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Response of Pea Seedlings on Multi-Walled Carbon Nanotubes Seed Treatments

Anna Gorczyca[1], Ewa Pociecha[2], Ewelina Matras[1]
[1]Department of Microbiology and Biomonitoring, University of Agriculture in Krakow
Mickiewicz Ave. 21, 31-120 Krakow, Poland
anna.gorczyca@urk.edu.pl; ewelina.matras@urk.edu.pl
[2]Department of Physiology, Plant Breeding and Seed Production, University of Agriculture in Krakow
Podluzna 3, 30-239 Krakow, Poland
rrchilmo@cyf-kr.edu.pl

Abstract **-** The aim of the study was to evaluate the effect of multi-walled carbon nanotubes (MWCNTs), a spore suspension of *Fusarium culmorum* (*FC*), MWCNTs together with a conidia suspension of *FC* and sterilization with sodium hypochlorite on germination coefficient, morphology, infection of seedlings by naturally occurring microorganisms, efficiency of photosystem II and amylolytic activity of α-amylase. MWCNTs limited germination of pea seeds and the length of hypocotyls. MWCNTs affected the morphology of pea seedlings by increasing the length of the shoots and roots but did not affect surface of shoots and the efficiency of photosynthesis. MWCNTs also limited infection of the seedlings caused by natural pathogens microbiome of kernels and *FC*.

Keywords**:** MWCNT, *Fusarium*, pea, morphology, physiology

Abbreviations: nanomaterials (NMs); carbon nanotubes (CNTs); multi-walled carbon nanotubes (MWCNTs); *Fusarium culmorum* (*FC*)

1. Introduction

Understanding the mechanism of interaction of NMs with plants is important not only from the point of view of ecological risk assessment, but also the possibility of using them in agriculture. Plant responses to NMs exposure are very variable. NMs can have positive, neutral or negative effects on plants. Intelligently designed NM have great potential for use as growth promoters, nanofertilizers, nanopesticides, soil conditioners or nanosensors. Numerous studies have highlighted the positive effect of NMs on plants, especially at low doses. The negative effect of NMs on plants was shown by reducing plant growth, photosynthetic efficiency, changes in gene expression, and the production of reactive oxygen species, increasing cellular oxidative stress, causing damage to DNA, protein and membranes. Large variability of plant responses to carbon nanotubes can be observed between different species and even plant varieties. The aim of the study was to evaluate the impact of MWCNTs on the efficiency of seed germination of pea, interaction between MWCNTs and natural seed microbiome and the ability to inhibit the development of phytopathogenic fungi as a result of MWCNTs treatment.

2. Materials and methods

MWCNTs were purchased from CNT Co. Ltd. Korea. The manufacturer's characteristics indicated the features of MWCNTs: diameter: 1-50 nm, length: 1-25 μm, total area: 150-250 m2·g-1, density: 0.03-0.06 g·cm-2, purity of material: 95% min., content of metal oxides: 5% max. Incineration of the MWCNT sample showed an average content of 3.3% of inorganic residues, and their analysis of the following elements content: Cr 98.0 mg·kg-1, Mg 53.2 mg·kg-1, Mn 25.0 mg·kg-1, Ca 57.0 mg·kg-1, Fe 360.3 mg·kg-1. The physicochemical characteristics of the MWCNTs used in the experiment included parameters such as: pH 6.62, κ - 3.3 μS/cm, ζ Sm. natural conditions -44 ± 2 mV, ζ Sm. c=10-2M NaCl – (-38±5) Mv and digestion time in HNO3 – 0 h.

The tested plant was pea Tarchalska variety. Seeds of pea in the same amount were placed in 100 ml Erlenmeyer flasks. The following variants of treatments were tested: (1) control - seeds with a natural microbiome and 20 mL of water; (2) sterilized seeds - sterilization with 10% sodium hypochlorite solution, 3-times washing and 20 mL of water; (3)

pathogen - 5 mL of *FC* spores suspension ($1.5 \cdot 10^7$ pcs / 1 mL) and 15 mL of water; (4) MWCNT - suspension was prepared using 100 mg MWCNTs, which were sonicated for 24h in 100 mL of water, treatment - 5 mL of MWCNTs suspension and 15 mL of water; (5) pathogen + MWCNT - 5 mL of MWCNTs suspension, 5 mL of *FC* spores suspension and 10 mL of water.

