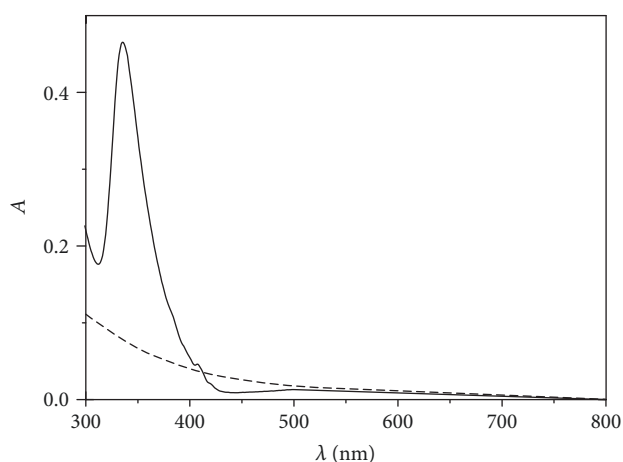


FIGURE 3: Absorbance spectrum of C_{60} solution in *o*-xylene ($C = 10^{-5}$ M) relative to *o*-xylene (solid line) and a solution of C_{60} -Thr ($C = 10^{-5}$ M) in water relative to water (dashed line).

contain an absorption maximum, which also agrees well with the literature data obtained for polyhydroxylated and carboxylated fullerene derivatives [69–71], as well as derivatives with amino acids [37]. Figure 4 shows the luminescence spectrum of an aqueous C_{60} -Thr solution obtained with a FluoroMax-4p. For comparison, Figure S1 presents the luminescence spectrum of a C_{60} solution in *o*-xylene. The decrease in fluorescence intensity of C_{60} -Thr compared to the individual fullerene is associated with oxygen quenching contained in water.

The Zantar fungicide consists of the following active substances: bixafen (N-(3,4-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide), 50 g·l⁻¹,

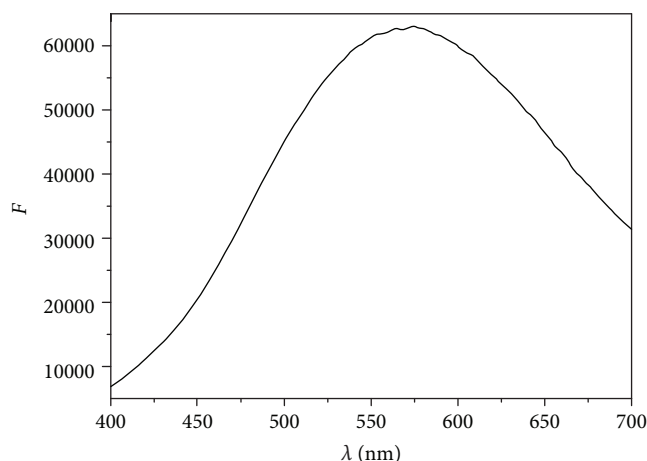


FIGURE 4: The emission spectrum of the C_{60} -Thr derivative solution ($C = 10^{-5}$ M) in water at a wavelength of 340 nm.

and tebuconazole (3-1-(4-chlorophenyl)-4,4-dimethyl-3-[1H-1,2,4-triazol-1-yl-methyl]pentan-3-ol), $166 \text{ g}\cdot\text{l}^{-1}$.

2.2. Biological Effect of the C_{60} -Thr Derivative on Plants. The estimation of biological activity of the C_{60} -Thr derivative has been performed by the methods previously described in ref. [72]. The experimental studies included determination of concentration ranges with positive, neutral, and inhibiting effects on the germination of Ajur variety cress salad test-culture seeds (*Lepidium sativum* L.). In addition, the lengths of roots and the sprout part were measured within 7 days after the seed soaking. After 3 days, we have determined the seed germination energy, and after 7 days, we determined the germination capacity of seeds and performed measurements on root and sprout lengths. Such an investigation was carried out according to International Seed Testing Association (ISTA) rules and generally accepted methods [73]. All experiments were repeated in triplicates.

The C_{60} -Thr plant growth-stimulating ability, influence on the photosynthetic apparatus function in leaves, and antioxidant properties were determined in two vegetation experiments carried out in a controlled environment with the spring barley variety Leningradsky (*Hordeum vulgare* L.). The plant growth was performed using the aeration tanks with a hydroponic solution and artificial illumination [74]. The C_{60} -Thr concentration in the hydroponic solution containing micro- and macroelements was equal to $C = 1 \text{ mg}\cdot\text{l}^{-1}$. The plants grown using the nutrient solution without C_{60} -Thr in the pH range 6.2-6.9 were used as a control. Changing of solutions, as well as pH monitoring, was performed every 3 days. The experiments were carried out under the following conditions: the duration of light period was equal to 14 hours, the temperature was maintained within $25 \pm 2^\circ\text{C}$, and the relative humidity was equal to $65 \pm 5\%$.

