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ARTYKUŁ ORYGINALNY

Wpływ mikrocząstek i nanocząstek tlenku miedzi na wzrost grzybni *Alternaria alternata*, *Botrytis cinerea* i *Fusarium oxysporum*

Effects of copper oxide micro- and nanoparticles on *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum* mycelium growth

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Streszczenie

Celem niniejszych badań było porównanie wpływu cząstek tlenku miedzi (Cu_xO) oraz nanocząstek tlenku miedzi (Cu_xO NPs) na wzrost *in vitro* wybranych patogenów roślinnych: *Alternaria alternata*, *Botrytis cinerea* i *Fusarium oxysporum*. W eksperymencie *in vitro* patogeny hodowane były na pożywce PDA zmodyfikowanej dodatkiem Cu_xO lub Cu_xO NPs w stężeniu 0, 100, 200, 500, 1000 lub 2000 mg/l. Zarówno Cu_xO, jak i Cu_xO NPs znacząco ograniczały wzrost grzybni wszystkich testowanych patogenów. W stosunku do *A. alternata* inhibicyjne działanie miało zastosowanie Cu_xO NPs w stężeniu 500 mg/l, ograniczające wzrost patogenu średnio o 76,64% w stosunku do kontroli. W przypadku *B. cinerea* odnotowano podobną zależność, zastosowane Cu_xO NPs w stężeniu 500 mg/l ograniczyło wzrost patogenu średnio o 70,94% i było najbardziej skuteczne. W przypadku *F. oxysporum* zastosowanie 1000 i 2000 mg/l Cu_xO NPs lub 2000 mg/l Cu_xO było skuteczne w ograniczeniu wzrostu grzybni patogenu, a inhibicja wyniosła blisko 100%. Chociaż Cu_xO NPs wykazały obiecujące właściwości przeciwgrzybicze, konieczne są dalsze badania w celu oceny ich aktywności *in vivo*, wpływu na środowisko, akumulacji w tkankach roślinnych i potencjalnej toksyczności dla innych organizmów. Zrozumienie, w jaki sposób te nanocząstki oddziałują na środowisko oraz jakie są ich szlaki degradacji ma kluczowe znaczenie dla ich bezpiecznego stosowania w rolnictwie.

Słowa kluczowe: oddziaływanie fungistatyczne, tlenek miedzi, nanotechnologia, zrównoważone rolnictwo, ochrona roślin

Abstract

The study aimed to compare the effect of Cu_xO micro- and nanoparticles (Cu_xO NPs) on the *in vitro* growth of selected plant pathogens: *Alternaria alternata*, *Botrytis cinerea*, and *Fusarium oxysporum*. In the *in vitro* experiment, pathogens were cultured on the PDA medium supplemented with Cu_xO or Cu_xO NPs at the concentration of 0, 100, 200, 500, 1000, or 2000 mg/l. Both Cu_xO and Cu_xO NPs significantly inhibited mycelial growth in all tested pathogens. In *A. alternata*, treatment with 500 mg/l of Cu_xO NPs was the most effective, reducing pathogen growth by an average of 76.64% compared to the control. A similar trend was observed in *B. cinerea*, where treatment with 500 mg/l Cu_xO NPs reduced pathogen growth by an average of 70.94%, indicating it as the most effective concentration. In contrast, *F. oxysporum* was only inhibited by 1000 and 2000 mg/l Cu_xO NPs or 2000 mg/l Cu_xO, which resulted in almost 100% growth inhibition. Although Cu_xO NPs showed promising antifungal properties, further study is necessary to evaluate their *in vivo* activity, environmental impact, plant tissue accumulation, and potential toxicity to non-target organisms. Understanding how these nanoparticles interact with the environment and their degradation pathways is vital for their safe application in agriculture.

Keywords: antifungal activity, copper oxide, nanotechnology, sustainable agriculture, plant protection

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Wstęp / Introduction

Nanoparticles (NPs) for their size below 100 nm at least in one of three dimensions exhibit distinctive physicochemical properties that differ significantly from those of their micrometre-sized particles or bulk materials (Nagarajan 2008; Strambeanu et al. 2014). This is due to the large surface-to-volume ratio, which has an effect on numerous properties of nanomaterials, i.e. surface, electrical, magnetic, optical properties, and/or even biological activity, etc., thus affecting the functionality of nanomaterials in many fields (Nalci et al. 2019).

To achieve global food security in the face of such issues as global climate change, extreme weather conditions and a depleting pool of registered pesticides, agriculture needs to expand its practices. Among sustainable practices that can be implemented, nanotechnology is a viable solution, improving the precision and efficiency of agrochemicals (Roy and Hossain 2024). Nanoparticles in agriculture can be used as nanofertilizers to release micro- and macronutrients in soil, reducing the fertilizer amount, the risk of overfertilization and fertilizer run-off, improving the uptake of nutrients and crop yield (Kumar et al. 2021; Yadav et al. 2023). Nanoparticles used as nanopesticides can be applied on stem and leaves, or to soil for root application (Shangguan et al. 2024). They increase the uptake of the pesticide by promoting its absorption and transport in the plant, increase the contact area between pesticide and target pest and regulate the release of pesticide (Kannan et al. 2023).

Copper is one of the most important microelements necessary for the proper development and functioning of human and animal bodies, and at the same time highly toxic if it occurs in excess (Swędrzyńska and Sawicka 2010; Lamichhane et al. 2018). It has a strong antimicrobial effect and plays an important role in integrated plant protection (copper-based preparations with a wide range of bactericidal and fungicidal effects). It is essential in organic farming, but its long-term use may have serious consequences due to its accumulation in the soil (La Torre et al. 2018). Metal-containing nanoparticles may provide an organic alternative to fungicides and a valuable tool for controlling plant resistance to pathogens. Copper nanoparticles and copper-based nanomaterials may have greater application potential compared to larger particles due to the high surface activity enabling their use in lower doses than other copper-based compounds. As an effect nanoparticles can be used in environmentally safe concentrations, and therefore, ensuring both a reduced risk of excessive accumulation and lower plant protection costs (Sardella et al. 2018; Ajilogba et al. 2021; Jomeyazdian et al. 2024). Mali et al. (2020) showed that eco-friendly green synthesed copper nanoparticles (using *Celastrus paniculatus* leaves extract) are a good antifungal agent against plant pathogenic fungi *Fusarium oxysporum*, whereby mycelial growth inhibition depends on NPs concentrations.