After 24 h shaking, all seeds in the flasks were rinsed three times with sterile distilled water. The seeds prepared in this way were placed in germination trays, which were incubated at 21°C and dark. Macroscopic evaluation of germination was performed. After 24 h, the sprout length was measured and the number of germinated seeds counted, while after 48 h the number of germinated seeds was also counted, and the hypocotyl length was assessed using a 5° scale, where: 1° - no sprout, 2° - hypocotyl length 1 mm, 3° - hypocotyl length 2-3 mm, 4° - hypocotyl length 4-7 mm, 5° - hypocotyl length above 7 mm. The germination coefficient was calculated according to formula:

$$GC = \frac{\sum_{i=2}^{5}[2(i-2)+1]n_i}{\sum_{i=1}^{5} n_i}$$

where n_i is the number of seeds for treatment assessed in the category (degree of scale).

The remaining seeds were placed in EQMM Easy Green Mikrofarm USA sprout trays. Seeds from each analysed variant were placed in separate containers. After 20 days, seedling morphology was evaluated. Shoots and roots length, seedling infection caused by natural microbiome or inoculated phytopathogens, surface of shoots and fresh and dry weight of seedlings were measured. PSII efficiency was measured using a Plant Efficiency Analyzer (PEA; Hansatech) with an excitation light intensity of 3 mmol $m^{-2} \cdot s^{-1}$. Measurements were made after 30 min adaptation to the dark. Amylolytic activity was measured in germinating seeds collected from each replicate after 24 and 72 h. Seeds were homogenised in 0.1 M phosphate buffer (pH 7.0), and then centrifuged at 16,000×g for 5 min. The obtained sample of tissue extract (70 µL) was treated with phosphate buffer (630 µL, pH 7.0), 2% Lugol reagent (35 µL) and 0.5% soluble starch solution. The whole was shaken and then placed in a cuvette in a spectrophotometer Ultraspec 2100 Pro and the absorbance was recorded for 5 min at 595 nm. The protein content was determined according to the Bradford method [1].

Data were analysed by ANOVA. Means and standard errors were calculated. A post hoc comparison was conducted using Duncan's multiple range test ($p \leq 0.05$). All calculations were carried out using the STATISTICA 12.0 (StatSoft, Inc., USA) software package.

3. Results

Table 1 shows the effect of applied treatments on seed germination and plant biometry. Our results indicate that MWCNTs limit the germination. When comparing the effect, it can be concluded that MWCNTs function fungistatically against the pathogenic spores of FC, because a smaller amount of germinated seeds was observed in the combined treatment with pathogen and MWCNTs than with the conidia of the pathogen alone. It was observed that the inhibition of pea seed germination is greater during the first 24 hours and is significantly reduced over the next 24 hours. Seeds treated with FC, MWCNTs and combined MWCNTs and FC suspension had the shortest hypocotyl length. It can be concluded from this that MWCNTs and pathogen cause a significant slowdown in the growth of sprouts of the tested plant in the early germination phase. Seeds subjected to sterilization also limited the length of the sprout, but to a slightly lesser extent. All treatments used decreased the germination coefficient. Pea seedlings treated with FC and MWCNTs suspensions significantly increase the average length of shoots and roots. It was shown that MWCNTs can stimulate the growth of plants with the previously found reduction in germination of seeds. Despite the significant influence on the elongation of shoots, the applied treatments do not increase their surface area. It can be concluded that the seedlings formed after treatment of MWCNTs seeds were significantly longer, but had a comparable assimilation surface for the seedlings of the control part. In our own research, the applied treatments caused significant differences in the dry matter content of shoots and roots of test plants. The highest content of dry matter was observed in seedlings in control. The remaining treatments reduced the dry matter content in shoots of the pea seedlings as compared to the control. The greatest decrease was observed in the dry matter after treatment of pea seeds with FC suspension. The combined treatment with the suspension of FC and MWCNTs had the most influence on the dry mass of roots. The average dry matter content in this variant was 4% higher than in the control. The lowest content of dry matter roots of seedlings of the examined plants was noted when seeds were treated with FC suspension.