To estimate the physiological state of plants, the spectroscopic method based on the reflection from the leaf surface was used. The reflective spectra were registered in situ using the fiber-optic spectroradiometric system Ocean Optics (USA) with an optical resolution of 0.065 nm. For the

spectroscopic investigation, well-formed and established leaves were used. All spectra were recorded for no fewer than 10 plants and repeated in duplicates. The reflective indexes [75, 76] chlorophyll (ChlRI), and anthocyanin (ARI) indexes were calculated according to the following equations:

$$\text{ChlRI} = \frac{(R_{750} - R_{705})}{(R_{750} + R_{705} - 2R_{445})}, \quad (1)$$

where ChlRI is the chlorophyll reflection index and R_{750} , R_{705} , and R_{445} are reflections at radiation wavelengths of 750 nm, 705 nm, and 445 nm, respectively [77],

$$\text{ARI} = R_{750} \left(\frac{1}{R_{550}} - \frac{1}{R_{700}} \right), \quad (2)$$

where ARI is the anthocyanin reflection index and R_{750} , R_{700} , and R_{550} are reflections at radiation wavelengths of 750 nm, 700 nm, and 550 nm, respectively [78].

ChlRI and ARI indexes are in accordance with the net productivity of plants, and they allow estimation of their physiological state [75, 76].

The assimilated surface area of leaves was calculated according to

$$S = \frac{P \cdot S_1 \cdot n}{P_1}, \quad (3)$$

where S is the assimilated surface area of leafage, S_1 is the surface area of one segment of a leaf, n is the number of a segment, P is the total mass of leaves, and P_1 is the mass of a leaf segment.

The estimation of the C_{60} -Thr ability to influence the antioxidant system of plants (i.e., intensity of lipid peroxidation, the total content of ROS, and superoxide dismutase (SOD) activity) was performed according to the methods described in references [65, 79, 80]. The intensity of lipid peroxidation in barley leaves was determined by the measurement of malondialdehyde-thiobarbituric acid adducts [79]. ROS content was evaluated according to the method based on the conversion of adrenalin to adrenochrome with a further spectrophotometric analysis of adrenochrome concentration at 480 nm [65]. The rate of superoxide radical formation was calculated using

$$\bar{V} = \frac{\Delta D}{t_1}, \quad (4)$$

where ΔD is the difference between the optical densities of homogenate-containing adrenalin and water solution of homogenate and t_1 is the incubation time (min) evaluated in relative units ($1 \text{ rel.un.} = 10^{-3} \text{ optical units}\cdot\text{min}^{-1}$).

To image ROS production in the spring barley roots under favorable conditions and irradiation with UV-B radiation, a fluorescent oxidant-sensing dye dihydrodichlorofluorescein diacetate was used. The spring barley roots were incubated with $50 \mu\text{M}$ dihydrodichlorofluorescein diacetate solution for 1 min and rinsed with water.

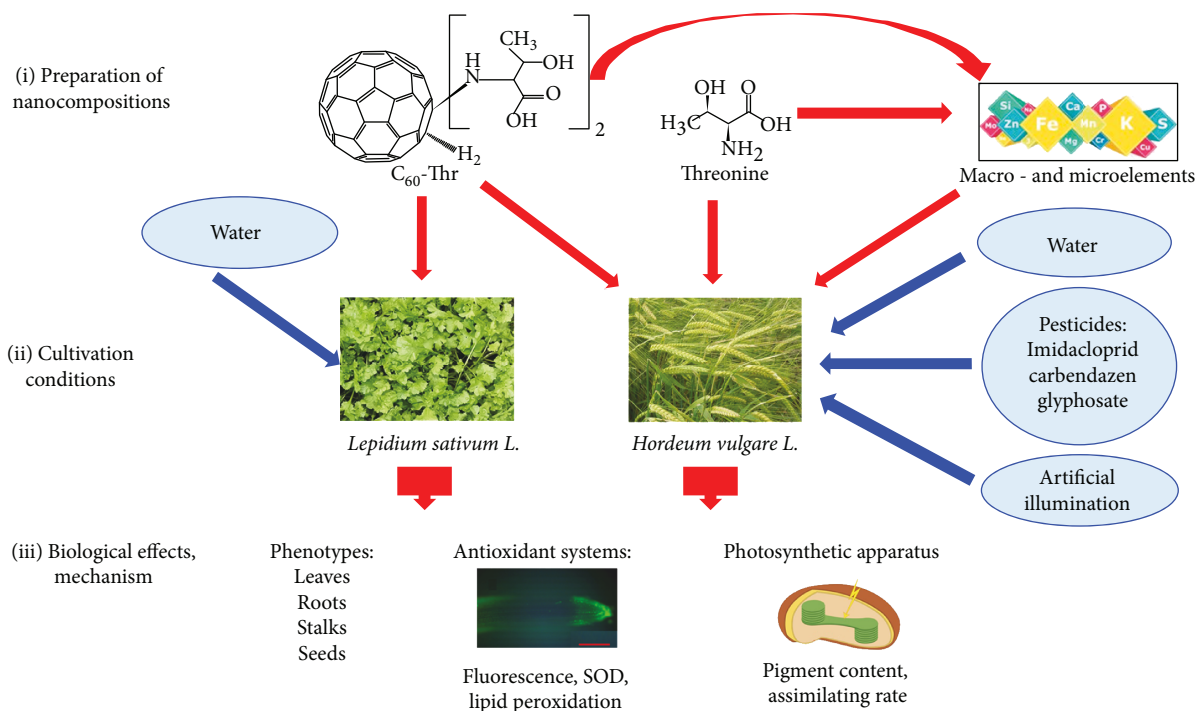


FIGURE 5: Experimental stages involving the preparation of nanocomposition, cultivation, biological effect evaluation, and measurements of biochemical parameters.

