Carbon Nanotubes Influence the Enzyme Activity of Biogeochemical Cycles of Carbon, Nitrogen, Phosphorus and the Pathogenesis of Plants in Annual Agroecosystems

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Abstract
We conducted pre-sowing seed treatment of spring wheat carbon nanotubes modified with thionyl chloride, ethylene diamine, azobenzole, and dodecylamine. CNTs did not disrupt the structure of the crop, but the activity of extracellular enzymes in the rhizosphere of plants in the flowering stage changed: laccase works more poorly in the variant of the CNTs with the amino groups exochitinase and phosphatase activity increased in the case of chlorinated CNTs, OH and COOH groups on the surface of the nanotubes twice accelerate work β-glucosidase. The changes observed in the biogeochemical cycles in the rhizosphere are a possible cause of the effect of nanotubes on the development of epidemic diseases of wheat.

1. Introduction

The expansion of the application sphere of nanomaterial led the humanity to deeper understanding that such materials are unique compositions with unpredictable physical and chemical properties. Contemporary nanotechnologized society absolutely needs the study of both the properties of these materials and the mechanisms of their behavior in natural environments [1-2]. In the course of interaction with bio-objects, carbon nanotubes—like toxicants—behave in an unconventional way, which complicates both the normalization of the content of nanomaterial in living environments and the assessment of their potential harmfulness [3-4]. For instance, the document of year 2010, GN 1.2.2633-10 "Hygienic standards of content of nanomaterial with high priority in environment", covers the standards only for one type of single-walled carbon nanotubes (CNTs) and only the value of Tentative Safe Exposure Levels (TSELs) in the air of work area that equals to 0.01 fiber per 1 cm^3, while this fiber has the length of more than 5 μm. Toxic properties of carbon nanotubes depend on the parameters of the material itself, on the length of nanotubes (the more the length, the more the toxic effect), on their capacity for aggregation and dispersion, on the presence of various metal particles on the surface of nanotubes, on the release by a cell of proteins that wrap nanotubes.

The least studied biological object is soil, which represents the most complex methodological subject to study. The analysis of biological activity of soil allows determining the character and degree of its alteration under any kind of anthropogenic exposure on topsoil. In the recent years, soil science

has experienced the dissemination of the concept of soil matrix. According to the existing concept, soil matrix represents the surface of solid soil particles. Near this surface, layers of adsorbed particles form in a specific way. The particles are organic and mineral substances, microorganisms, gases, ions and molecules. The matrix includes three subsystems: mineral, organic and organic-mineral. The most important property of active centers of the soil matrix is their catalytic activity occurring in soil processes. In this case, we consider the enzymes immobilized on mineral, organic and organic-mineral components of soil particles. These particles also adsorb nanomaterial present in soil. In 2009, the research has demonstrated that COOH-modified single-layer CNTs have low sensitivity to the ionic strength of a solution (as compared to fullerenes and small nanoparticles), since they are strongly held by a soil matrix. The enzyme activity was preferably chosen as a diagnostic indicator due to low experimental error rate, simplicity of evaluation, high sensitivity to external exposures and proven role of enzyme activity indicators in assessing the effect of pollution induced by various techno-genic products.

The analysis of the biological aspects of safe application of carbon nanotubes (CNTs) for cultivating strategic grain crops is of particular importance [5]. The work has the aim of studying the influence of various modifications of CNTs on the pathogenesis and activity of extracellular soil enzymes catalyzing the biogeochemical cycles of carbon, nitrogen and phosphorus in the rhizosphere of spring wheat.

2. Methods

Carbon nanomaterial was produced at scientific and educational innovative center "Nanotechnologies and nanomaterials" of Vladimir State University named after Alexander and Nikolay Stoletovs. The synthesis involved the pyrolysis of propane-butane mix on copper-nickel catalyst at the temperature of 600 ˚C. According to electron microscopy results, the diameter of CNTs varied from to 20 to 40 nm; the length was about 100 nm (figure 1-2).

