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Biogenic copper oxide nanoparticles from *Trichoderma harzianum*: a novel approach for managing wheat blast disease

Nanopartículas de óxido de cobre biogénicas de *Trichoderma harzianum*: un nuevo enfoque para el manejo del brusone del trigo

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ABSTRACT: Wheat blast, caused by the fungus *Pyricularia oryzae Triticum* pathotype (PoT), is a devastating disease in South America, Asia and Africa due to limited fungicide effectiveness and a lack of resistant varieties. More recently, it has also been detected in Africa, further exacerbating the global threat posed by this pathogen. In Argentina, it has been detected since 2012, but although no outbreaks have yet been recorded, this pathogen represents an imminent risk due to its presence in the nearby countries. For that, new strategies should be considered for controlling the disease and proper surveillance. Nanotechnology can contribute to protecting crops since it offers different mechanisms of action against pathogens. Thus, metallic oxide nanoparticles obtained by physicochemical or biogenic methods can act as antimicrobials. This study involved the biosynthesis of green copper oxide nanoparticles (CuONPs) from the fungus *Trichoderma harzianum* and evaluation of their ability to reduce fungal mycelium growth and wheat blast disease symptoms in plants. Physicochemical characterization of the CuONPs performed by TEM and EDS showed elongated fibers in shape and an average size of 397 ± 55 nm in length and 124 ± 13 nm in width, as good physico-chemical stability. *In vitro* and *in vivo* experiments to evaluate the potential of CuONPs against PoT showed that they were effective in strongly inhibiting the mycelial growth of PoT native aggressive strains PY15, PY22 and PY34 by 74, 72 and 67% respectively, at a concentrationof 1000 ppm. Moreover, CuONPs at a concentration of 500 ppm applied as a foliar spray on wheat plants inoculated with PY34 caused a reduction of 95% in disease severity. Further, wheat plants in which their seeds were previously pelletized with 500 ppm CuONPs showed a disease symptom reduction of 90%. These findings confirm that the biosynthesized CuONPs have a promising antifungal activity, which could be used as protection of wheat against PoT pathogen.
KEYWORDS: biogenic nanoparticles, PoT, wheat blast management, copper oxide.

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RESUMEN: El brusone del trigo, causado por el hongo *Pyricularia oryzae* patotipo *Triticum* (PoT), es una enfermedad devastadora en América del Sur, Asia y África debido a la limitada efectividad de los fungicidas y la falta de variedades resistentes. En Argentina, el patógeno ha sido detectado desde 2012, aunque no se han registrado brotes hasta la fecha, representa un riesgo inminente dada su presencia en países limítrofes. Por lo tanto, se deben considerar nuevas estrategias para controlar la enfermedad y una vigilancia adecuada. La nanotecnología puede contribuir protegiendo cultivos agrícolas, al ofrecer diferentes mecanismos de acción contra los patógenos. Así, las nanopartículas de óxidos metálicos obtenidas por métodos fisicoquímicos o biogénicos pueden actuar como antimicrobianas. Este estudio involucró la biosíntesis de nanopartículas de óxido de cobre (CuONPs) biosintetizadas a partir del hongo *Trichoderma harzianum* y la evaluación de su capacidad para reducir el crecimiento del micelio fúngico y los síntomas de la enfermedad del brusone de trigo en plantas bajo condiciones controladas. La caracterización fisicoquímica de las CuONPs realizada por TEM y EDS mostró fibras alargadas en forma y un tamaño promedio de 397 ± 55 nm de largo y 124 ± 13 nm de ancho, con una buena estabilidad fisicoquímica. Los experimentos *in vitro* e *in vivo* para evaluar el potencial de las CuONPs contra PoT demostraron ser efectivas en inhibir fuertemente el crecimiento micelial de las cepas nativas de PoT PY15, PY22 y PY34 en un 74%, 72% y 67%, respectivamente, a una concentración de 1000 ppm. Además, las CuONPs a una concentración de 500 ppm aplicadas en aspersión foliar sobre plantas de trigo inoculadas con PY34 causaron una reducción del 95% en la severidad de la enfermedad. Además, las plantas de trigo cuyas semillas fueron previamente pelletizadas con 500 ppm de CuONPs mostraron una reducción del 90% en los síntomas de la enfermedad. Estos hallazgos confirman que las CuONPs biosintetizadas poseen una actividad antifúngica, que podría utilizarse para optimizar la protección del bruzone del trigo causado por PoT.
PALABRAS CLAVE: nanopartículas biogénicas, PoT, manejo del brusone del trigo, óxido de cobre.