The images were acquired using a fluorescent microscope (excitation filter BP 450-490, emission filter LP 520) Axiostar plus (ZEISS, Jena, Germany) equipped with a video camera (SONY DXC-950P, Sony, Tokyo) [60].

SOD activity was measured by a method based on the ability of SOD to compete with nitroblue tetrazolium dye for superoxide radicals [80].

After finishing off the vegetation experiments, the plant growth biometrics were measured: the length of roots and aboveground parts of the plant, the number of culms and leaves, and the total mass of the plants, as well as the masses of the plant organs (culms, leaves, and roots). The root length values correspond to the length of the longest root and the length of the aboveground parts correspond to the highest plant shoots.

Evaluation of the foliar C_{60} -Thr derivative treatment effect on the leaf disease development caused by the *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur (dark brown spot disease), *Pyrenophora teres* Drechsler (barley net blotch), *Pyrenophora graminea* (S. Ito & Kurib.) Anamorph Drechslera graminea (Rabenh.) Shoemaker (striped spot disease), *Blumeria graminis* (DC) Speer f. sp. hordei Marchal (powdery mildew), *Rhynchosporium secalis* (Oudem.) J. J. Davis (rhynchosporium), *Puccinia hordei* G.H. Otth. (dwarf rust), and *Puccinia graminis* Pers. f. sp. hordei (Erikss. et Henn.) (stem rust) phytopathogens was conducted in a field as a part of a small-scale experiment (the Russian Federation, Leningrad Region, Men'kovo village; an experimental field of the Agrophysical Institute) on podzolic and sod-podzolic soils of the Taiga forest region (northwest, a region of cultivation) in two varieties of barley: Leningradsky and Ataman, which differ in resistance to the main phytopathogens. The

leaves of the barley variety Leningradsky refer to the group that is medium-resistant to helminthosporium patches, while the barley variety Ataman is susceptible. In addition, they differ significantly in the duration of the vegetative period, morphological features, and intended purpose.

The barley Leningradsky is an ultrapremature ripening, yielding variety referred to as the pallidum subvariety. These plants are flexible and resistant to lodging straw with a 70-90 cm height and are characterized by cold resistance at the first stages of development, acid resistance, drought resistance, high productive stooling (2-4 stems), leveled plant stand, even germination, resistance to strewing, grain germination on the root, ear fragility, technological effectiveness in the cultivation and refinement of grains, and persistence to pathogens of the mesh and dark brown leaf spot. In addition, the variety effectively uses spring soil moisture because of active growth in the period of sprouting—tillering.

The Belarusian selection barley variety Ataman is medium late with the growing season from 79 to 98 days. The barley variety Ataman is characterized by a medium size with a stem height of 70-75 cm, high tillering coefficient, moderate susceptibility to helminthosporium and mesh patchy lesions, and strong susceptibility to the barley smut. The barley variety Ataman ear is two-rowed, medium length, well-grained, cylindrical, and of medium density, with a weak waxy coating. In addition, the grain is large, semilong, yellow-gray, and lined.

Foliar treatment of barley plants with C_{60} -Thr derivative nanocompounds was carried out using the Solo hand sprayer during the plant development phase: tillering, tube exit, and stalking. The foliar treatment by Zantar fungicide was performed during the stalking period (BBCH 39). The flow rate

TABLE 1: The effect of C₆₀-Thr water solutions on morphological and physiological characteristics of seeds of cress *Lepidium sativum* L.

C ₆₀ -Thr concentration (mg·l ⁻¹)	Germination energy		Germination capacity		Shoot length		Root length	
	%	% to control	%	% to control	cm	% to control	cm	% to control
0 (water)	61	100	70	100	3.9 ± 0.3	100	5.5 ± 0.5	100
0.001	58	95	72	103	4.1 ± 0.5	105	5.4 ± 0.5	98
0.01	46*	75*	69	99	4.0 ± 0.4	103	5.5 ± 0.7	100
0.1	36*	59*	67	96	4.4 ± 0.6	113	5.6 ± 0.8	102
1	35*	57*	69	99	4.6 ± 0.4*	118*	5.9 ± 0.7	107
10	39*	64*	74	106	4.7 ± 0.5*	121*	6.4 ± 0.6	116
25	39*	64*	74	106	4.7 ± 0.3*	121*	6.2 ± 0.7	113
50	30*	49*	66	94	4.4 ± 0.3	113	6.0 ± 0.6	109
100	27*	44*	62	89	3.9 ± 0.3	100	5.6 ± 0.5	102
500	22*	36*	52*	74*	3.0 ± 0.4*	77*	4.0 ± 0.6*	73*

* $p < 0.05$ versus the corresponding value for control.

of the working fluid of the fungicide or nanocompounds corresponded to 300 l·ha⁻¹.