Plant pathogens may contribute to a significant reduction in the size and quality of crops and their commercial value. Depending on weather conditions and the phytosanitary condition of plants, the incidence of diseases may range from 70 to 80% of the total plant population, and in some cases, yields may decline by as much as 80–98% (Nazarov et. al 2020). The most economically important phytopathogens include species such as *Alternaria alternata* (DeMers 2022), *Botrytis cinerea* (Bi et al. 2023) and *F. oxysporum* (Zuriegat et al. 2020), which attack many crop plants, including cereals, ornamental and forest plants. *Fusarium oxysporum* causes Fusarium wilt and the fatal vascular syndrome. It is a difficult pathogen to combat because its spores can remain dormant in the soil for up to several dozen years (Joshi 2018). *Botrytis cinerea* can cause significant losses, and due to the variety of attack methods, controlling this disease is difficult (Williamson et al. 2007; Bi et al. 2023). *Alternaria alternata* has a wide host range, including several hundred plant species, including many important crop plants. Symptoms of black spot appear late during cold storage, which may lead to significant postharvest losses (Troncoso-Rojas and Tiznado-Hernández 2014; DeMers 2022).

The aim of this study was to investigate and compare the effect of Cu_xO and Cu_xO NPs on the response of three plant pathogens, i.e. *A. alternata*, *B. cinerea*, and *F. oxysporum*, under *in vitro* culture conditions. Further *in planta* tests are planned with the use of Cu_xO and Cu_xO NPs at the most optimal concentrations selected in this preliminary experiment.

Materiały i metody / Materials and methods

The copper nitrate hydrate $Cu(NO_3)_2 \cdot xH_2O$ (≥ 99.9% trace basis) as a source of copper was supplied from the Merck, Darmstadt, Germany. Ascorbic acid and NaOH with analytical purity were supplied from Warchem Sp. z o.o., Warsaw, Poland. Water was purified with HYDROLAB water filtering system supplied from Gliwice, Poland.

The synthesis of particles was performed with wet precipitation method, where 1 g of $Cu(NO_3)_2$ was dissolved into the 100 ml of deionized water (DI) using magnetic stirrer. Next, the 500 mg of ascorbic acid was dissolved into 50 ml of DI and added slowly to the beaker containing copper ions. The solution was continuously stirred using magnetic stirrer with the stirring rate of about 1200 rpm at room temperature and the 1 M NaOH was added till pH 8.5, where the greenish solution turned orange-brick color forming colloidal suspension. The stirring was continued for 30 minutes. After that time, the suspension centrifuged with 4000 rpm, where the solution was replaced with DI several times till reaching neutral pH to remove the salts from the solution. The sample was labeled as Cu_xO. Obtained particles were vortexed between centrifuging. Next, the same procedure was implemented to fabricate Cu_xO NPs, while the additional pulsed sonication for 10 min using ultrasonic

homogenizer in pulsed mode (5 s pulses) was used after the first washing of nanoparticles with DI. Then, the washing was performed with the same procedure like for the previous sample. Lack of homogenization leads to the formation of the agglomerates that needs to be disassembled into NPs ultrasonically.

The morphology of obtained particles was confirmed by Scanning Electron Microscopy (SEM) ZEISS Crossbeam 350 and FE-SEM Merlin (ZEISS, Stuttgart, Germany), where the sample was immobilized onto the carbon-based conducting tape glued to the aluminum sample holder. More detailed morphology of nanoparticles was studied using Transmission Electron Microscopy (TEM) Zeiss Libra 120 Plus, Stuttgart, Germany, where the colloidal suspension was drop-casted onto the copper mesh covered with Formvar layer and dried under the fumehood prior to the analysis. Elemental composition of the prepared NPs was investigated using EDS (Energy dispersive X-ray spectroscopy), while the Fourier Transform Infrared Spectroscopy (FTIR) using ATR Spectrum Two in the range from 400 to 4000 cm was used to determine the specific vibrations in the NPs. The optical properties of the NPs was investigated with UV-vis spectrometry Lambda 1050+ in the range from 200 to 800 nm in the transmission mode using quartz cuvette with d = 10 mm. Sonication was performed using ultrasonic homogenizer 150 W model TF-150N (Tefic Biotech Co., Limited) supplied from ABChem, Olsztyn, Poland.