Table 1: Effect of applied treatments on seed germination and plant biometry.

Treatment	Germination [%]		Length of hypocotyl [mm]	Germination coefficient	Length of shoots [mm]	Length of roots [mm]	Surface of shoots [cm^2]	Dry matter of shoots [%]	Dry matter of roots [%]
	24 h	48 h							
Control	92 a*	95 a	4.25 a	5.25 a	98.36 c	118.92 b	6.44 a	17.45 a	16.37 b
Sterilized seeds	65 b	85 ab	3.03 b	4.45 b	109.68 bc	102.88 c	6.12 a	16.84 ab	15.59 bc
Pathogen	59 c	80 b	1.31 c	2.50 c	115.92 b	143.68 a	7.07 a	16.63 b	14.98 c
MWCNT	33 e	80 b	0.95 c	4.30 b	127.76 a	139.28 a	6.20 a	16.84 ab	15.56 bc
Pathogen + MWCNT	43 d	85 ab	0.92 c	2.05 c	97.56 c	140.84 a	6.14 a	17.01 ab	20.37 a
Mean	58.4	85.0	2.09	3.71	109.86	129.12	6.39	16.95	16.57

The results of the disease index (DI) for individual treatments in own experience are presented in Fig. 1. The largest number of seedlings with symptoms of blight were observed in the control. In turn, the smallest scale of symptoms of seedling blight was found for plants whose seeds were exposed to MWCNTs. These results indicate that MWCNTs may have a strong effect limiting the pathogenesis of seedling blight caused by microorganisms of the natural seed microbiome. No significant differences were found between sterilized seeds and FC suspension.

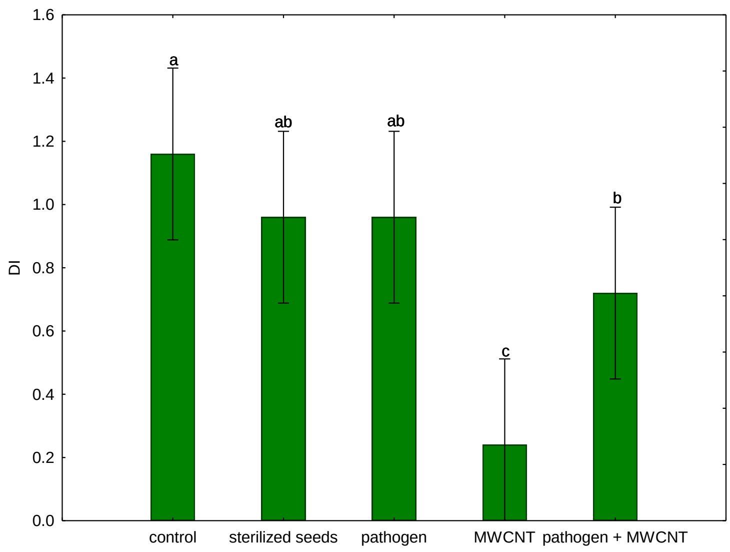

Fig. 1: The effect of applied treatments on the index infection (DI) by blight of seedlings.

The conducted analysis of photosynthetic efficiency including PSII allows to conclude that the treatment used in the own experience does not modify this feature statistically significantly. The calculated PSII parameters are shown in Fig. 2. The observed tendency was that the values of the parameters examined, i.e. the ratio of the energy flow absorbed to the active area of the leaf section (ABS/CS), energy flux for electron transport (energy transferred to reaction center)

(TR0/CS), energy flux for electron transport (Et0/CS), the trapping of photons by the antenna system (DI0/CS) and density of the active reaction centers CS0 (RC/CS0) were the highest in seedlings whose seeds were exposed to MWCNTs, only the number of active parameter reaction centers (RC/CSm) reached the highest value after treatment of seedlings together with the suspension of FC and MWCNTs. The values for the other treatments were similar to those in the control.

