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CROP PROTECTION

Nanoparticles in inhibiting *Pantoea ananatis* and to control maize white spot

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ABSTRACT: *Maize white spot (MWS) caused by Pantoea ananatis is one main maize leaf diseases, and nanoparticles (NPs) are an innovative approach for bacterial disease control. This research evaluated the toxicity of pure NPs and doped NPs with different elements in inhibiting bacterial growth and to control MWS. Pure NPs and ZnO NPs doped with silver (Ag), gold (Au), copper (Cu), iron (Fe), manganese (Mn), and nickel (Ni) at different concentrations were used to determine the toxicity for P. ananatis in vitro, evaluating the bacterial growth inhibition zone. To assess the control of MWS, in the preventive application, maize plants were sprayed with NPs of ZnO:0.1Cu, ZnO:0.05Fe, ZnO:0.2Mn and ZnO:0.7Ni at 10, 5 or 2.5 mg mL^{-1}, and after 3 days, the plants were inoculated with bacterial suspension. To assess the curative application, plants were inoculated with the bacteria, and 3 days later sprayed with the NPs. The disease severity was assessed and the area under the disease-progress curve (AUDPC) was calculated. The doped ZnO NPs with different elements, and at different concentrations inhibited bacterial growth in vitro. NPs of ZnO:0.1Cu and ZnO:0.2Mn at 5 or 2.5 mg mL^{-1}, in both applications reduced the severity of MWS, showing potential for use in the disease management.*
Key words: *bactericidal, nanocrystals, severity, Zea mays.*

Nanopartículas na inibição de *Pantoea ananatis* e no controle da mancha branca do milho

RESUMO: *A mancha branca do milho (MBM) causada por Pantoea ananatis é uma das principais doenças foliares da cultura, e as nanopartículas (NPs) surgem como inovação no controle de doenças bacterianas. O objetivo do presente trabalho foi avaliar a toxidez de NPs puras e dopadas com diferentes elementos, na inibição do crescimento bacteriano e no controle da MBM. NPs puras e NPs de ZnO dopadas com: prata (Ag), ouro (Au), cobre (Cu), ferro (Fe), manganês (Mn), e níquel (Ni) em diferentes concentrações foram usadas para determinar a toxidez à P. ananatis, avaliando-se o halo de inibição do crescimento bacteriano. Para avaliar o controle da MBM, na aplicação preventiva, plantas de milho foram pulverizadas com NPs de ZnO:0.1Cu, ZnO:0.05Fe, ZnO:0.2Mn e ZnO:0.7Ni a 10, 5 e 2.5 mg mL^{-1}, e três dias depois foram inoculadas com a suspensão bacteriana. Na aplicação curativa, as plantas foram inoculadas com a suspensão bacteriana e três dias após pulverizadas com as NPs. A severidade da doença foi avaliada e calculada a área abaixo da curva de progresso da doença (AACPD). NPs de ZnO dopadas com os diferentes elementos e concentrações inibiram o crescimento bacteriano in vitro. As NPs de ZnO:0.1Cu e ZnO:0.2Mn a 5 e 2.5 mg mL^{-1}, nas duas aplicações reduziram a severidade da MBM, apresentando potencial de uso no manejo da doença.*
Palavras-chave: *bactericida, nanocristais, severidade, Zea mays.*

Maize white spot (MWS) caused by the bacterium *Pantoea ananatis* (PACCOLA-MEIRELLES et al., 2001) can cause losses of over 60% in production, mainly in conditions of moderate temperature (14 °C) and high relative humidity (> 60%) (CASELA et al 2006). The use of resistant hybrids and the application of fungicides is recommended as measures of disease control (PEDRO et al., 2012).

Bacterial diseases are difficult to control once established in the field (COSTA et al., 2012), and innovative strategies should be evaluated for their control, such as the use of nanoparticles (NPs). NPs have reduced size (less than 100 nm), high reactivity (KAH & HOFMANN, 2014), and biocidal efficacy, interacting with the microbial membrane due to the high surface/volume ratio (ALLAKER, 2010).

Several NPs have been synthesized in the search for an efficient, biocompatible, and specific material, such as oxides of inorganic metals (SILVA et al., 2018). Zinc oxide (ZnO) NPs have high catalytic activity, chemical/physical stability, and biocompatibility (HE et al., 2019), generating interest in formulations for agricultural purposes. Its semiconductor nature induces the formation of

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reactive oxygen species (ROS), leading to oxidative stress and cell death (SABIR et al., 2014) .