Introduction

Nanotechnology is an emerging area of research with the potential to be applied in different fields of analytical chemistry and environmental science, such as medicine, agriculture, and the pharmaceutical industry (Sajid and Płotka-Wasylka, 2020). Particularly, nanoparticles (NPs) are material with unique properties, ranging in size and at least one spatial dimension within a size scale from 1-100 nm (Selmani *et al*., 2022). Currently, there is a wide variety of metallic NPs, such as silver, gold, copper, titanium and zinc, which have particular properties that depend mainly on their size, shape, geometry and surface area (Slavin and Bach, 2022). In agriculture, the application of innovative technological solutions based on nanotechnology has been an emerging topic. The main advantages of exploring the use of nanoparticles against plant pathogens are the decreased toxicity of soil and reduced frequency of application when compared with synthetic antifungal agents (Ali *et al*., 2023; Vanti *et al*., 2020), which allows the partial substitution of agrochemicals and reduces the negative effects associated with their continuous use.

Wheat is one of the most important cereals worldwide, and blast caused by the ascomycete fungus *Pyricularia oryzae Triticum* pathotype (PoT) is an emerging and preoccupying disease in tropical and subtropical crop production regions currently confined to South America and Asia, although there is a potential risk of expansion into other regions (Ceresini *et al*., 2019). In Argentina, PoT was identified for the first time in 2007 (Cabrera and Gutiérrez, 2007). The pathogen significantly reduces wheat yields and grain quality, ranging from 40 to 100% under favorable climatic conditions, as temperatures between 25-29 ºC, and relative humidity up to 70% (Castroagudín *et al*., 2016). In this sense, traditional management using fungicides is not effective in controlling wheat spike blast if warm, rainy weather occurs during the heading stage (Wang *et al*., 2018); therefore, other alternatives should be considered as quarantine measures, cultural practices, resistant cultivars and non-fungicidal chemical treatments, which are also effective components of integrated wheat blast management.

Copper is an essential trace element for crop growth and has traditionally been used in agriculture as an alternative method for controlling a broad spectrum of phytopathogenic microorganism (Hasanin *et al*., 2022), due to copper's interaction with nucleic acids, interference with energy transport, and disruption of enzyme activity and integrity of cell membranes playing an important role in integrated pest management (Pscheidt and Ocamb, 2022). However, because of its accumulation in the soil, innovative formulations with reduced copper content could be more attractive. One strategy to maximize its effectiveness is to reduce the particle size to improve the coverage of the treated surface by reducing the total amount of copper required to obtain the same effect. Thus, the use of nanoparticles (NPs) could be an alternative method with greater effects than copper salts (Khan *et al*., 2023).

Fungal green biosynthesis of NPs is a promising eco-friendly method for mass-scale production since physicochemical methods require the use of toxic solvents, which generate hazardous products for the environment. Particularly, NPs made from the genus *Trichoderma* have been used successfully against different fungal phytopathogens. As example, Guilger *et al*. (2017) synthesized silver NPs from *T. harzianum* with potential against *S. sclerotiorum*. Joshi *et al*. (2019) obtained selenium NPs from *T. atroviride* that showed antifungal activity against *Pyricularia grisea* under *in vitro* conditions. On the other hand, Consolo *et al*. (2020) reported that both Ag and CuONPs caused a significant reduction in mycelial development of *A. alternata* and *P. oryzae* in a dose dependent concentration. Recently, Sanguiñedo *et al*. (2023) reported that Cu and Ag NPs from *T. harzianum* showed antifungal activity against *Sclerotium oryzae*, *Rhizoctonia oryzae-sativae*, *Fusarium graminearum* and *P. oryzae*.

More broadly, research carried out in Argentina warns about the aggressiveness of the PoT pathogen, the reports show that the pathogen can attack plants at any growth stage, with a wide range of aggressiveness levels, ranging from 10-100% depending on the strain/cultivar interaction and the physiological stage of the crop (Martínez *et al.,* 2019, 2021; Martínez and Perelló, 2024).