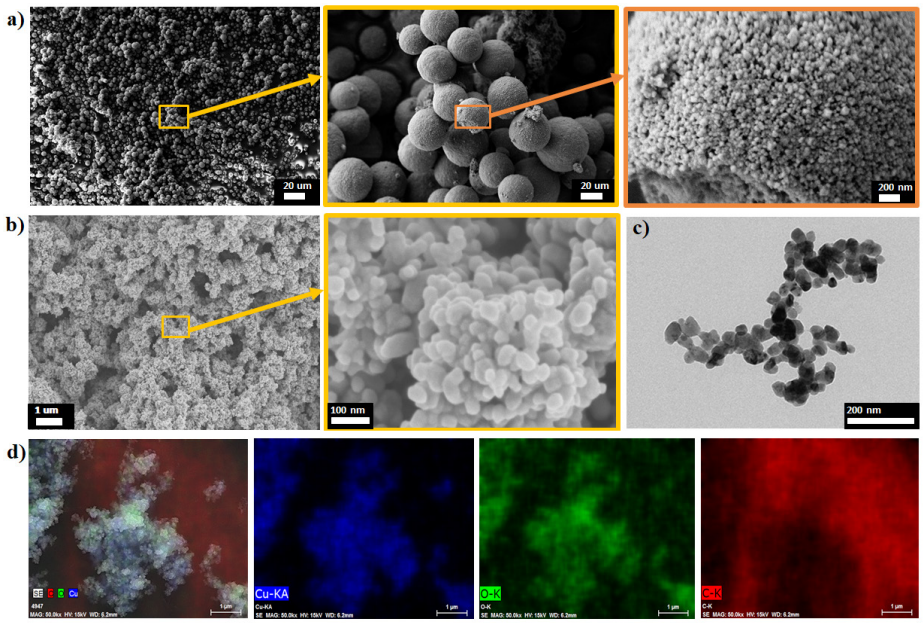
Plant pathogens were obtained from the Bank of Pathogens of Institute of Plant Protection – National Research Institute (Poznań, Poland) collection. *Botrytis cinerea* 2235 was isolated from a tomato stem (*Solanum lycopersicum*) grown in Chwaszczowo, Poland, and stored in glycerol at –20°C; *F. oxysporum* 2105 was isolated from *Triticum vulgare* grown in Winna Góra, Poland, and preserved in liquid nitrogen; *A. alternata* 2115 was isolated from tomato leaves (*S. lycopersicum*) collected in Plewiska, Poland, and stored in liquid nitrogen. All pathogens were inoculated on the potato dextrose agar (PDA) medium (BDDifcoTM, New Jersey, USA) with the addition of Cu_xO or Cu_xO NPs at the following concentrations: 0, 100, 200, 500, 1000, 2000 mg/l. Cu_xO and Cu_xO NPs were added into the PDA medium, and such obtained suspensions were sterilized in an autoclave at 121°C and 1 atm for 15 minutes, and next placed for 30 minutes in the Elmasonic S80(H) Ultrasonic Cleaner (37 kHz, 150 W; Elma Schmidbauer GmbH, Singen, Germany) for proper particles dispersion. Media were immediately poured onto 90 mm Petri dishes. Fresh discs of seven-day-old pathogens cultures: *A. alternata*, *B. cinerea*, and *F. oxysporum* (5 mm diameter) were cut and separately transferred onto a medium (one disc in the centre of each Petri dish). Cultures were incubated at 23°C in the darkness. The diameter (mm) of mycelium was measured every 24 h until it reached the edge of the dish in one of the treatments (for each pathogen separately).

The experiment was set up in a completely randomized design. Each experimental object consisted of 5 repetitions. The obtained data were presented as mean ± standard deviation (SD) and subjected to two-way analysis of variance (ANOVA) and a post hoc Fisher test at the significance level of $p \leq 0.05$. Tables with results provide real numerical data, while alphabet letters point to homogenous groups of the statistical calculations based on transformed data. All statistical analyses were performed with the use of the Statistica 12.0 software (StatSoft Polska, Cracow, Poland).

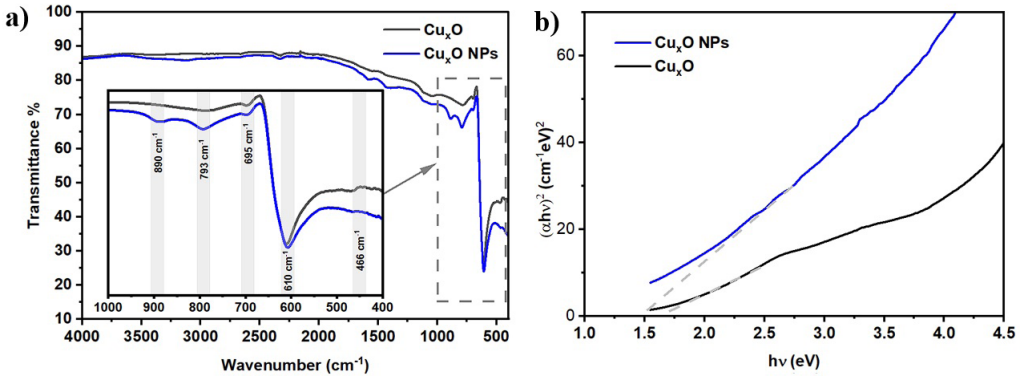
Wyniki i dyskusja / Results and discussion

Prior to the application of the particles against plant pathogens, morphology and composition studies were performed. Photo 1a presents Cu_xO composed of small particles that form aggregates of nanostructures. Particles can be disassembled ultrasonically into smaller structures, and the average size of nanoparticles below 20 nm. Following images presented at photo 1b show the Cu_xO NPs with the similar size, while photo 1c presents more detailed morphology of the nanoparticles. Images obtained with TEM are similar to the morphology presented in the literature (Jeong and Kim 2009; Pourahmad et al. 2023). The EDS maps presented in photo 1d show the distribution of the copper and oxide elements in the nanoparticles. The uniform distribution of both elements contributes to the copper oxide in the obtained particles, however do not reveal the full chemical formula of the copper oxide. Therefore, FTIR was applied to determine the presence of vibrations that refer to the specific functional groups, see figure 1a.

The bands below 700 cm refer to the presence of copper oxides in the sample, where the band at 466 cm corresponds to the stretching of the Cu-O bond in the crystal lattice of the copper oxide particles. A vibrational mode with the highest intensity among Cu-O-related bands observed at 610 cm is attributed to the Cu_2O stretching. The band observed around 695 cm corresponds to the vibrational mode characteristic of CuO. The presence of bands at 466 and 695 cm for the Cu-O and the broadening of the band located at 610 cm in the obtained NPs can suggests the presence of mixed phases indicating that NPs are predominantly composed of cuprous oxide Cu_2O (Chang 2011; Sahai et al. 2016; Gherasim et al. 2022). It can be concluded that obtained material has a minor contribution from cupric oxide CuO phase resulting in the Cu_xO formula to be used to describe the prepared system in this work. The following bands are characteristic to the presence of organic groups onto the surface of material from the organic reducing agents residues to be possibly adsorbed onto the NPs' surface. The minor bands at 789, 885, and 1419 cm can come from the C-H bending. The broad band between 1000–1150 can come from the C-O stretching, while 1572 cm can be caused by the presence of C = C,