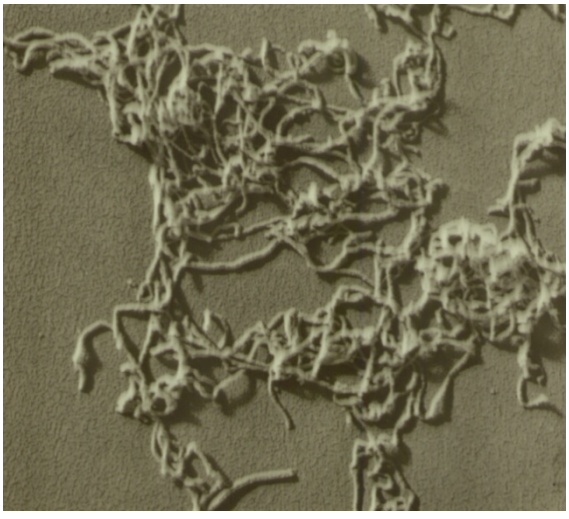

Figure 1. SEM image of CNTs on carbon substrate (70000x magnification)

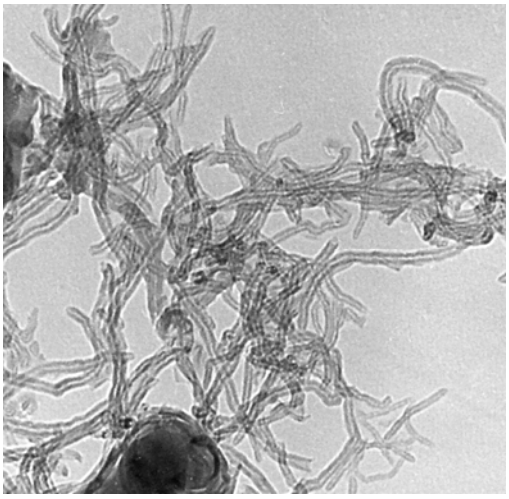

Figure 2. TEM image of CNTs (150000x magnification)

The specific area of material's surface was 232 m^2/g; the poured density of dry material was 0.03 g/cm^3. The content of carbon nanotubes, the content of amorphous carbon and their percentage ration were determined by X-Ray diffractometry (figure 3). According to the analysis of obtained diffractogram and the interpretation of carbon peaks using PDF-2 Release 2011 database, the content of CNTs in the obtained material was 77% (figure 4). The oxidation of CNTs was performed by two methods: a conventional method with the use of the mix of mineral acids and a mechanochemical method. Carboxylation of CNTs was performed using a mix of concentrated nitric and sulfuric acid

(1:1). A subsample of CNTs was placed into a flask that was consequently filled with a mix of mineral acids. The proportion of masses was 1:5. The mix was intensively stirred at the temperature of 70 ˚C for 24 hours. Then, the material was filtered off, rinsed with water and ethanol and air dried.

Fabrication of CNTs modified by hydroxyl group was carried out using a mechanic-chemical method. The method involved the milling of equal-weight subsamples of CNTs and alkali (KOH) in a ball mill for 60 minutes. Consequently, the produced material was washed to remove alkali and air dried. In the process of functionalization, CNTs were cleaned from amorphous carbon. The purity degree was determined by interpreting the diffractogram of modified CNTs with the use of PDF-2 Release 2011 databases (figure 3-4). After adding carboxyl and hydroxyl groups to the surface of CNTs, the fraction of amorphous carbon was less than 5 wt%.