In this context, taking into account that the disease caused by PoT represents a potential threat to wheat cultivation and it is necessary to identify disease control strategies, we carried out the biogenic synthesis and characterization of copper oxide nanoparticles (CuONPs) from a cell filtrate of *T. harzianum* strain. The CuONPs were characterized according to their size and morphology and their effects against PoT fungi were evaluated under *in vitro* conditions in a dose response curve. Moreover, this study aimed to investigate the possible effects of NPs applied as foliar spray or after seed treatment on plants inoculated with the phytopathogen.

Material and methods

Fungal strains

Trichoderma harzianum strain IB-363 from the INBIOTEC culture collection and PoT strains PY15, PY22 and PY34 from CIDEFI culture collection were grown on potato dextrose agar (PDA) medium at 24 ºC for 5-7 days.

Biosynthesis of copper nanoparticles

To obtain fungal biomass, two agar discs of 5 mm of mycelia from *T. harzianum* grown for 7 days on potato dextrose agar (PDA, Britania) were transferred to an Erlenmeyer flask containing 50 ml of culture broth (KH_2PO_4 (7 g L^{-1}), K_2HPO_4 (2 g L^{-1}), $MgSO_4.7H_2O$ (0.1 g L^{-1}), $(NH_4)_2SO_4$ (1 g L^{-1}), yeast extract (0.6 g L^{-1}), glucose (10 g L^{-1})), according to Consolo *et al*. (2020). The cultures were incubated on an orbital shaker at 150 rpm at 24 ºC for 5 days. Further, the mycelium was harvested, washed with sterile double distilled water, and then transferred to a flask containing 50 ml of sterile double distilled water for 72 h on an orbital shaker at 24 ºC. After incubation, the fungal biomass was separated with a spatula, and the aqueous filtrate was stored. Synthesis of copper nanoparticles was carried out using 50 ml of aqueous culture filtrate in flasks, adding $CuSO_4.5H_2O$ at a final concentration of 50 mM solution. The reaction was carried out in dark conditions at 40 ºC, stirring vigorously, and adjusting to pH 11 with NaOH. Simultaneously, a positive control composed of the culture aqueous filtrate without copper, as well as its respective negative control, 50 mM of metal salt in deionized water, was checked. The nanoparticles were separated by centrifugation (8,000 rpm for 10 min), washed twice with double distilled water and then lyophilized. NPs were stored in polypropylene tubes under dark conditions at room temperature. The experiment was performed in triplicate.

Physicochemical characterization

TEM and EDS

In order to characterize the biosynthesized NPs, transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDS) were performed. Previously, NPs were suspended in 1 ml of deionized water by sonication for 1 minute at 25% amplitude at room temperature and deposited onto a copper grid with a lacey carbon film coating. Observation of NPs was carried out under a transmission electron microscope equipped with an Oxford X-

MAX 65 T EDS (JEOL JEM-2100 Plus, Tokyo, Japan) in the HRTEM and HAADF mode, and X-ray images and spectra were also obtained using an acceleration voltage of 200 kV.

Biological activity under *in vitro* assay

To determine the biological activity of CuONPs on the growth of the PoT fungus, a culture medium poisson assay was carried out according to Kalia *et al*. (2020). PoT strains PY15, PY22 and PY34 were replicated on PDA medium and grown at 26 ºC for 7 days. Once the mycelium had developed, a disc of 5 mm of young mycelium was placed in the center of a new Petri dish containing PDA supplemented with a range of 100 to 1000 ppm of CuONPs. The plates were kept at 26 ºC under dark conditions. The assays were performed in triplicate, and as a negative control, each fungal strain was grown in Petri dishes containing only PDA. To determine the inhibitory effect of NPs on fungal growth, the colony radius was determined by measuring the average length of the PoT mycelium taken from the 5 mm disc seeded at the center of the plate to the edge of the colony. Determinations were made 6 days after seeding the mycelial disc in the culture medium. The percentage of growth inhibition was determined following the formula 1 described by Vera-Reyes (2019):

$$\%\ \text{fungal growth inhibition} = [(c-t)/c] \times 100 \qquad (1)$$

where *c* is the average of the control growth and *t* is the average growth with the treatment.

To identify morphological fungal effects caused by the NPs, strains were grown as described above, adding 500 ppm of CuONPs in the culture media. Then, mycelium was stained with trypan blue, and observed under an optical microscope and registered with a digital camera (Arcano), and the hyphal thickness was measured using Image-J software.