The registration of harmful objects on the barley leaves was carried out according to the method presented in Ref. [15]. During the vegetation period, the following parameters of the plants were determined:

- (i) *R*: disease onset reflecting the average level of injury of plot or agricultural substation according to

$$R = \frac{\sum(a \cdot b)}{N}, \quad (5)$$

where *R* (%) is the disease development, $\sum(a \cdot b)$ is a sum of the product of diseased plants (*a*) by the corresponding percent of injury (*b*), and *N* is the total quantity of plants (healthy and injured)

- (ii) *BE*: biological effectiveness (%) of the plant foliar treatment by nanocompositions and fungicide calculated on the basis of

$$BE = \frac{P_c - P_{\text{exp}}}{P_c} \cdot 100, \quad (6)$$

where *P_c* indicates the injury development of the plant in control conditions and *P_{exp}* indicates the injury development of the plant under fungicide treatment

Wilcoxon rank test was used for statistical analysis. Differences between mean values were considered significant when $p < 0.05$. All calculations were performed in STATISTICA 8 software. The overview of the experimental stages including the preparation of nanocompositions, cultivation conditions, biological effect evaluation, and measurements of biochemical parameters is presented in Figure 5.

3. Results and Discussion

3.1. The Effect of C₆₀-Thr Water Solutions on Morphological and Physiological Characteristics of Seeds. The results of the biological investigation performed using the cress salad (*Lepidium sativum* L.) and spring barley (*Hordeum vulgare* L.) reveal that the C₆₀-Thr derivative has significant biological activity. The treatment of seeds of the cress salad (*Lepidium sativum* L.) by water solutions of C₆₀-Thr leads to the following effects (see Table 1):

- (i) The values of germination energy determined after 3 days of seeds steeping in water solutions of C₆₀-Thr showed retarding of seed germination in the concentration range of $C = 0.01\text{--}500$ mg·l⁻¹. Additionally, in the concentration range of $C = 0.01\text{--}100$ mg·l⁻¹, the effect of retarding of seed germination was temporary, and after 6 days, the germinating capacity characteristics were the same as for the control experiments
- (ii) In the concentration range of $C = 1\text{--}25$ mg·l⁻¹, the positive influence of the C₆₀-Thr derivative on plant growth during the early stages of ontogeny was determined; in the concentration range up to $C = 1$ mg·l⁻¹, we have not observed any noticeable effects of C₆₀-Thr on plant growth; treatment of plants by C₆₀-Thr solutions with concentrations equal to $C = 100\text{--}500$ mg·l⁻¹ leads to a decrease of germinating capacity and lengths of roots or sprouts (see Table 1). For carrying out further vegetation experiments (for introducing C₆₀-Thr to the plant root zone), we used the water solutions of C₆₀-Thr with the lowest concentration equal to $C = 1$ mg·l⁻¹, which had a significant positive effect on the plant

3.2. The Effect of C₆₀-Thr as a Part of the Nutrient Solution on Physiological Parameters and Net Productivity. Analysis of Table 2 shows that addition of C₆₀-Thr solution ($C = 1$ mg·l⁻¹) to the aerated root-inhabited medium of spring

TABLE 2: The effect of C₆₀-Thr** as a part of the nutrient solution on physiological parameters and net productivity of spring barley *Hordeum vulgare L.* (results from two vegetation experiments).

Experimental conditions	Parameters						
	Index of pigment content (relative units)		Area of assimilating leaf surface (cm ²)	Dry mass (g)			
	ChlRI	ARI		Roots	Stalks	Leaves	Total
Control (absolute values)	0.41–0.45	0.55–0.72	168–374	0.10–0.19	0.24–0.25	0.35–0.41	0.69–0.85
C ₆₀ -Thr (absolute values)	0.48*–0.52*	0.41*–0.55*	212*–424*	0.20*–0.33*	0.41*–0.42*	0.48*–0.51*	1.06*–1.26*
C ₆₀ -Thr, deviation to control (%)	+16*–+17*	-25*–-24*	+13*–+26*	+74*–+100*	+68*–+71*	+24*–+37*	+48*–+54*
Thr (absolute values)	0.47*–0.49	0.51–0.57*	154–209*	0.19*–0.24*	0.25–0.29*	0.36–0.42	0.84*–0.92
Thr, deviation to control (%)	+9–+15*	-21*–-7	-44*–-8	+26*–+90*	+4–+16*	+2–+3	+8–+22*

* $p < 0.05$ versus the corresponding value for control; **the average parameters are presented.

TABLE 3: The effect of C₆₀-Thr as a part of the nutrient solution on parameters of the antioxidant system of spring barley *Hordeum vulgare L.* under artificial illumination.