Fot. 1. Obraz SEM dla Cu_xO (a), Cu_xO NPs (b), obraz TEM dla Cu_xO NPs (c), mapa EDS obrazująca rozkład pierwiastków dla Cu_xO NPs (d)
Photo 1. SEM image for Cu_xO (a), Cu_xO NPs (b), TEM image for Cu_xO NPs (c), elemental maps obtained with EDS for Cu_xO NPs (d)



Rys 1. Widmo FTIR (a) i wykres Taca (b) dla Cu_xO i Cu_xO NPs
Fig. 1. FTIR spectra (a) and Tauc plot (b) for Cu_xO and Cu_xO NPs

while the broad minor band around 2315 cm and 3000 cm is characteristic to the atmospheric CO_2 and O-H, respectively (Bujok et al. 2019; Almond et al. 2020; Reza et al. 2020). Complementary to FTIR analysis, the UV-vis spectrophotometry was used to determine the optical bandgap. The bandgap energy E_g for Cu_xO NPs is about 1.47 eV, while for the Cu_xO is about 1.65 eV, see Tauc plot presented in figure 1b. However, as different slopes can be distinguished resulting in different E_g values the results are in the good agreement with the FTIR studies that reveal mixed copper oxides in the obtained particles.

After the characterization, the activity of obtained copper oxide particles against plant pathogens was studied. Hao et al. (2017) described CuO NPs' potential as antifungal agents against *B. cinerea*. In their study copper oxide nanoparticles demonstrated strong antifungal activity at the concentration of 50 mg/l. In the presented research, the inhibition of *B. cinerea* mycelium growth was observed with the use of copper oxide nanoparticles (Cu_xO NPs) and copper oxide (Cu_xO) at higher concentrations. For Cu_xO mycelium growth was inhibited only at high concentrations of 1000 and 2000 mg/l. For Cu_xO NPs more effective inhibition was achieved at the concentration of 500 mg/l or higher. The concentration of 500 mg/l reduced mycelium growth by an average of 70.94%, while doubling this concentration (to 1000 mg/l) resulted in a further 22.72% reduction, achieving a total reduction of 93.66%. The reduction observed with Cu_xO NPs at 500 mg/l surpassed the inhibition observed with Cu_xO at 1000 mg/l. Generally, the use of Cu_xO NPs at the concentration of 500 mg/l emerged as the most effective approach in the reduction of the *B. cinerea* mycelium growth. Further increases in concentration offered incremental benefits. Regardless of the type of sample used, only the use of 500 mg/l and higher concentrations resulted

in the reduction of the pathogen growth, and regardless of the concentration, the use of Cu_xO NPs resulted in the stronger reduction of *B. cinerea* growth than the use of Cu_xO (tab. 1, 2, fig. 2). Pathogen completely overgrown the plate in just three days on medium with the addition of Cu_xO at the concentrations from 100 to 500 mg/l, and with Cu_xO NPs at the concentrations of 100 and 200 mg/l. The pathogen showed the highest growth dynamics between the first and second day, suggesting that in the initial phases, both Cu_xO and Cu_xO NPs had limited inhibitory effects (tab. 1, 2, fig. 2, photo 2).

The study of Ismail et al. (2023) demonstrated that chitosan-decorated copper oxide nanocomposite (CH@CuO NPs) effectively inhibited *B. cinerea* in both *in vitro* and *in vivo* experiments. At the concentrations of 100 and 250 mg/l, CH@CuO NPs provided 100% control efficacy for grey mould on leaves, whole plants, and fruits without toxicity, outperforming the conventional fungicide Teldor 50% SC, which achieved 80–97% control efficacy. Similarly, Sadek et al. (2022) reported that copper nanoparticles significantly suppressed *B. cinerea* growth at relatively low concentrations (5–100 µg/ml), outperforming $CuSO_4$, and a chemical fungicide Topsin-M under both *in vitro* and *in vivo* conditions, and in the study by Bikdeloo et al. (2021), 15 mmol/l copper nanoparticles added into the culture medium completely inhibited *Botrytis* growth.

Tabela 1. Wpływ wybranych stężeń Cu_xO i Cu_xO NPs [mg/l] na średnicę grzybni *Botrytis cinerea* [mm]

Table 1. The influence of selected concentrations of Cu_xO and Cu_xO NPs [mg/l] on mycelia diameter of *Botrytis cinerea* [mm]

Stężenie próbek Samples concentration [mg/l]	Próbki – Samples		Średnia Mean
	Cu_xO	Cu_xO NPs	
0	82.00 ± 0.00 a	82.00 ± 0.00 a	82.00 A
100	82.00 ± 0.00 a	82.00 ± 0.00 a	82.00 A
200	82.00 ± 0.00 a	82.00 ± 0.00 a	82.00 A
500	82.00 ± 0.00 a	27.38 ± 7.30 c	54.69 B
1000	47.13 ± 3.49 b	9.88 ± 1.92 d	28.51 C
2000	9.00 ± 2.84 d	7.63 ± 1.78 d	8.32 D
Średnia – Mean	64.02 A	48.48 B	

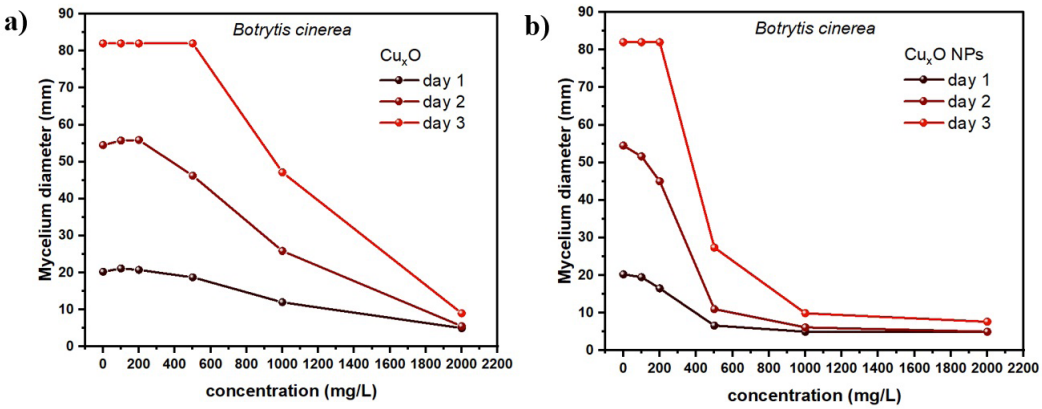
Średnie oznaczone tą samą literą nie różnią się istotnie przy $p \leq 0{,}05$ (test Fishera). Wielkie litery odnoszą się do głównych efektów (niezależnie), a małe litery do interakcji między dwoma badanymi zmiennymi niezależnymi
Means followed by the same letter do not differ significantly at $p \leq 0.05$ (Fisher test). Upper-case letters refer to the main effects (irrespectively), lower-case letters refer to the interaction between the two studied independent variables