In vivo effects of CuONPs on wheat plants infected by PoT

For plant assay, wheat seeds (*Triticum aestivum* L. var. Baguette) were surface sterilized in a NaClO solution 1% (vol/vol) for 2 min and then rinsed three times with sterile water.

To evaluate the effect of CuONPs on wheat blast disease progress, we selected PY34, which is one of the most aggressive fungal strains assayed (Martínez *et al*., 2021; Martínez and Perelló, 2024). We carried out two independent experiments: one of them included plants whose seeds were pelletized with NPs, and the other one was carried out by spraying NPs on the leaves and plant aerial parts.

For pelletizing treatment, seeds were coated in 50 ml of 1‰ agar water solution containing 0 (control), 250 and 500 ppm of CuONPs in continuous stirring for 2 min. After that, seeds were pre-germinated in Petri dishes containing moist filter paper, and kept in the dark for 4 days. Plastic pots were filled with 1kg of a substrate composed of commercial soil and vermiculite at a proportion of 2:1. Subsequently, three seeds were placed per pot, and then plants were grown for 15 days at Z1.3 stage (Zadoks *et al*., 1974) and maintained in a growth chamber (26 ± 2 ºC, 16/8 h light/dark and watered regularly) until fungal inoculation.

Another group of plants was cultivated in a growth chamber during 15 days as described above, and then individually foliar sprayed after inoculum application with CuONPs at a dose of 0 (control), 250, and 500 ppm. A completely randomized design was used for pot arrangement, and all experiments were performed in triplicate.

Inoculum preparation

PoT strain PY34 was grown on oat-meal agar and incubated at 26 ºC for 10 days under alternating cycles of 12/12 h light/dark conditions to increase the rate of sporulation (Martínez *et al*., 2019). Subsequently, sporulating plates were flooded with 25 ml of a solution composed of distilled water and 1% tween 20 per Petri dish. Spores were removed by scraping with a metal spatula and the mycelia and conidia were separated by filtration through a fine mesh nylon cloth. Finally, the solution was adjusted to a concentration of 5.5 x 10^4 conidia/ml using a Neubauer chamber.

Pathogenicity assay

Plants from pelletized or non-pelletized seeds were inoculated with PoT PY34 fungus by spraying leaves with a fungal suspension. The inoculum was applied directly as a spray with a hand sprayer at a volume of approximately 3 ml per pot. The plants were covered with polyethylene for 24 h and maintained at 26 ± 2 ºC in dark conditions to ensure infection with the pathogen. Then, they were kept at 25 ºC under a 16/8 light/dark photoperiod, and watered every 48 h, maintaining a humidity of up to 90%. We evaluated plants daily to determine the development and progression of lesions according to Urashima *et al*. (2005), in which phenotypic reactions were recognized as 0 = no visible reaction; 1 = pinhead-sized spots; 2 = small brown to dark brown lesions with no distinguishable centers; 3 = small eyespot shaped lesions with gray centers; 4 = typical blast lesions, elliptical with gray centers; 5 = complete blighting and leaf death. When different types of lesions were found on a single leaf, the largest lesions were considered. Fifteen days after inoculation, the percentage of leaf area showing the characteristic disease symptoms was determined. Disease severity was calculated as a percentage, according to the following formula 2:

$$\text{Severity (\%)} = [\text{diseased leaf area}/\text{total leaf area}] \times 100 \qquad (2)$$

Statistical analysis

For *in vitro* assay and plant pathogenicity experiments, completely randomized block designs with three replicates were conducted. The experimental data were statistically analyzed by one way analysis of variance (ANOVA). Means and standard deviations were calculated and statistically examined using analysis of variance and Tukey's multiple range test at $p < 0.05$. For graphics, GraphPad Prism 5.0 was used.