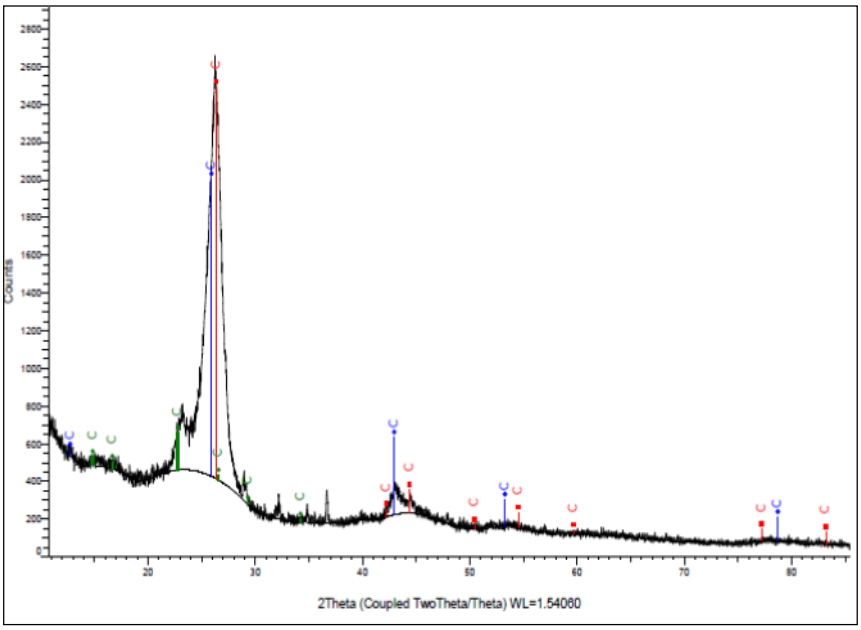

Figure 3. Diffractogram of CNTs after production

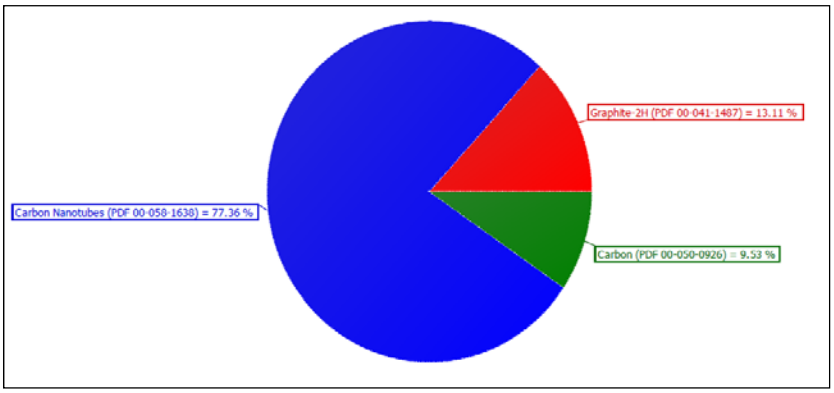

Figure 4. Diagram of carbon state distribution in the material containing CNTs after pyrolysis

The fabricated material was studied by a complex of physicochemical methods (IR and Raman scattering spectrometry, XRD analysis and thermo-gravimetric analysis).

IR spectra were recorded by PerkinElmer Spectrum 100 FT-IR spectrometer (PerkinElmer, USA, 2006). IR spectra of oxidized CNTs demonstrate the peaks that correspond to oxygen-containing functional groups and various vibrations of aromatic ring double bond (figure 5 and 6). The peaks of oxidized CNTs are identified in table 1.

Table 1. Peak identification for oxidized CNTs of IR spectra.

Groups	ν [cm^{-1}]	Identification
C–O(H)	1295˚см$^{-1}$, 1400˚см$^{-1}$	Distinctive for phenol
C=C	1456˚см$^{-1}$, 1540˚см$^{-1}$	Various vibrations of aromatic ring double bond
C=O	1567˚см$^{-1}$, 1636˚см$^{-1}$, 1701˚см$^{-1}$	Corresponds to various carbonyl compounds
–C(O)–O–C(O)–	1861˚см$^{-1}$	Anhydride group
R(OH)	2324˚см$^{-1}$, 2843˚см$^{-1}$ 3276˚см$^{-1}$, 3746˚см$^{-1}$	Corresponds to the vibrations of hydroxyl group in various compounds

The recording of Raman scattering spectra was performed by confocal Raman and fluorescence microscopy with the use of atomic force microscope (AFM) and by spectroscopy with the use of NTEGRA Spectra Probe NanoLaboratory.

The spectra of Raman scattering of CNTs (figure6) modified by hydroxyl and carboxyl groups demonstrate the increased intensity of D and G modes in the regions of 1362 cm^{-1} and 1584 cm^{-1}. This evidences the violation of graphene hexagonal symmetry induced by the occurrence of covalent bond on the sidewall of the external layer of carbon nanotubes.

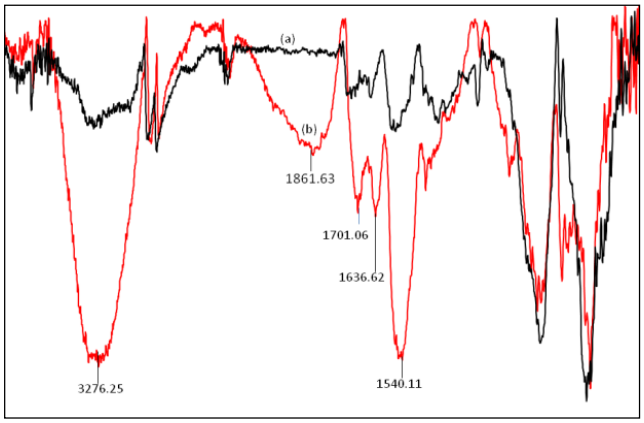

Figure 5. IR spectra. a) pure CNTs and b) CNTs oxidized by mineral acids.