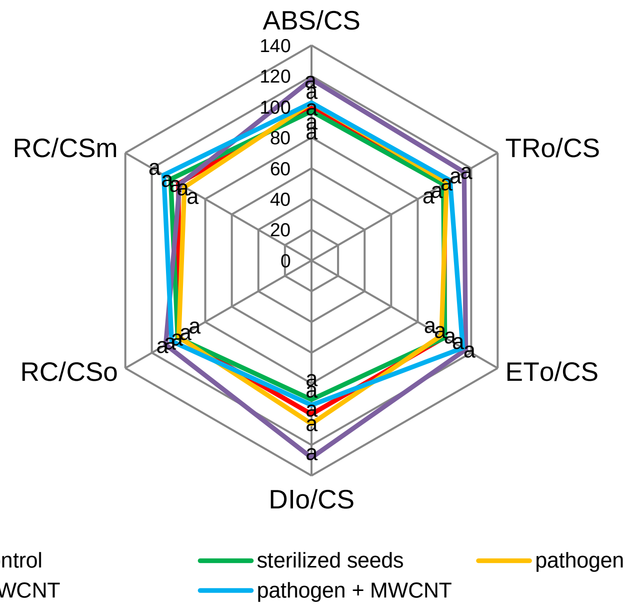

Fig. 2: The effect of applied treatments on efficiency of photosystem II.

The influence of applied treatments on amylases activity showed Fig. 3. Amylase activity in seeds increased with time in the case of controls, sterilized seeds, pathogen and MWCNTs. In the variant (pathogen and MWCNT), the higher value of amylases was noted after 24 hours treatment, whereas after 72 hours the amylase activity decreased.

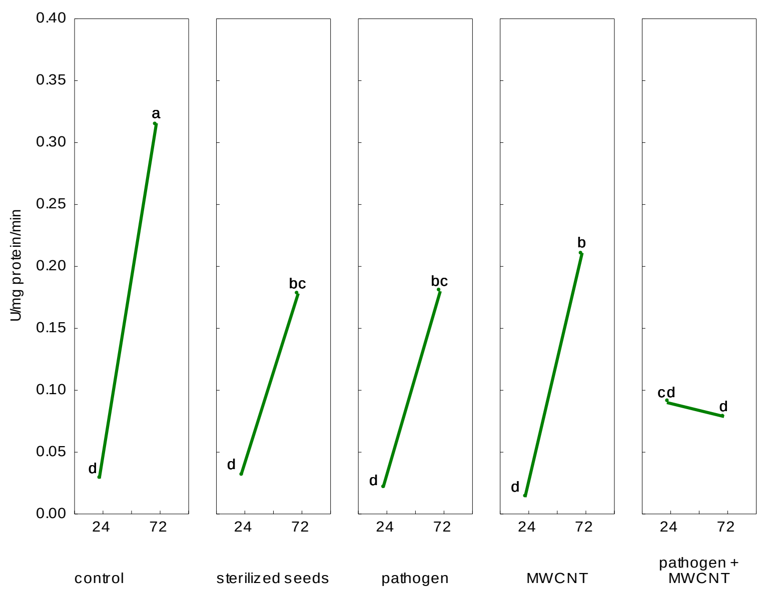

Fig. 3: The effect of applied treatments on the amylases activity in seeds of pea.