Parameters	Leaves			Roots		
	Control	C ₆₀ -Thr		Control	C ₆₀ -Thr	
	(absolute values)	Absolut values	Deviation to control (%)	(absolute values)	Absolute values	Deviation to control (%)
Lipid peroxidation ($\mu\text{M}\cdot\text{g}^{-1}$)	6.50	5.20*	-20*	6.90	6.40	-7
SOD (relative units)	1.07	1.08	+1	1.08	1.26*	+17*
ROS (relative units)	3.33	6.23*	+87*	0.33	1.03*	+212*

* $p < 0.05$ versus the corresponding value for the control.

barley (*Hordeum vulgare L.*) under hydroponic conditions leads to the increase of leaf surface area and chlorophyll content. Thus, the addition of C₆₀-Thr promotes the increase of the photosynthetic apparatus activity and the accumulation of dry matter in the plant. Moreover, the presence of C₆₀-Thr water solution in the barley root-inhabited medium leads to a decrease of the anthocyanin index and an increase of plant growth parameters. The latter fact indirectly confirms the improvement of the physiological state of the plants (Table 2). In addition, the presence of C₆₀-Thr in nutrient solution leads to the increase of total plant biomass by 48–54%, in particular, due to the rise of the mass of leaves, stems, and roots. We can conclude that, in comparison with pure L-threonine, the C₆₀-Thr derivative has a more pronounced influence on the physiological state of barley plants.

3.3. The Effect of C₆₀-Thr on Parameters of the Plant Antioxidant System and on Plant Resistance to Stresses. Table 3 shows that the presence of C₆₀-Thr in nutrient solution affects plant antioxidant system activity. In particular, the level of lipid peroxidation in barley leaves decreased by 20%, the SOD activity did not change, and ROS generation increased by 87%. At the same time, the level of lipid peroxidation in barley roots did not change, the SOD activity slightly increased (by 17%), and ROS generation significantly increased (212%). Additionally, we performed an *in vitro* investigation of the antioxidant properties of the C₆₀-Thr derivative under stress conditions. For this purpose, the inhibition of the oxidative burst reaction assisted by the dihydrodichlorofluorescein diacetate fluorescence assay was

examined (Figure 6). It was shown that UV-B irradiation of barley roots may induce subapical swelling, probably due to the oxidative stress [81]. Figure 6(c) clearly demonstrates that subapical swelling was accompanied by a significant increase of dihydrodichlorofluorescein fluorescence. The latest fact indicates the accumulation of ROS in the root tip. Dihydrodichlorofluorescein fluorescence in unirradiated root tips was observed mostly in the apex (in the quiescent center) and the inside of dead cells of the root cap (Figures 6(a) and 6(b)). Pretreatment of barley roots by C₆₀-Thr water solution ($C = 1 \text{ mg}\cdot\text{l}^{-1}$) one day before the irradiation inhibited subapical swelling and decreased the intensity of dihydrodichlorofluorescein fluorescence (Figure 6(d)). Therefore, at low concentrations, the C₆₀-Thr derivative acted as a free radical scavenger and decreased oxidative stress in irradiated roots.

During the vegetation experiment, the influence of the C₆₀-Thr derivative and its nanocomposition on the stability of spring barley in the presence of insecticides (imidacloprid), fungicides (carbendazim), and herbicides (glyphosate) was studied. The foliar treatment of spring barley by the C₆₀-Thr solution ($C = 0.1 \text{ mg}\cdot\text{l}^{-1}$) and nanocomposition containing the fullerene derivative, as well as macro- and microelements (see Table 4) in the absence of pesticides, promoted the activation of plant metabolism, lipid peroxidation, and SOD (see S1). The abovementioned effects led to an increase in grain productivity of spring barley by 27 and 8%, as well as increased the total mass by 33 and 10% (see S2). The foliar treatment of control plants with the pesticides imidacloprid and carbendazim led to a decrease in grain productivity by 13% and 7%.