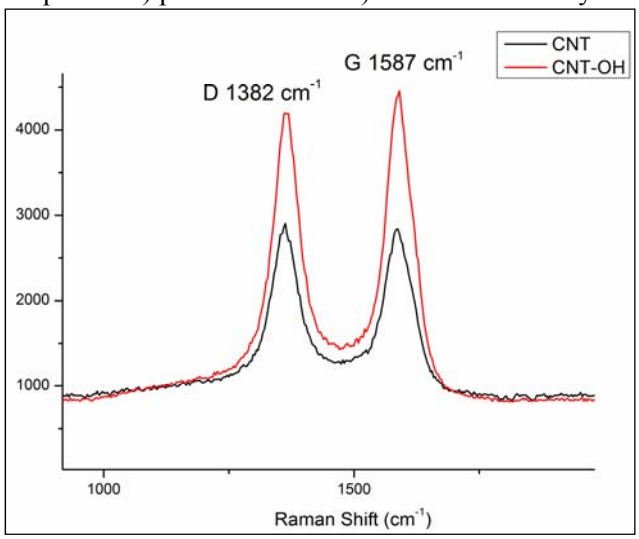

Figure 6. Raman scattering spectrum of CNTs after mechano-chemical treatment

XRD analysis was carried out with the use of D8 ADVANCE X-ray powder diffractometer (Bruker AXS, Germany) and X-ray tubes with copper anode. The parameters of the systems were as follows: the power was 1.6 kW, Bragg Brentano geometry, 2theta scanning angles from about 15˚ to 90˚, the step of 0.02, the exposure of 0.3 seconds per point, PDF-2 Release 2011 database.

According to the analysis of the obtained diffractogram and the identification of carbon peaks through PDF-2 Release 2011 database, the content of CNTs in the produced material is 77% (figure 3 and 4). After oxidation, the fabricated sample, according to the identification of the diffractogram of carbon peaks, contains 95% of CNTs (figure 7 and 8).

We used the following variations and designations: reference sample without CNTs and carbon, M-200 stands for activated carbon, S-0 stands for initial CNTs, S-1 stands for carboxylated CNTs, S-2 stands for carboxylated nanotubes after chlorination (chlorinating agent was thionyl chloride), S-3 stands for CNTs modified by ethylenediamine (OH-group), S-4 stands for CNTs modified by azobenzene, S-5 stands for CNTs modified by dodecylamine.