4. Discussion

In our own research we have shown a negative impact of MWCNTs on germination of pea seeds. MWCNTs not only reduced the number of germinating seeds, but also limited the length of the hypocotyl. These results do not confirm the expected positive impact of MWCNTs on germination efficiency, which, according to the literature, may be the result of damage to the seed, so-called scarification. The positive effect of MWCNTs on germination of wheat and rapeseed seeds was also not demonstrated by Larue et al. [2] and Hamdi et al. [3] for lettuce seeds. According to the research of Villagarcia et al. [4] modified MWCNTs have a more favourable effect on seed germination than the same MWCNTs used in raw form. Studies show that tomato seeds germinate better when treated with MWCNTs, which have been previously purified and dispersed, or subjected to a functionalization process. Under the same conditions, tomato seeds treated with raw MWCNTs do not show an improvement in germination efficiency. Mondal et al. [5] showed that oxidized-MWCNTs only in low doses increased germination of mustard compared to pristine MWCNTs. In turn, Ratnikova et al. [6] showed that only the combination of MWCNTs with ultrasound increases the germination efficiency and early growth of tomato seedlings. Seed germination can also be affected by impurities embedded in the side walls of carbon nanostructures. This is confirmed by the results of studies conducted on alfalfa and wheat presented by Miralles et al. [7]. The authors showed that industrial MWCNTs and impurities located on their surface have a positive effect on the germination of test plants only at doses below 2560 $mg \cdot L^{-1}$.

There are many factors that determine the germination of seeds, such as water availability, access to oxygen, temperature conditions and the intensity of light. Increased seed germination after using CNTs is associated with better water uptake [8]. According to the authors, tomato seeds treated with SWCNTs had a higher moisture level, which can be explained by the scarification of the seed coat allowing the penetration of larger amounts of water, and thus, the initiation of germination. In another study, seeds of barley, soybean and maize treated with MWCNTs showed accelerated germination, which is associated with increased expression of aquaporin genes [9]. The same results were noted by Khodakovskaya et al. [10] conducting research on tobacco. According to the authors, overexpression of aquaporin genes may contribute to better seed germination, cell growth and improved photosynthesis efficiency.

MWCNTs used in own research, despite limiting seed germination, stimulated the shoots and roots of pea. However, despite the significant impact on faster growth of shoots did not increase their surface. Similar results were obtained by Ghodake et al. [11], who proved the increased elongation of mustard seedlings after using MWCNTs despite the previously stated lack of effect on mustard germination and mungo beans. The positive impact of MWCNTs on the test plant was also confirmed by the results of Mondal et al. [5] who observed an increase in mustard shoots and roots. Lahiani in [9] showed a 26% increase in soybean root length, while in the case of maize a 40% increase in shoot length was observed. Other studies using industrial MWCNTs have shown that they had a positive effect on the extension of wheat root and alfalfa [7]. Wang et at. [12] also showed a 50% and 32% increase in wheat root length after 3 and 7 days of treatment with 40–160 $mg \cdot L^{-1}$ oxidized-MWCNTs. In turn, in studies conducted by May and Patlolla [13] using peas it was found that the functionalized MWCNTs did not affect the morphology of seedlings. SWCNTs was also used in these studies for comparison, which in turn resulted in a significant reduction in the length of shoots and roots. Similar results were obtained by Haghighi and Teixeira da Silva [14], who proved the lack of SWCNTs effect on the growth of turnip seedlings. Cañas et al. [15] proved that non-functionalized SWCNTs increased onion and cucumber root length, while reducing tomato growth compared to PABS (poly-3-aminobenzenesulfonic acid) coated SWCNTs. In turn, SWCNTs subjected to functionalization negatively affected lettuce root elongation. Cabbage and carrots were resistant to modified and unmodified SWNCTs. Khodakovskaya et al. [8] showed that SWCNTs had a positive effect on the growth of shoots of tomato, which were longer and better developed, but the roots of these seedlings were shorter than those of control plants. MWCNTs adversely affected soybean growth [16].

Our own research found a reduction in the dry matter content in the shoots and roots of pea seedlings after the application of MWCNTs. The opposite results were obtained by Haghighi and Teixeira da Silva [14], who showed that the dry mass of tomato and radish shoots increased after the application of SWCNTs in all the concentrations used, while the fresh weight of radish seedlings decreased with the increasing concentration of SWCNTs. Khodakovskaya et al. [8] also proved that SWCNTs caused an increase in vegetative biomass of leaves, stems and roots of tomato, in all concentrations used. The growth of biomass of tomatoes grown on artificial media (Murashige and Skoogmedium) after treatment with 50 μgmL SWCNTs and MWCNTs was also noted by the team of Khodakovskaya et al. [17]. Tiwari et al. [18] also observed an increase in fresh corn biomass (43%) and an increased nutrient uptake (calcium, iron) after treatment with 60 $mg \cdot L^{-1}$ MWCNTs compared to control. The opposite results were obtained by Stampoulis et al. [19] who reported a 60% reduction in zucchini biomass after 15-day MWCNTs exposure at a concentration of 1000 $mg \cdot L^{-1}$ under hydroponic conditions.