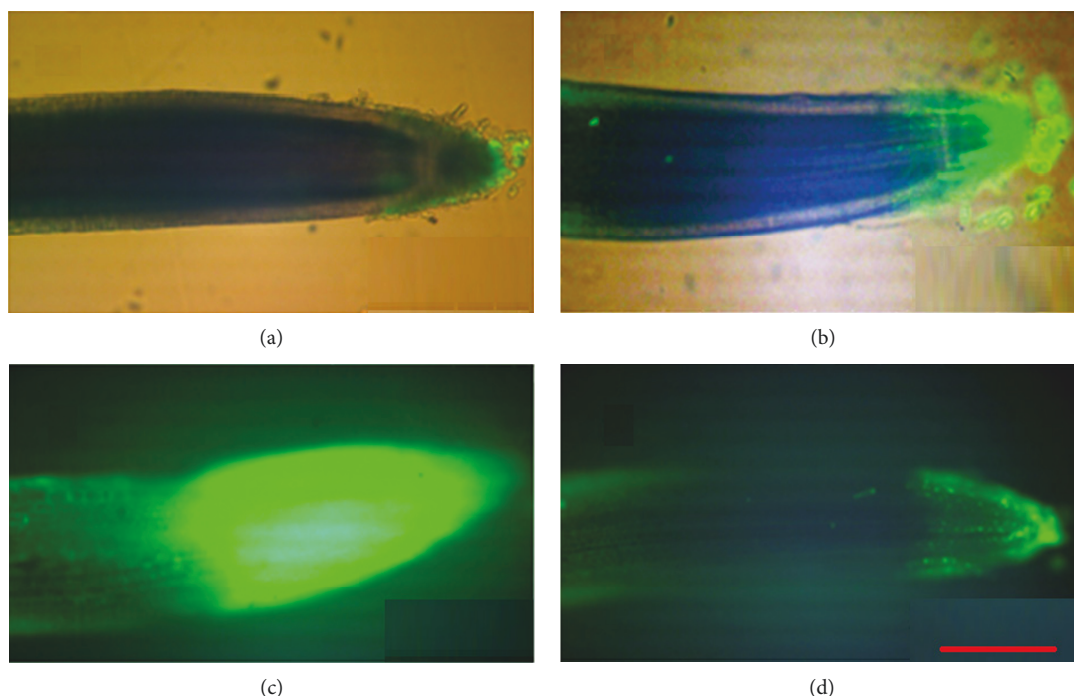


FIGURE 6: Effect of the C_{60} -Thr derivative on the fluorescence of dichlorofluorescein in tips of spring barley roots. (a) The root growing in the water (illumination by visible light); (b) the root preexposed one day with $C = 0.1 \text{ mg}\cdot\text{l}^{-1}$ C_{60} -Thr derivative (illumination by visible light); (c) the root growing in water after 3 hours of irradiation with UV-B radiation (dose $1 \text{ kJ}\cdot\text{m}^{-2}$); (d) the root preexposed (during one day) with $C = 0.1 \text{ mg}\cdot\text{l}^{-1}$ C_{60} -Thr derivative after 3 hours of irradiation with UV-B (dose $1 \text{ kJ}\cdot\text{m}^{-2}$).

TABLE 4: Composition of nutrient solution.

Chemicals	$C \text{ (g}\cdot\text{l}^{-1})$	g (300l)
KNO_3	0.050	15.0
KH_2PO_4	0.050	15.0
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.050	15.0
CaCl_2	0.088	26.4
NH_4NO_3	0.066	19.8
$\text{Fe}(\text{NH}_4)_3(\text{C}_6\text{H}_5\text{O}_7)_2$	0.0017	0.510
H_2SO_4	0.0002	0.060
H_3BO_3	0.0006	0.180
$\text{MnSO}_4\cdot 5\text{H}_2\text{O}$	0.0004	0.120
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	0.00004	0.012
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	0.00004	0.012
$\text{K}_2\text{SiO}_3/\text{Na}_2\text{SiO}_3$	0.0002	0.060
$(\text{NH}_4)_2\text{MoO}_4$	0.0006	0.180
C_{60} -Thr	0.0001	1.5

Additionally, the significant decrease (by 63%) of grain productivity was detected under the condition of exposure by glyphosate (see S2).

The pretreatment of plants with C_{60} -Thr, L-threonine, and nanocomposition containing the fullerene derivative, as well as macro- and microelements, did not have a significant effect on the state of plants in the presence of imidacloprid and carbendazim. At the same time, negative influence of

glyphosate herbicide on the grain productivity of plants was reduced by 29%. The leaves of plants treated with C_{60} -Thr containing nanocomposition and glyphosate had a maximal increase of SOD activity with a concomitant decrease of the lipid peroxidation level in comparison with control plants (treated by water and water solution of macro- and microelements with or without glyphosate herbicide treatment; see Table 5). The obtained experimental results of the C_{60} -Thr and C_{60} -Thr derivative influence on the plant antioxidant system can be explained by the immunomodulatory effect of these substances. The action of C_{60} -Thr can be compared with a vaccine which starts the immune response to a hazardous factor before its affection and simultaneously significantly increasing the plant resistance. For example, pathogen-derived molecules, nonpathogenic plant-associated microorganisms, or physiologically active natural or synthetic chemical compounds can act as plant vaccines providing the nonspecific defensive response [7, 62, 72].

Reducing the pesticide dose on the environment and, at the same time, maintaining the effectiveness of plant protection against disease is one of the priorities of modern agricultural production. The field-condition investigations of the C_{60} -Thr influence on plant resistance to the leaf diseases showed that foliar treatment of growing plants with C_{60} -Thr solutions is significantly less effective than chemical fungicide treatment. However, the combined application of C_{60} -Thr solutions and fungicide led to an increase in the plant protection effectiveness. The level of synergistic effect depends on the plant variety; for example, the consistent foliar treatment of the spring barley Leningradsky variety

TABLE 5: The influence of the spring barely treatment by Zantar fungicide and C₆₀-Thr nanocompositions on the spring barley crop under open ground conditions.

Options of the plant foliar treatment	Concentration of fungicide (g·ha ⁻¹)	Grain weight from the ear		Weight of 1000 grains		g·m ²	Yield % to the control
		g	% to the control	g	% to the control		
Leningradsky variety barley							
Control (without treatment)	—	0.62	—	31.1	—	352.4	—
Zantar	0.8	0.62	100	33.0	106	381.2	108
Zantar	0.4	0.68	110	33.3	107	373.6	106
C ₆₀ -Thr	—	0.59	95	30.8	99	316.8	90
C ₆₀ -Thr+Zantar	0.8	0.85*	137*	34.9*	112*	527.6*	150*
C ₆₀ -Thr+Zantar	0.4	0.68	110	32.4	104	391.6	111
HCP ₀₅		0.23		2.47		132.8	
Ataman variety barley							
Control (without treatment)	—	0.59	—	39.8	—	395.2	—
Zantar	0.8	0.66	112	44.5*	112*	475.2*	120*
Zantar	0.4	0.66	112	43.8*	110*	425.2	108
C ₆₀ -Thr	—	0.61	103	42.9*	108*	407.6	103
C ₆₀ -Thr+Zantar	0.8	0.64	108	42.8	108	463.8	117
C ₆₀ -Thr+Zantar	0.4	0.62	105	43.6*	109*	445.6	113
LSD ₀₅		0.08		3.06		79.8	

* $p < 0.05$ versus the corresponding value for control.