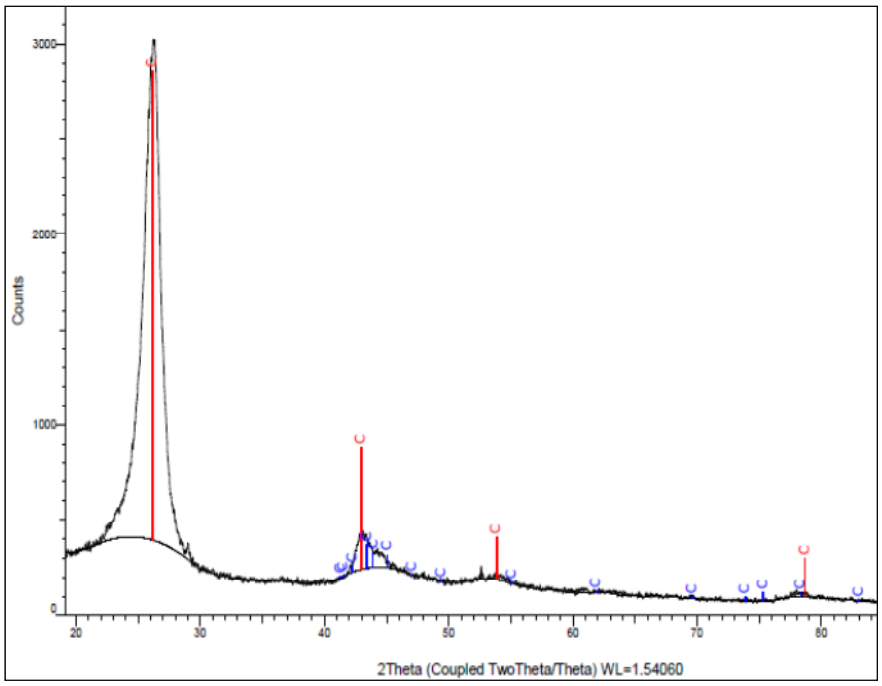

Figure 7. Diffractogram of CNTs after oxidation and cleaning

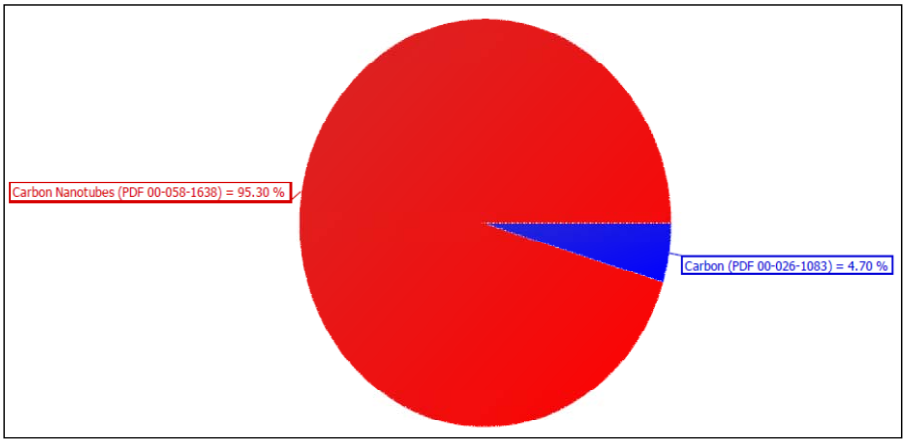

Figure 8. Diagram of carbon state distribution in the material containing CNTs after oxidation and cleaning

Studied enzymes contribute to mobilize nutrients sequestered in soil organic matter trough the lysis of macromolecules such as cellulose, hemicelluloses, lignin, chitin, proteins, polyphenols, etc.1–2 g of fresh rhizosphere soil and 60 ml acetate buffer (pH 5.0) were blended on the highest speed for 2 minutes. Then we poured the homogenate into two conical retort, closed and put them in a shaker at the regime 180 rpm for 2 or 3 hours at room temperature. After this we removed the retorts and put them in the refrigerator (+5 by Celsius) for overnight. In the morning we put the retorts on a shaker again for 15-20 minutes or more. For enzyme analysis we diluted the suspension by 10 times while stirring, centrifuged at 2000 rpm for 2 minute; supernatant was kept in Eppendorf-tubes in the refrigerator (+5 by Celsius). The activity of laccase was determined by colorimetric method. The activity of ß-glucosidase, cellobiohydrolase, acid phosphatase, exochitinase, xylosidase, glucuronidase and leucine-aminopeptidase were determined by a fluorimetric method, which is a novel approach to analyzing soil enzymes [6]. The method is based on the fact that the substrates being specific for each enzyme, are connected with fluorochrome, methylumbelliferone or aminomethyl coumarin. Fluorochrome, being excited by a light with the wavelength of 355 nm, re-emits the light with the wavelength of 460 nm. Thus, the measurement of the number of re-emitted photons allows evaluating the amount of substrate reacted with an enzyme.

3. Experimental

The field experiment was carried out at the same region of West Siberia as in the previous research and we obtain the same results that no modifications of CNTs damaged the production process of spring [7]. Crop structure was analyzed according to the standard procedure. In terms of agro-climatic zoning, the experimental area belongs to moderately cold and moderately moist climatic region of a forest-steppe zone. The sum of air mean daily temperatures over the period with the temperature of air above 10 °C is 1700–1800; the amount of precipitation over the period is 225–250 mm, while the annual precipitation is 400–450 mm. This area is characterized by late-spring and early-autumn frost and drought conditions during early spring-summer period. The zone exhibits podzolized grey forest loamy soil. Each kilogram of topsoil contains 5.3 % of humus, 100–164 mg of mobile phosphoric acid, 34-43 mg of available potassium and 0.95 mg of mobile nitrogen.