Information on the impact of MWCNTs on plant physiology is very important because NMs, due to their small size, can cause many changes in plant organisms. In our research, we did not find any significant impact of MWCNTs on selected parameters of photosystem II performance. In turn, we noted an increase in amylase activity in pea seeds after 72 h treated with MWCNTs. Karami and Sepehri [20] proved that MWCNTs especially at a dose of 500 mg kg^{-1} in the presence of 100 μM exogenous sodium nitroprusside (SNP, as NO donor) significantly improved the photosynthesis parameters of barley grown under conditions of salinity stress and increased the content of chlorophyll and relative water content (RWC). In addition, plants showed higher activity of antioxidant enzymes, i.e. superoxide dismutase (SOD), catalase (CAT), ascorbic peroxidase (APX) and lower content of malonyldialdehyde (MDA) and hydrogen peroxide (H2O2). Similar results were noted by Ghorbanpour and Hadian [21], who showed that MWCNTs can activate antioxidant signaling pathways, thereby protecting the plant from salinity stress. Khodakovskaya et al. [17] showed that MWCNTs at a dose of 100 $mg \cdot L^{-1}$ had a positive effect on tobacco cell cultures. CNTs interactions with plant cells can also cause plant growth inhibition by reducing the concentration of endogenous plant hormones [22]. Lin et al. [23] proved that MWCNTs reduce cell viability, chlorophyll content and superoxide dismutase (SOD) activity in *Arabidopsis thaliana*. Higher toxicity of smaller particles was observed in this study, thus confirming that the size of NMs is an important determinant of their toxicity. MWCNTs in high concentration [125-1000 $mg \cdot L^{-1}$] caused damage to the cells and tissues of spinach leaves, changes in root and leaf morphology and an increase in reactive oxygen species (ROS) [24]. Wild and Jones [25] noted an internal MWCNTs translocation in wheat. They observed that MWCNTs could penetrate the wheat skin wall and then penetrate into the cytoplasm of the hair of the roots of the test plant. In turn, Larue et al. [26] found the presence of MWCNTs in parts of young wheat and rapeseed plants, but did not show any negative changes in the development and physiology of these plants.

The reactions of plants treated with CNTs are very variable. The available literature reported both positive and negative effects. The interaction of CNTs with plants is a very complex process in which the plant (species), CNTs (type of carbon nanomaterial, including the type of nanotubes and how they function or disperse in solution and concentration) and the type of substrate are closely related. Changing one of these elements can completely disrupt CNTs-plant interactions and cause various unpredictable reactions. From an ecological-toxicological point of view, a better assessment of CNTs behaviour and their impact on ecosystems is needed. Further research is urgently needed to understand the mechanisms of interaction between CNTs and plants, as plants are an important component of ecosystems, showing close interactions with other living organisms.

4. Conclusion

The assessment of the impact of CNTs on plants remains ambiguous. The research shows that MWCNTs cause an increase in the length of shoots and roots of pea, despite the reduction in seed germination. In addition, MWCNTs reduce the pathogenesis of seedlings blight caused by the organisms of the natural seed microbiome and *FC*. MWCNTs do not significantly affect the photosynthesis intensity of pea seedlings, but they increase the activity of amylases over the time. Based on the own results obtained and data from the available literature, we are not able to determine whether CNTs will be able to be innovative material stimulating the germination and growth of plant seedlings, or whether they will be considered harmful. Therefore, further assessment of the effects of CNTs on plants at all possible levels is necessary.

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