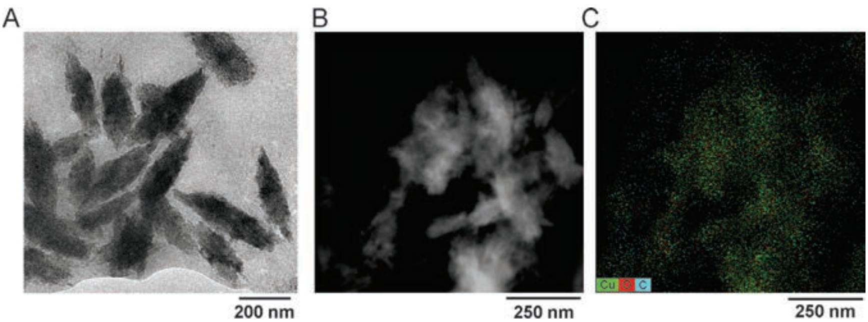
Results

Biosynthesized nanoparticles and characterization

From the aqueous filtrate of *T. harzianum*, CuONPs were successfully synthesized within 1 h after adding 50 mM copper sulfate and alkalinization with NaOH at pH 11. The formation of NPs was evidenced by the change in the color suspension of the aqueous filtrate from blue to a colloidal dark brown color.

The morphology and size of the NPs determined by TEM images showed that CuONPs were dispersed and elongated fibers. The average size was 397 ± 55 nm in length and 124 ± 13 nm in width (figures 1A and 1B). The atomic composition determined by EDS analysis revealed Cu (> 26%) and O (> 36%) (figure 1C).

FIGURE 1. Characterization of CuONPs biogenically synthesized with *Trichoderma harzianum* strain IB-363.



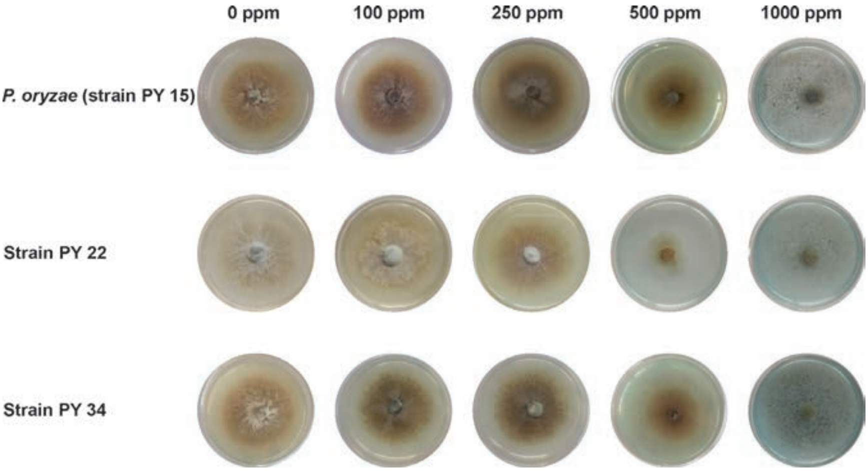
(A, B) transmission electron microscopy (TEM) and (C) energy dispersive X-ray spectroscopy (EDS) analysis.
Source: Author's elaboration.

In vitro biological activity of CuONPs

The inhibitory effect of CuONPs supplemented in the fungal growth culture media observed after 6 days of treatment showed a significant reduction in mycelia development in a dose dependent concentration (figure 2). Thus, supplementation of the culture medium at a final concentration between 100-200 ppm showed a low mean inhibitory percentage in mycelial growth for all strains (table 1). However, when the concentration of NPs in the culture medium was increased to 500 ppm, strains PY15, PY22 and PY34 showed mean growth inhibition values of 42, 27 and 18%, respectively. In turn, the

1000 ppm dose of CuONPs showed a percentage inhibition of up to 74% for strain PY15, 72% for PY22 and 67% for PY34 (table 1). Additionally, fungal mycelia of PoT strains grown for 7 days in culture medium, supplemented with 500 ppm of CuONPs, showed morphological alterations, exhibiting vacuolization and a significant reduction in hyphal size in comparison with their respective control (figure 3, supplementary table 2).

FIGURE 2. Inhibitory effect of biosynthesized CuONPs against PoT strains.



Fungal colonies in PDA supplemented with 0-1000 ppm.
Source: Author's elaboration.

TABLE 1. Efficacy of biogenic CuONPs on the growth of pathogenic fungi *in vitro*.