The study involved the seeding of the first-grade Novosibirskaya-29 spring wheat. The seeding rate amounted to 200 kilograms of viable seeds per a hectare. The soil was subjected by fall tillage, harrowing by BDT-7 disk harrow and pre-seeding cultivation by KPS-4 ripper. The area of plot was 1.2 m^2; the accounting area was 1.0 m^2. The location of analytical replication of variants was random. The experiment had three replications. The soil was not fertilized; weeding and crop tending were manual. Potatoes was the previous culture, pesticides were not used. The selection of samples for phytopathological expert examination was carried out as per GOST 12044-93**.**

In 2009, it was shown that COOH-modified single-walled CNTs were weakly sensitive, compared to fullerenes and small nanoparticles in the ionic strength of the solution as effectively retained by the soil matrix [5, 8]. In this case, a number of experiments proved the role of CNTs as regulators of growth [8-9]. Currently, living organisms and enzyme activity are used as important diagnostic indicators assessing the impact of soil contamination by various products' of techno-genesis [10-12]. Previously, it was shown that the CNTs affect plant phenotype and microbiota, maximal development of which are determined at the flowering stage [5, 9].

The examination involved the study of the activity of hydrolase and oxidase taking part in biogeochemical cycles of carbon, nitrogen and phosphorous. Enzyme activity depends on different plant-microbe interactions. For example, in the rhizosphere of wheat plants infected by Fusarium, the possibility of *in vitro* depolymerization and polymerization of humic substances by fungus laccase was shown. The possibility of synthesizing humic substances in soils by oxidative condensation of low-molecular predecessors on the surface of loamy minerals under the action of immobilized laccase is well known fact. We studied the activity of key enzymes of the cycles include the degradation of plant cell wall – cellulose, hemicellulose, phenol compounds, and pectin; the breakdown of organic nitrogen

– proteins, peptides, and chitin; and of the degradation of organic phosphorous – nucleotides, phospholipids (table. 2).

Table 2. General potential catalytic activity of soil enzymes in the rhizosphere of wheat plants at blossom stage [pmol of substrate per min per mg of dry soil]

Enzyme name	Control	M – 200	S – 0	S – 1	S – 2	S – 3	S – 4	S – 5
Leucine-aminopeptidase	2.25±0.19	*	1.27±0.04	2.08±0.01	4.39±0.24	4.03±0.12	1.34±0.02	1.66±0.02
Acid phosphatase	5.8±0.3	5.2±0.3	8.0±0.6	5.7±0.1	15.8±1.3	10.7±0.2	5.1±0.1	5.2±0.3
Xylosidase	20.3±1.4	*	23.5±1.8	36,9±1.8	*	38,7±2.3	17.1±0.8	*
β-Glucosidase	11.3±0.2	10.9±0.3	10.0±0.4	27.3±0.6	12.7±1.3	20.3±0.5	10.5±0.3	18.7±0.2
Cellobio-hydrolase	195±12	148±8	159±12	135±11	358±29	193±17	148±13	239±12
Exochitinase	18.1±0.9	9.5±0.6	14.5±0.5	5.9±0.7	46.1±2.7	27.0±0.3	16.2±0.9	9.9±0.5
Glucuronidase	89.3±9.7	71.4±3.9	75.2±6.5	39.2±3.0	32.8±4.6	97.6±2.2	92.1±5.2	87.7±3.4
Laccase	77.1±8.3	79.1±5.9	42.9±1.3	62.7±3.8	88.0±5.2	92.1±5.7	45.7 ± 1.4	52.9 ± 3.1

* Sample/reference sample < 1.2, i.e. the results are non-informative, since they are too close to the values of non-transformed substrate fluorescence.

It is worth mentioning that OH modification of CNTs leads to increased activity of all studied enzymes of nitrogen, carbon and phosphorus cycles, except those of cellobiohydrolase activity, which does not change. Perhaps this may be due to an increase in the intensity of the D and G modes in the 1362 cm^{-1} and 1584 cm^{-1} in the Raman spectra of CNTs-OH, which constitutes a violation of the hexagonal symmetry of graphene, caused by the appearance of a covalent bond in the side wall of the tubes. Laccase works poorly in the variants of the CNTs with the amino groups, and exochitinase and phosphatase activity increased in the case of chlorination of CNTs. OH- and COOH-groups on the surface of the nanotubes twice accelerate the work of β-glucosidase. There is no unequivocal opinion on this point: in soil both single and multi-walled CNTs with azo-group increased the enzyme activity in the soil [12] and single-layer inhibited the activity of alkaline phosphatase and invertase for 14 days [13]. This is a possible reason for the differences in the development of fungal diseases as extracellular soil enzymes play a role of elicitors. At the tillering stage symptoms of root rot were significantly decreased in the variants of CNTs with COOH-, OH- and the azo-groups, and all variants of CNTs reduced the incidence of Septoria by 5-9 times. Blight disease was decreased in plants treated with pure CNTs and CNTs with azo-group (table 3).