To increase the bactericidal effect, ZnO NPs may be doped with different elements, which consists of the integration of new elements in the NP, enhancing and changing its physical, electronic, and chemical structure (SILVA et al., 2018). In the management of plant diseases the doped ZnO NPs have already been described for the control of *Fusarium* spp, *Xanthomonas axonopodis* pv. *citri, Xanthomonas oryzae* pv. *oryzae*, *Clavibacter michiganensi, Xanthomonas perforans*, *Pseudomonas syringae* and *Xanthomonas gardneri* (ELMER & WHITE, 2016; BALLOTTIN et al., 2017; RIVAS-CÁCERES et al., 2018; LIAO et al., 2019; ELSHARKAWY et al., 2020; FRAGA et al., 2021).

However, studies of NPs doped with different chemical elements and concentrations are required for suggesting alternatives for MWS control. Therefore, this study evaluated the toxicity of pure NPs, and ZnO NPs doped with silver, gold, copper, iron, manganese, and nickel to inhibit *Pantoea ananatis* growth *in vitro,* and the efficacy of ZnO NPs doped with copper, iron, manganese, and nickel in preventive and curative applications for the control of MWS.

The *Pantoea ananatis* strain (UFU A18) preserved and maintained in the research collection of the Laboratório de Bacteriologia Vegetal (LABAC), Instituto de Ciências Agrárias, Universidade Federal de Uberlândia (UFU), Minas Gerais was grown using the 523 growth media (KADO & HESKETT, 1970) for 48 hours at 28 °C. The bacterial suspension was prepared in sterile filtered water and adjusted to OD_{550} = 0.5 (10^8 CFU mL^{-1}) using a spectrophotometer. The *Pantoea ananatis* was confirmed by PCR using the species-specific primer pair ANAf/ANAr, that amplify a fragment of 389-bp (FIGUEIREDO & PACCOLA-MEIRELES 2012; SAUER et al. 2015)

To evaluate its bacterial activity, ZnO NPs doped with silver (Ag), gold (Au), copper (Cu), iron (Fe), manganese (Mn), and nickel (Ni) at different concentrations, as well as the pure NPs of AgO, Au, CuO, FeO, MnO, NiO, and ZnO were tested (Table 1). The NPs approximate size was 20 nm, and they were synthesized at the Laboratory for New Insulating and Semiconductor Materials, at UFU's Physics Institute, according to the methodology described by SILVA et al. (2018). Each NP was prepared using distilled sterile water at concentrations 100 and 10 mg mL^{-1}.

The NPs were used to evaluate the inhibition zone of *P. ananatis in vitro*. A basic layer of 2% agar-water medium and semi-solid nutrient medium (0.8%), supplemented with 10% of the bacterial suspension (10^8 CFU mL^{-1}), was added to Petri dishes (8 cm). Five sterile filter paper disks (6 mm) were placed onto the medium and soaked with 10 µL of each NP solution, 500 µg mL^{-1} of streptomycin (positive control), and filtered sterile water (negative control). After incubation at 28 °C for 48 hours the diameter of the inhibition zone was measured in centimeters.

The experiment was performed in a factorial scheme with completely randomized design, with 6 doped NPs at different concentrations, 7 pure NPs, in 2 dilutions (100 and 10 mg mL^{-1}), plus 2 additional (ZnO and pure NP), and streptomycin and sterile filtered water as control treatments, each

Table 1 - Concentrations of doped and pure nanoparticles (NP).

Doped NP	----------Concentration of the doped element (%)----------						Pure NP
ZnO: Ag	0.7	1	3	7	9	11	Ag_2O
ZnO: Au	0.1	0.5	1	5	10		Au
ZnO: Cu	0.1	0.4	1	4	12		CuO
ZnO: Fe	0.05	0.5	3	7	11		FeO
ZnO: Mn	0.2	0.4	0.8	4	8	12	MnO
ZnO: Ni	0.7	1	3	11			NiO
							ZnO

ZnO: (Zinc oxide) doped with: Ag (Silver), Au (Gold), Cu (Copper), Fe (Iron), Mn (Manganese), Ni (Nickel), Ag_2O (Silver oxide), CuO (Copper oxide), FeO (Iron oxide), MnO (Manganese oxide), NiO (Nickel oxide).

with 3 replicates. The data obtained were subjected to analysis of variance and the means were compared using the Tukey test, and Dunnett test at 5% probability using R v 4.0.2 (R CORE TEAM, 2020).