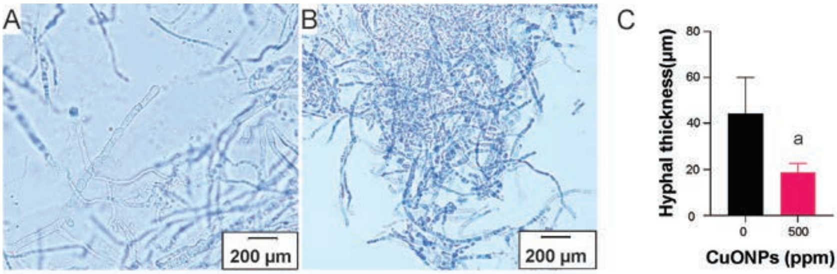
CuONPs (ppm)	*P. oryzae* (strain PY15)		*P. oryzae* (strain PY22)		*P. oryzae* (strain PY34)	
	Colony diameter (mm)	MGI%	Colony diameter (mm)	MGI%	Colony diameter (mm)	MGI%
0	1.64 ± 0.13		1.15 ± 0.16		1.51 ± 0.12	
100	1.7 ± 0.09	0	1.01 ± 0.03	12 (a)	1.38 ± 0.12	8
250	1.62 ± 0.18	2	1.02 ± 0.09	11 (a)	1.31 ± 0.12	13 (a)
500	0.96 ± 0.05	42 (a)	0.84 ± 0.09	27 (b)	1.23 ± 0.10	18 (a)
1000	0.43 ± 0.14	74 (b)	0.32 ± 0.06	72 (c)	0.5 ± 0.17	67 (b)

Values represent means of three biological replicates ± SD. Different letters within each column show a significant difference among the treatments at $p \leq 0.05$, derived from Tukey's multiple range test at $p < 0.05$. MGI: mycelia growth inhibition.
Source: Author's elaboration.

Effect of CuONPs on plant disease progress

The effect of CuONPs on wheat plants after 15 days of fungal inoculation with strain PY34 showed that both plants whose seeds were treated before sowing with CuONPs, or were sprayed on leaves, presented a reduction in leaf

FIGURE 3. Mycelial growth effect of CuONPs on PoT.



(A) PY 34 strain in PDA; (B) in PDA supplemented with 500 ppm; (C) hyphal thickness in control (0 ppm) and 500 ppm after 7 days of growth (n = 15). Different letters represent significant differences compared to the control at $p < 0.05$, derived from Tukey's test.
Source: Author's elaboration.

Supplementary material:

TABLE 2. Effect of biogenic CuONPs on the thickness of PoT pathogenic hyphae *in vitro*.

CuONPs (ppm)	*P. oryzae* (strain PY34)
	Hyphal width (μm)
0	44.28 ± 15.74 (a)
500	18.89 ± 3.82 (b)

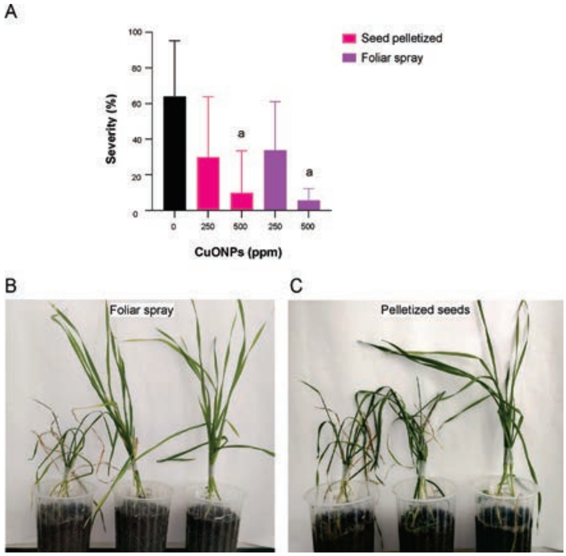
The values represent the mean thickness of 15 randomly selected PoT hyphae, which was determined using Image-J software ± SD. Different letters within each column represent a significant difference among the treatments derived from T-Test at $p < 0.05$.
Source: Author's elaboration.

blast symptoms. The application of 250 ppm of CuONPs through foliar spraying or seed pelletizing showed a reduction of the blast disease of 67% and 70%, respectively (figure 4A); meanwhile, at a concentration of 500 ppm, the disease severity was reduced by 90% and 95%, respectively. Further, both ways of CuONPs application not only delayed the appearance of disease symptoms, but also plants showed a better performance resulting in healthy plants, mainly at a concentration of 500 ppm (figures 4B and 4C). Control plants inoculated only with PoT inoculum showed typical blast lesions 4-5 and around 75% severity.