Tabela 2. Wpływ wybranych stężeń Cu_xO i Cu_xO NPs [mg/l] na średnicę grzybni *Fusarium oxysporum* [mm]

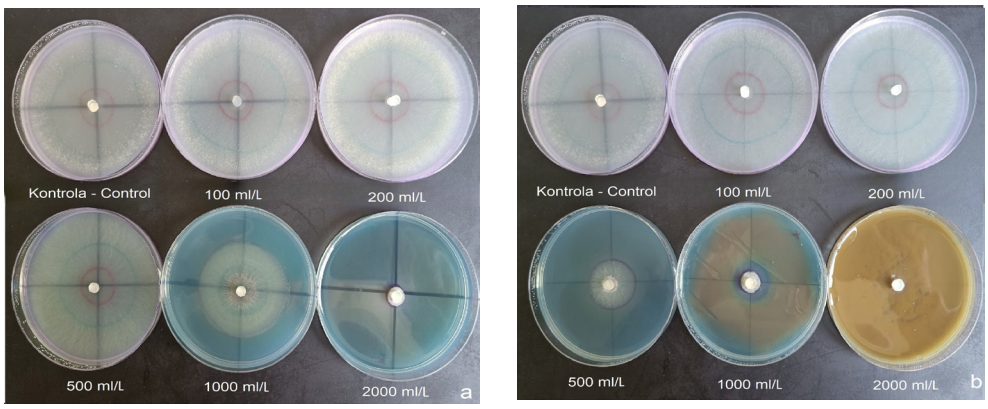
Table 2. The influence of selected concentrations of Cu_xO and Cu_xO NPs [mg/l] on mycelia diameter of *Fusarium oxysporum* [mm]

Stężenie próbek Samples concentration [mg/l]	Próbki – Samples		Średnia Mean
	Cu_xO	Cu_xO NPs	
0	51.75 ± 2.84 b	51.75 ± 2.84 b	51.75 A
100	43.50 ± 6.83 c	45.25 ± 0.83 bc	44.38 BC
200	45.13 ± 2.79 bc	51.00 ± 10.80 b	48.07 AB
500	50.13 ± 5.30 bc	44.63 ± 2.41 bc	47.38 AB
1000	78.50 ± 1.84 a	5.00 ± 0.00 d	41.75 C
2000	5.00 ± 0.00 d	5.00 ± 0.00 d	5.00 D
Średnia – Mean	45.67 A	33.77 B	

Średnie ± SD, oznaczone tą samą literą, nie różnią się istotnie przy $p \leq 0{,}05$ (test Fishera). Wielkie litery odnoszą się do głównych efektów (niezależnie), a małe litery do interakcji między dwoma badanymi zmiennymi niezależnymi
Means ± SD followed by the same letter do not differ significantly at $p \leq 0.05$ (Fisher test). Upper-case letters refer to the main effects (irrespectively), lower-case letters refer to the interaction between the two studied independent variables



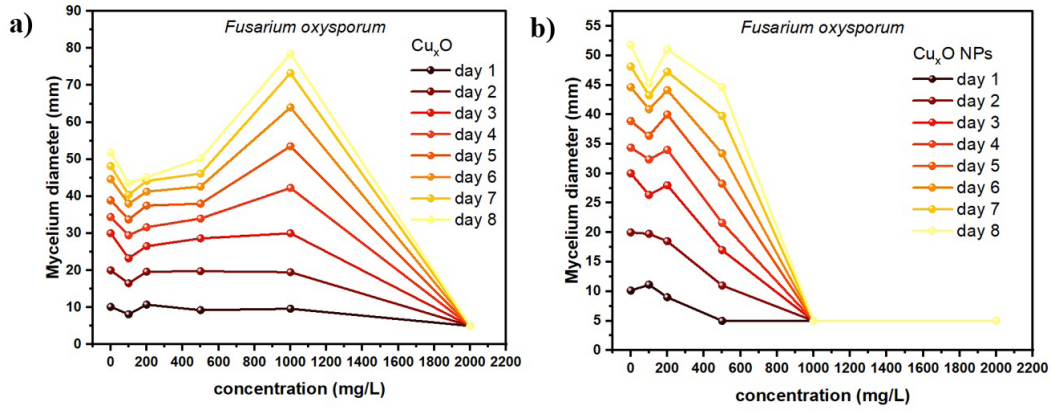
Rys. 2. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na dynamikę wzrostu grzybni *Botrytis cinerea* w zależności stężenia
Fig. 2. The influence of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on the growth dynamics of *Botrytis cinerea* mycelia