In the annual agro-ecosystems 75% of fungal and 89% of bacterial pathogens are transferred through seeds, which are the first to occupy ecological niches in the rhizosphere of sprouting seedlings [11]. Phytopathological analysis of seeds showed a two-fold reduction of infection by Fusarium in variants with COOH- and OH- groups; reduction in the incidence of Helminthosporium by three times in almost all cases, and a two-fold decrease in infection of plants by Alternaria in versions with nitrogen, and chloranhydrid groups (table. 4).

It is known that during the growing season the main infection agents of root rot of wheat are spread by airborne droplets using conidia. Germination of conidia is stimulated by exudates of root hairs.

Table 3. Phytopathological expert examination of Nobosibirskaya-29 wheat seeds

CNT modification	Affected by diseases [%]					
	Total	Fusariosis	Helmintho-sporiosis	Alternaria blight	Bacteriosis	Fungi
Reference	43.0	8.0	10.0	25.0	0	0
M – 200	25.0	8.0	5.0	12.0	0	0
S – 0	41.0	15.0	3.0	20.0	1.0	2.0
S – 1	37.0	4.0	6.0	27.0	0	0
S – 2	28.0	11.0	4.0	13.0	0	0
S – 3	46.0	3.0	10.0	33.0	0	0
S – 4	29.0	12.0	3.0	14.0	0	0
S – 5	35.0	15.0	5.0	15.0	0	0

At the same time plants with reduced turgor of cells are more infected, especially on heavy clay soils. Perhaps just the ability of CNTs to work in a cell plasma membrane as a water pump [5, 9] is one of the reasons why the infection of root rots in wheat seedlings treated by CNTs is decreasing.

Table 4. Analysis of the development and dissemination of diseases affecting Novosibirskaya-29 wheat plants at tillering stage*.

CNT modification	Root rot [points]			Septoria spot [points]			Helmintho-sporiosis [points]			Powdery mildew [points]			Brown rust [%]		
	P, %	R	I	P [%]	R	I	P [%]	R	I	P [%]	R	I	P [%]	R	I
Reference	22.70	0.27	1.20	27.20	0.27	1.00	13.60	0.18	1.33	18.18	0.18	1.00	50.00	0.59	1.18
M – 200	18.20	0.22	1.25	4.50	0.04	1.00	13.60	0.13	1.00	18.20	0.22	0.71	36.30	0.80	2.25
S – 0	22.50	0.48	2.14	6.45	0.06	1.00	9.67	0.09	1.00	22.58	0.22	1.00	61.20	0.60	1.00
S – 1	13.50	0.21	1.60	2.70	0.05	2.00	18.90	0.18	1.00	37.80	0.37	1.00	64.80	1.32	2.04
S – 2	25.00	0.50	2.00	3.57	0.04	1.00	14.28	0.14	1.00	17.86	0.25	1.40	64.28	28.90	45.00
S – 3	12.50	0.12	1.00	0.00	0.00	0.00	29.10	0.29	1.00	25.00	0.29	1.16	62.50	0.80	1.40
S – 4	13.50	0.24	1.80	2.70	0.02	1.00	10.80	0.10	1.00	13.50	0.16	1.20	48.60	0.78	1.60
S – 5	36.36	0.86	2.30	4.50	0.04	1.00	18.10	0.18	1.00	9.00	0.09	1.00	77.20	27.90	36.10

*P is the disease extension, the ratio between the number of diseased plants and total number of plants in a testing sample (percent). R is the development of a disease reflecting the averaged degree of affection of the plot or the whole field (points). I is the intensity or degree of plant affection (points).

Thus, the CMTs functionalized by different groups have a significant influence on rhizosphere microbiota of spring wheat, changing the activity of extracellular enzymes of the main biogeochemical cycles and phyto-sanitary state of the soil.

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