TABLE 6: Efficiency of the spring barely treatment by Zantar fungicide and C₆₀-Thr nanocompositions against leaf disease under open ground conditions.

Options of the plant foliar treatment	Concentration of fungicide (g·ha ⁻¹)	Helminthosporium				Powdery mildew			
		Leningradsky variety barley		Ataman variety barley		Leningradsky variety barley		Ataman variety barley	
		R (%)	BE (%)	R (%)	BE (%)	R (%)	BE (%)	R (%)	BE (%)
Control	—	37.9	—	12.8	—	2.00	—	0.59	—
Zantar	0.8	11.7	69.1		73.4	0.08	96.0	0.29	50.8
Zantar	0.4	14.9	60.7	4.8	62.5	0.13	93.5	0.49	16.9
C ₆₀ -Thr	—	29.7	21.6	7.4	42.2	2.40	0.0	1.8	0.0
C ₆₀ -Thr+Zantar	0.8	10.3	72.8	3.3	74.2	0.03	99.9	0.54	58.5
C ₆₀ -Thr+Zantar	0.4	12.6	66.8	3.6	71.9	0.25	87.5	0.34	42.4
LSD ₀₅		7.4		2.9		2.09		1.09	

R: disease development; BE: biological effectiveness of the plant foliar treatment; LSD: least significant difference.

by C₆₀-Thr solutions and fungicide led to a decrease in the development of helminthosporium patches on the upper plant leaves by 67-73% (depending on the rate of fungicide consumption) and powdery mildew by 88-99% (see Table 6). The biological efficacy of only fungicidal treatment against helminthosporium and powdery mildew was equal to 61-69% and 94-96%, correspondingly. Additionally, the biological effect of the combinational treatment by fungicide (in a half dose) followed by C₆₀-Thr aqueous solutions had the efficiency similar to the total dose of fungicide.

A similar situation is also observed in the foliar treatment of Ataman variety barley plants by individual C₆₀-Thr derivative and fungicide or in the case of combinational treatment. The highest biological efficiencies (exceeding the results of

fungicidal treatment) were observed in the case of simultaneous treatment by C₆₀-Thr and fungicide: we detected the development of helminthosporium patches by 72-74% and powdery mildew by 42-59% (see Table 6). It should be noted that the biological effect of the combined treatment by fungicide (in a half dose) followed by C₆₀-Thr aqueous solutions also had high values: 71.9% for helminthosporium patches and 42.4% for powdery mildew.

The yield structure analysis of the two varieties of spring barley revealed a clear advantage (in the Leningradsky variety) or similarity (in the Ataman variety) of its indicator values in the variant with a combined treatment by C₆₀-Thr and fungicide compared with the variant where only fungicide treatment is performed (see Table 5).

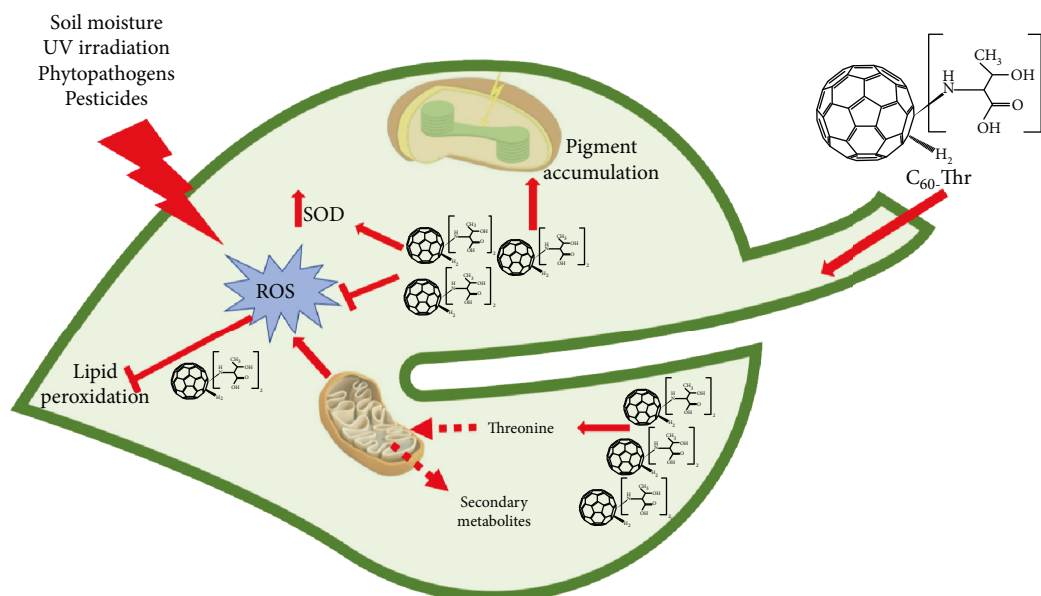


FIGURE 7: A scheme showing the molecular mechanisms of the C_{60} -Thr derivative contribution to the nonspecific resistance of plants to stress action.