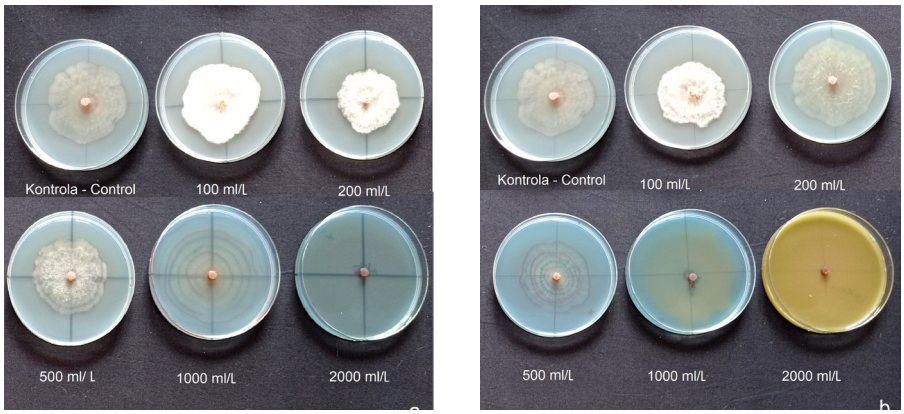
Fot. 2. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na wzrost grzybni *Botrytis cinerea*
Photo 2. The effect of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on the growth of *Botrytis cinerea*

In the presented study the highest concentrations of Cu_xO NPs, specifically 1000 and 2000 mg/l, resulted in the complete inhibition of *F. oxysporum* growth (100%). For Cu_xO results were more variable. Mycelial growth was limited at both 100 and 2000 mg/l, but unexpectedly, growth stimulation was observed at 1000 mg/l, making the effect of Cu_xO less consistent compared to Cu_xO NPs. Regardless of the concentration, the application of Cu_xO NPs consistently led to a stronger reduction in the pathogen growth than Cu_xO. Overall, Cu_xO NPs demonstrated superior and more reliable effectiveness in inhibiting *F. oxysporum* growth compared to Cu_xO, especially at higher concentrations. In *F. oxysporum* the most advantageous method seemed to be the use of 1000 mg/l Cu_xO NPs, which completely limited the mycelium growth (tab. 2, fig. 3). *Fusarium oxysporum* grew most intensively during the initial days of culture, but slowed over time (tab. 2, fig. 3, photo 3). Although concentrations of 100–500 mg/l Cu_xO NPs in our experiment did not significantly inhibit pathogen growth, other studies have reported effectiveness at similar or lower concentrations. For instance, López-Lima et al. (2021) observed a 67% reduction in pathogen growth at a concentration of 500 mg/l. Ashraf et al. (2021) reported that applying CuO-CFNPs to the roots and leaves of tomato plants infected with *F. oxysporum* significantly reduced disease occurrence across a range of concentrations (5–350 µg/ml). This treatment also improved plant growth rates and fruit quality. CuO-CFNPs at the concentration of 300 µg/ml reduced disease occurrence by 26.7%, and disease severity by 31.1%. In the study by Tiwari et al. (2024) 10 ppm CuO NPs reduced Fusarium wilt by 75%, through a combination of direct antimicrobial effects and induced defence responses, while also improving plant growth by mitigating oxidative stress.

The inhibition of *A. alternata* mycelium growth was effectively achieved with the use of Cu_xO NPs and Cu_xO. The most beneficial seems to be the use of CuO NPs at the concentration of 500 mg/l, which reduced the growth of the pathogen by an average of 76.64% compared to the control (with Cu_xO by an average of 29.43%), while the reduction in the pathogen growth was observed to a similar extent as



Rys. 3. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na dynamikę wzrostu grzybni *Fusarium oxysporum*
Fig. 3. The influence of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on the growth dynamics of *Fusarium oxysporum* mycelia



Fot. 3. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na wzrost grzybni *Fusarium oxysporum*
Photo 3. The effect of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on the growth of *Fusarium oxysporum*

when using a higher concentration (1000 mg/l Cu_xO NPs and 1000 mg/l Cu_xO). On average, mycelial growth was slower with Cu_xO than in the control, but the effect was less pronounced compared to Cu_xO NPs. Starting from the second day of culture, mycelium growth was consistently slower with Cu_xO NPs compared to Cu_xO, averaging 4.71 mm/day with Cu_xO NPs and 5.95 mm/day with Cu_xO. At equivalent concentrations, Cu_xO NPs consistently resulted in smaller mycelial diameters than Cu_xO. Regardless of the sample type, increasing concentrations of Cu_xO or Cu_xO NPs consistently reduced pathogen growth, with Cu_xO NPs demonstrating superior efficacy and reliability. *Alternaria alternata* overgrew the plates at a rate comparable to *F. oxysporum* (8 days) and more than twice as long as *B. cinerea* (3 days), growth was slower starting on the second day when treated with Cu_xO NPs compared to Cu_xO (tab. 3, fig. 4, photo 4). Banik and Pérez-de-Luque (2017) found that only the highest concentrations of Cu NPs significantly inhibited the growth of *A. alternata* mycelium, with complete inhibition achieved at 800 mg/l, while growth reduction began at 200 mg/l. Our findings revealed a similar trend: the inhibition of *A. alternata* growth increased with higher concentrations of CuO NPs, but complete inhibition was observed only at the higher concentration of 2000 mg/l. This aligns with observations by Al Abboud and Alawlaqi (2011), who reported that *A. alternata* tolerated copper ions in the growth medium up to 800 mg/l, but growth ceased entirely at 1000 mg/l. Abou-Salem et al. (2022) further demonstrated that the antimicrobial effectiveness of Cu NPs increased with concentration; applying 70 and 90 µg/ml Cu NPs resulted in 100% antifungal efficiency. Also in the study by Consolo et al. (2020), CuO NPs significantly inhibited mycelial development of *A. alternata* in a concentration-dependent manner.