Discussion

Green synthesis of metal nanoparticles is considered an advantageous methodology when compared to traditional synthesis, because it can minimize the use of harmful chemical compounds and simultaneously reduce energy consumption (Mantuano *et al*., 2020). In this sense, fungal-mediated extracellular biosynthesis could be an excellent approach to produce NPs at a low cost in an eco-friendly manner.

FIGURE 4. Effect of CuONPs on wheat blast disease after inoculation with PY34 PoT strain.



(A) Disease severity of wheat plants after foliar spray or seed pelletizing at 0, (control) 250 and 500 ppm; (B) foliar damage caused by the phytopathogenic fungus in plants after spray application of NPs or, (C) under seed pelletizing treatment. Letters within each column represent a significant difference among the treatments derived from Tukey's multiple range test at $p < 0.05$.
Source: Author's elaboration.

In agriculture, the application of NPs is a novel approach that could be considered as a very effective tool against plant pathogens or as growth promoters (Zhao *et al*., 2020). In this work, we successfully synthesized CuONPs from *T. harzianum* strain IB-363 under controlled conditions of temperature, pH and metal salt concentration. As reported, bioactive metabolites produced by different fungal species would be involved in the reduction of metallic salts for nanoparticle synthesis (Brenelli *et al*., 2019; Qamar and Ahmad, 2021; Zaki *et al*., 2021). Hence, the cell filtrate obtained by *T. harzianum* strain IB-363 was able to reduce blue copper sulfate pentahydrate to brown copper oxide in nanoscale particles. They were synthesized as elongated fibers (figures 1A and 1B) at a final concentration of 50 mM of copper sulfate salt, under alkaline conditions at 40 ºC. There are still few reports on the synthesis of CuONPs from *T. harzianum*. In this sense, Consolo *et al*. (2020) reported a similar morphology when conditions of 1-2 mM and 45 ºC were used. This highlights the great ability of the fungus filtrate to reduce the copper salt to a concentration 100 times higher, generating similar nanoparticles. On the other hand, recently Gaba *et al*. (2022) and Sanguiñedo *et al*. (2023) reported small spherical CuONPs of around 31-45 and 3.5 nm, respectively adding $CuCl_2$ to cell filtrates. Moreover, from T. viride and *T. asperellum*, Saravanakumar *et al*. (2019) and Sawake *et al*. (2022) obtained CuONPs with spherical shape using $Cu(NO_3)_2.3H_2O$ and $CuSO_4.5H_20$, respectively. Thus, we emphasize that to date, the particular morphology of copper NPs as elongated fibers has not been reported from *Trichoderma* or other fungal filtrates used as reducing agents.

In this work, we focused on the use of copper NPs because bulk copper compounds are naturally used as fertilizers and antifungals in agriculture due to their low cost, protective activity, and reduced risk for resistance development (Keller *et al*., 2017). Moreover, it was reported that copper NPs are more fungitoxic than copper sulfate. According to previous precedents, CuONPs have been shown to have a biocidal effect on the growth of different phytopathogenic fungi (Malandrakis *et al*., 2019; Consolo *et al*., 2020; González Merino *et al*., 2021; Mohamed *et al*., 2021; Gaber *et al*., 2023). However, to date, it has not been reported that biosynthesized CuONPs inhibit PoT mycelial growth under *in vitro* conditions. In this sense, although differences in susceptibility at a dose-dependent (0-1000 ppm) were demonstrated against the aggressive argentinian strains (PY15, PY22, PY34), as shown in figure 2 and table 1, a dose of 500 ppm was sufficient to inhibit in a range of 18-42% the growth of all strains. It was reported that deleterious effects could be induced by metallic NPs in the fungus, such as alterations in morphology (deformation, roughness, and weakening of the mycelium). Thus, these effects could be attributable to the disruption of cell wall compound synthesis, such as chitin, leading to a loss of cell wall stability and resulting in deformations and osmotic imbalances facilitating the permeation of CuNPs (Pariona *et al*., 2019). In this study, the use of CuONPs resulted in vacuolization and thinning of PoT fungal hyphae, demonstrating similar cellular alteration (figure 3), as shown in *Bipolaris* sp. after treatment with AgNPs (Al-Otivi *et al*., 2022). Additionally, Hashem *et al*. (2022) demonstrated that the use of biosynthesized AgNPs causes structural contraction, affecting various organelles and leading to an increase in vacuole volume, a decrease in cell size, and deformation of several organelles.