The analysis of the barley variety Leningradsky yield revealed the higher efficiency of combined treatment with the C_{60} -Thr derivative and fungicide (at half or full dose) in comparison with the control experiment (by 11 and 50%). At the same time, the fungicide treatment (at half or full dose) led to the increase of the yield by 6 or 8%, respectively.

The obtained results confirm the perspectives on the application of water-soluble fullerene derivatives in the cultivation of spring barely due to the possibility of pesticide load decrease on the environment.

4. Conclusions

The biological study of C_{60} -Thr revealed the plant growth-stimulating ability due to its influence on the photosynthetic apparatus activity and antioxidant properties (see Figure 7). Thus, C_{60} -Thr leads to the nonspecific resistance effect of plants to the action of stress factors (deficiency of soil moisture and phytopathogens). This fact allows us to assume the universal use of L-threonine as an intermediate product in various biosynthesis pathways of protective substances under stress action. As it is known, L-threonine is converted to L-leucine or L-valine after pyruvic acid is formed. Under aerobic conditions, pyruvic acid metabolizes to acetyl-coenzyme A participating in Krebs cycle reactions, followed by the formation of various secondary metabolites with protective, signaling, and other properties [82].

A positive effect of the developed nanocomposition containing C_{60} -Thr on plant resistance to glyphosate is obviously associated with the significant activation of protective plant antioxidant systems. It is known that treatment with pesticides promotes ROS generation in plant cells and leads to an imbalance between ROS formation and antioxidant defense system activity. However, the biological effect of the C_{60} -Thr and the molecular mechanisms of its action require

further detailed studies. The urgency of such complex investigation is closely connected with the development of the water-soluble fullerene derivative application in agriculture.

Data Availability

The data used to support the biological effect of the C_{60} -Thr derivative on the state of plants are included within the article.

Conflicts of Interest

The authors of this work declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Table S1: the effect of foliar plant treatment with the C_{60} -Thr solution and nanocomposition of C_{60} -Thr with macro- and microelements, as well as L-threonine on the activity of the antioxidant system of spring barley *Hordeum vulgare L.* growing under controlled favorable conditions

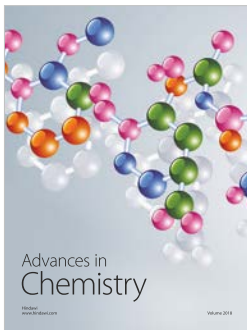
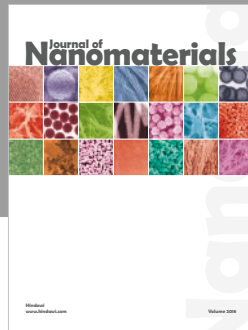
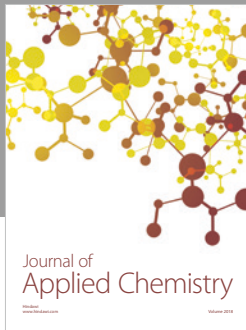
and under the influence of pesticides (P) imidacloprid (P1), carbendazim (P2) and glyphosate (P3). Sm: solution of macro- and microelements (Table 4); Thr+Sm: solutions of L-threonine ($0.057 \text{ mg}\cdot\text{l}^{-1}$) and Sm; C_{60} -Thr+Sm: solutions of C_{60} -Thr ($0.1 \text{ mg}\cdot\text{l}^{-1}$) and Sm; Thr+Water: water solution of L-threonine ($0.057 \text{ mg}\cdot\text{l}^{-1}$); C_{60} -Thr+Water: water solution of C_{60} -Thr ($0.1 \text{ mg}\cdot\text{l}^{-1}$). Table S2: the effect of foliar plant treatment with the C_{60} -Thr solution ($0.1 \text{ mg}\cdot\text{l}^{-1}$) and nano-composition of C_{60} -Thr with macro- and microelements, as well as L-threonine on the grain productivity of spring barley *Hordeum vulgare L.* growing under controlled favorable conditions and under the influence of pesticides (P) imidacloprid (P1), carbendazim (P2), and glyphosate (P3). Sm: solution of macro- and microelements (Table 4); Thr+Sm: solutions of L-threonine ($0.057 \text{ mg}\cdot\text{l}^{-1}$) and Sm; C_{60} -Thr+Sm: solutions of C_{60} -Thr ($0.1 \text{ mg}\cdot\text{l}^{-1}$) and Sm; Thr+Water: water solution of L-threonine ($0.057 \text{ mg}\cdot\text{l}^{-1}$); C_{60} -Thr+Water: water solution of C_{60} -Thr ($0.1 \text{ mg}\cdot\text{l}^{-1}$). Figure S1: the emission spectrum of C_{60} solution ($C = 10^{-5} \text{ M}$) in o-xylene at a wavelength of 360 nm (*Supplementary Materials*)

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