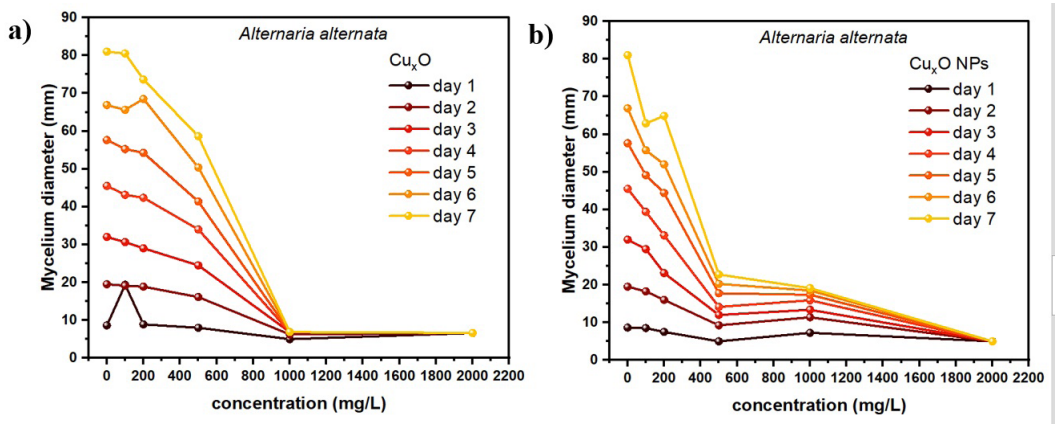
Tabela 3. Wpływ wybranych stężeń Cu_xO i Cu_xO NPs [mg/l] na średnicę grzybni *Alternaria alternata* [mm]
Table 3. The influence of selected concentrations of Cu_xO and Cu_xO NPs [mg/l] on mycelia diameter of *Alternaria alternata* [mm]

Stężenie próbek Samples concentration [mg/l]	Próbki – Samples		Średnia Mean
	Cu_xO	Cu_xO NPs	
0	81.00 ± 1.00 a	81.00 ±1.00 a	81.00 A
100	80.50 ± 2.06 a	62.88 ±10.85 bc	71.69 B
200	73.62 ± 1.43 ab	64.88 ± 2.38 bc	69.25 B
500	58.63 ± 20.99 c	22.75 ± 0.83 d	40.69 C
1000	6.88 ± 1.43 ef	19.13 ± 9.90 de	13.01 D
2000	6.63 ± 1.02 f	5.00 ± 0.00 f	5.82 D
Średnia – Mean	51.21 A	42.61 B	

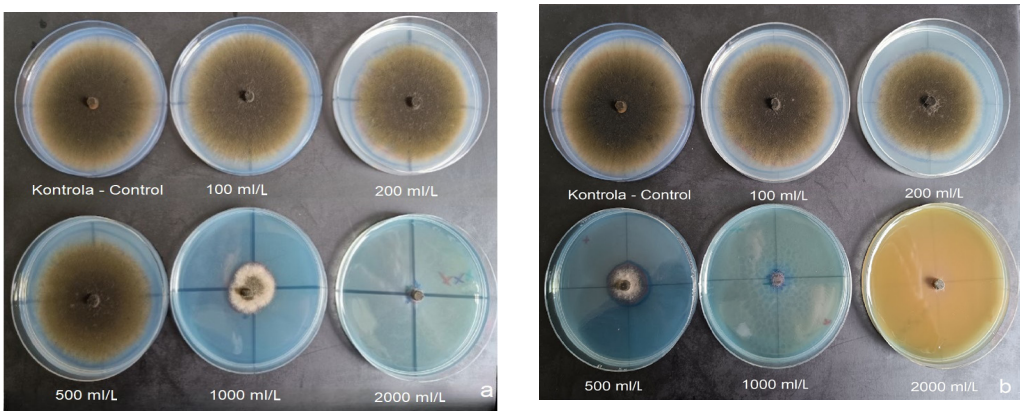
Średnie oznaczone tą samą literą, nie różnią się istotnie przy $p \leq 0,05$ (test Fishera). Wielkie litery odnoszą się do głównych efektów (niezależnie), a małe litery do interakcji między dwoma badanymi zmiennymi niezależnymi
Means followed by the same letter do not differ significantly at $p \leq 0.05$ (Fisher test). Upper-case letters refer to the main effects (irrespectively), lower-case letters refer to the interaction between the two studied independent variables

Overall, the inhibition of all three pathogen's mycelium growth varied depending on the type and concentration of copper oxide samples used, with Cu_xO NPs consistently demonstrating more effective inhibition than Cu_xO across all pathogens. Cu_xO NPs showed faster, more potent, and more reliable inhibitory effects compared to Cu_xO, particularly at higher concentrations, making them a more effective tool in managing pathogen development.

In the studies conducted by Banik and Pérez-de-Luque (2017), the enhancement in mycelial growth was observed in some tested pathogens, including *Botrytis fabae*, *F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *melonis*, and *A. alternata*, when treated with copper nanoparticles (Cu NPs) at the concentration of 100 mg/l. Differences in susceptibility can be associated with each fungus's intrinsic morphological and structural characteristics. This suggests that individual



Rys. 4. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na dynamikę wzrostu grzybni *Alternaria alternata*
Fig. 4. The influence of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on growth dynamics of *Alternaria alternata* mycelia



Fot. 4. Wpływ wybranych stężeń [mg/l] Cu_xO (a) i Cu_xO NPs (b) na wzrost grzybni *Alternaria alternata*
Photo 4. The effect of selected concentrations [mg/l] of Cu_xO (a) and Cu_xO NPs (b) on the growth of *Alternaria alternata*

pathogens may respond differently to copper nanoparticles, potentially even in a strain-specific manner, highlighting the need for further research in this area. The mechanisms of antifungal action for copper nanoparticles are complex and involve several key processes that enhance their antifungal efficacy. The primary mechanism is the generation of oxidative stress through the production of reactive oxygen species (ROS). ROS damage fungal cellular components, including lipids, proteins, and DNA, leading to cellular dysfunction and death. Cu_xO NPs' pro-oxidative activity, driven by their inorganic copper content, contributes to increased oxidative stress in fungal cells, enhancing their antifungal activity. Additionally, Cu_xO NPs interact with the fungal cell membrane, altering its organization and structure. The small size and spherical shape of the Cu_xO NPs facilitate their penetration into fungal cells, where they affect membrane rigidity, making it more prone to damage. This disruption compromises the integrity of the fungal cell membrane, causing leakage of vital intracellular components and ultimately leading to cell death. The surface charge of Cu_xO NPs also plays a crucial role in their antifungal mechanism. The positive zeta potential of Cu_xO NPs promotes their interaction with the negatively charged cell wall components of many fungal species. This interaction enhances the accumulation of Cu_xO NPs at the cell surface, facilitating their penetration and increasing their efficacy as antifungal agents. Additionally, Cu_xO NPs plays a crucial role in enhancing the plant's antioxidant defence mechanisms by increasing H_2O_2 scavenger activity, which helps mitigate oxidative damage caused by both biotic and abiotic stressors. This reinforces the plant's resilience against various types of environmental and physiological stress, further improving its overall health and defensive capabilities. Further research is still needed to focus on a deeper understanding of the interactions between Cu_xO NPs and various molecules, as well as their selectivity, to minimize their potential toxicity (Mali et al. 2020; El-Abeid et al. 2024; Krumova et al. 2024).