The application of biogenic copper nanoparticles in plants for the control of phytopathogens has been reported with promising results (Chakraborty *et al*., 2022), however, to date, no applications of biogenic metallic NPs have been documented in the pathosystem wheat-PoT which underscores the originality and relevance of our work.

This is the first report in which *in vivo* experiments were carried out on wheat plants inoculated with a higher virulent PoT strain (PY34) showing that both, plants whose seeds were treated before sowing with CuONPs, or were sprayed on leaves, had a drastic reduction in blast disease symptoms in a dose dependent manner. The effect was more noticeable when NPs were sprayed over leaves (figure 4). Similar results have been reported in finger millet-*P. grisea* pathosystem where the foliar application of copper-chitosan (Cu-Ch)NPs delayed the appearance of blast symptoms (Sathiyabama and Manikandan, 2018) or CuONPs in the pathosystem rice-*M. oryzae*, revealed a reduction of blast lesions around 15% (Chen *et al*., 2022). In this sense, other studies should be carried out to elucidate which is the target mechanism exerted by CuONPs if either the fungus loses its ability to colonize the host or it induces a plant defense signaling cascade that reduces the severity of the disease.

While CuONPs are emerging as a tool that offers a sustainable solution to mitigate the adverse effects of excessive use of pesticides and chemical fertilizers, it is crucial to recognize them as potential emerging pollutants, especially for water and soil. Although the toxic dose of CuONPs has been shown to be 10 times lower than Cu^{+2} in different organisms such as rodents, plants, aquatic organisms and cell cultures (Ameh and Sayes, 2019) it is necessary to achieve a balance in their application, ensuring agricultural efficiency and environmental health protection. Different studies carried out on tomato, lettuce, wheat, rice plants among others, in which different doses of CuNPs were applied have shown a promoting effect on plant growth in general at low doses, demonstrating that an appropriate concentration of nanoparticles can positively affect plants at a biochemical, morphological and physiological level, improving their productivity (Chen, 2018). In the same way, the control of fungal diseases by NPs will depend exclusively on the plant-pathogen relationship and must be adjusted for each particular pathosystem to optimize their use as well reduce its accumulation in the environment (Noman *et al*., 2023).

Particularly, although the effect of CuONPs on wheat plants has been little studied, beneficial effects have been reported after foliar application, improving yield, plant height, chlorophyll content, biomass as well as a reduction in the activity of oxidative stress enzymes (Alhaithloul *et al*., 2023). In other hand, and regarding toxicity, Zhang *et al*. 2021 compared the effect of CuONPs or Cu^{+2} in wheat plants at the same molar concentration demonstrating that NPs were less toxic since the release of Cu^{+2} from nanoparticle suspensions is very low and accumulates in smaller amounts in the roots and shoots. In view of this background, it is crucial to specify appropriate levels of copper nanoparticles for different crops in large-scale field applications to assess the potential hazardous effects on other non-target organisms.

Conclusions

In this study, CuONPs were successfully biosynthesized using the aqueous filtrate of a strain of *T. harzianum* as the reducing agent. This method is a simple, low-cost, and environmentally friendly alternative compared to conventional physicochemical methods. These metallic NPs exhibited *in vitro* antifungal activity against virulent PoT strains PY15, PY22 and PY34 inhibiting the mycelium growth between 67-74% at 1000 ppm. In other hand, wheat plants inoculated with PY34 showed a 95% reduction in disease severity when CuONPs were applied foliarly as well as through seed treatment at a concentration of 500 ppm.

This is the first report on demonstrating the potential of CuONPs as a new tool for successfully control the emerging disease wheat blast, replacing conventional antifungal compounds.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

Micaela Belén Gallo, *Andrés Torres Nicolini*, *Sergio Iván Martínez*: methodological development, analysis, interpretation and review.
Analía Edith Perelló: conceptualization, article design, interpretation, drafting of the original version.
Vera Alejandra Álvarez, *Verónica Fabiana Consolo*: conceptualization, article design, methodological development, analysis and interpretation, drafting of the original version, review, and final editing of the text.

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