Copper-based antimicrobial compounds are effective tools for managing crop diseases; however, excessive cop-

per use can lead to its accumulation in soil, contamination of surface and subsurface waters, and eventual entry into the food chain (Lamichhane et al. 2018). Since copper oxide NPs seem more effective, and might be used in smaller quantities, they seem to be a viable alternative to CuO. For example, CuO NPs improved wheat seed germination at concentrations as low as 0.1 mg/l, and were more efficient than bulk Cu fertilizers, with the potential to reduce traditional fertilizer use by 80%. Even though both forms of Cu reduced root growth, CuO NPs showed lower toxicity compared to bulk Cu, suggesting that CuO NPs may be a safer alternative for plant growth under certain conditions (Ibrahim et al. 2022). Similarly, although both bulk and copper oxide nanoparticles negatively affected barley growth in the concentration-dependent manner, with higher concentrations reducing seed germination, root length, and shoot length, CuO NPs exhibited less morphological and anatomical damage compared to bulk CuO at lower concentrations (Rajput et al. 2021). However, future studies need to address the impact of NPs on yield, human health and the environment, and the quantity used needs to match the stage of the crop and disease intensity (Rajput et al. 2020). For example, it was found that CuO NPs had a concentration-dependent effect on pea seeds, with 0.1 mg/l promoting root and sprout growth, while concentrations ≥ 1 mg/l exhibited potential toxicity, becoming significant at 10 mg/l (Chidiamassamba et al. 2024; Nagdalian et al. 2024). It is also important to take into consideration NPs' impact on non-target organisms such as aquatic invertebrates (Ghosh et al. 2024).

Parada et al. (2024) proposed combining CuO NPs with fungicides at lower concentrations to limit its impact. Their findings suggested that this approach not only reduced the amount of both agents required to inhibit fungal growth, but also limited the release of copper into the environment. However, limited research exists regarding the extent to which antifungal activity is affected when copper is used in nanoparticle form. Moreover, they demonstrated that combining CuO NPs with fungicides is more efficient than using each agent independently. Specifically, the amount of CuO NPs needed to inhibit the mycelial biomass of *B. cinerea* and *F. oxysporum* was reduced several-fold. CuO NPs were also tested with fungicides for controlling cucumber root rot disease caused by *F. solani*, showing enhanced antifungal activity compared to untreated control plants. When combined with fungicides, NPs improved plant resistance by boosting defence enzyme activity and gene expression, while also promoting better growth and yield in cucumbers (Kamel et al. 2022). The study by Malandrakis et al. (2020) demonstrated that copper NPs in combination with conventional fungicides were effective in suppressing *B. cinerea* isolates, including those resistant to multiple fungicides. NPs showed additive or synergistic effects when combined with fungicides like thiophanate methyl and fluazinam, suggesting their potential in combating drug resistance and sustainably reducing fungicide use. In the study by Sardar et al. (2022) combined zinc oxide and copper oxide nanoparticles demonstrated strong antifungal activity against *Alternaria citri*, the pathogen responsible for citrus black rot.

Currently, one of the primary challenges of using copper oxide-based NPs in the field is the ongoing evaluation of regulations regarding the use of nanomaterials in agriculture (Parada et al. 2024). Our results confirm the promising potential of Cu_xO NPs in controlling pathogen growth, offering significant prospects for the sustainable development of plant protection strategies. Future studies should focus on further explanation of the mechanisms of action of these nanomaterials, their interactions with various pathogens, and their environmental and human health impacts, contributing to the development of effective plant protection approaches.

Wnioski / Conclusions

1. The synthesized and used by us Cu_xO and Cu_xO NPs samples were found to significantly inhibit the mycelial growth of *A. alternata*, *B. cinerea*, and *F. oxysporum* during *in vitro* experiment.
2. In *A. alternata*, the most beneficial was the use of Cu_xO NPs at the concentration of 500 mg/l, which reduced mycelium growth by an average of 76.64%.
3. In *B. cinerea*, the application of Cu_xO NPs at the concentration of 500 mg/l also seemed to be the most optimal, because it reduced the growth of mycelium at the levelof over 70% compared to the control.
4. In *F. oxysporum*, only the use of 1000 and 2000 mg/l Cu_xO NPs or 2000 mg/l Cu_xO was effective in the limiting of mycelium growth, and the reduction reached 100%.
5. Plant pathogens responded differently to Cu_xO NPs, which requires further detailed research, especially regarding the determination of optimal concentrations to limit their growth. However, it is worth noting the undeniable advantage of Cu_xO NPs over Cu_xO in inhibiting the growth of some of them.
6. The efficacy of Cu_xO NPs was dependent on the pathogen type and concentration. Concentration optimization, particularly for each pathogen and specific application context, is critical in maximizing antifungal efficacy, while minimizing potential toxicity.

Zgłoszenie patentowe / Patent application

Opracowana metoda została zgłoszona do Urzędu Patentowego Rzeczypospolitej Polskiej jako zgłoszenie patentowe P.448515 „Sposób ograniczania wzrostu grzybni patogenów roślinnych *Alternaria alternata*, *Botrytis cinerea* oraz *Fusarium oxysporum* z zastosowaniem nanocząstek tlenku miedzi".

The elaborated method was sent to the Patent Office of the Republic of Poland as a patent application no. P.448515 „Method of limiting the growth of mycelium of plant pathogens *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum* using copper oxide nanoparticles".

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