NPs of ZnO:0.1Cu, ZnO:0.05Fe, ZnO:0.2Mn, ZnO:0.7Ni were selected for subsequent assessments due to the amount of the product available in the laboratory, and the cost of synthesis.

To evaluate the control of MWS, two assays were carried out at different times, in a greenhouse. Hybrid maize plants P1630H were grown in 500 mL pots containing soil, sand, and vermiculite (3: 1: 1). After 15 days of sowing (at 3 to 4 leaf stage), for assessing preventive application, leaves were sprayed until runoff with NPs solutions ZnO:0.1Cu, ZnO:0.05Fe, ZnO:0.2Mn, ZnO:0.7Ni NPs at 10 or 5 mg mL^{-1} (first assay), and 5 or 2.5 mg mL^{-1} (second assay), and water. After 3 days, the plants leaves were sprayed with bacterial suspension (10^8 CFU mL^{-1}). Similarly, the plants used for assessing the curative application were sprayed with bacterial suspension and, after 3 days, were sprayed with the treatment solutions described above. The plants were kept in a humid chamber 24 hours before and after inoculation.

The severity of MWS was assessed at 3, 6, 9, and 12 days after inoculation, using a scale ranging from 0 to 4, where: 0 = leaf without symptoms, 1 = 1 to 25% of the leaf is injured, 2 = 26 to 50% of the leaf is injured, 3 = 51 to 75% of the leaf is injured, 4 = above 75% of the leaf is injured (SILVA & TEBALDI, 2018).

The area under the disease-progress curve (AUDPC) was calculated using the formula: AUDPC = $\Sigma ((Y_i + Y_{i+1})/2) (t_{i+1} - t_i)$, in which Y is disease intensity; t is time (interval between evaluations, in days); and i is the number of assessments made over time (CAMPBELL & MADDEN, 1990).

The experimental design comprised an 8x2+1 factorial scheme with 8 treatments (4 NPs x 2 concentrations), 2 applications (preventive and curative) + 1 additional (water application), and 4 replicates. The data obtained were subjected to an analysis of variance, and the means were compared using the Scott-Knott test, and Dunnett test at 5% probability using R v 4.0.2 (R CORE TEAM, 2020).

The different concentrations of ZnO NPs doped with Ag, Au, Cu, Fe, Mn or Ni at 100 mg mL^{-1} and 10 mg mL^{-1} inhibited bacterial growth, the halos ranging from 0.6 to 1.1 cm (Table 2). As well as, pure NPs of CuO, NiO, Au, or Ag_2O at 100 mg mL^{-1} with inhibition zone ranging from 0.23 to 0.63 cm. The largest inhibition zone was observed using the ZnO:Ag NPs, even though at different concentrations of the doping elements. In the controls there was bacterial growth for water, and inhibition halo for streptomycin.

The antibacterial action of NPs can occur due to the destabilization, degradation or dissolution of cell membranes and bacterial biofilm; thereby, reducing protection, and enabling interaction with internal compounds, causing deregulation or promotion of the release of toxic ions; which can lead to the physical blocking of the cell transport channels or the oxidation of membrane lipids by ROS (DURÁN et al., 2016; BALLOTTIN et al., 2017; WANG et al., 2017). Previous studies have shown that the application of different concentrations of Ag NP can inhibit the growth or kill microorganisms as *Xanthomonas perforans* (OCSOY et al., 2013), *Pseudomonas* sp. (ARAÚJO et al., 2015), *Xanthomonas axonopodis* pv. *citri* (BALLOTTIN et al., 2017), and *Clavibacter michiganensis* subsp. *michiganensis* (RIVAS-CÁCERES et al., 2018).

The structural damage caused by Ag NPs occurs due to the dissipation of the protons from the bacterial membrane, which leads to cell death (DURÁN et al., 2016). The antibacterial property of Ag NPs have been described to *Xanthomonas perforans,* with morphological changes like cell deformation, and destruction of the membrane(OCSOY et al., 2013).

The antimicrobial action of ZnO:Au NPs was observed in several concentrations of the doping element, except for ZnO:10Au NPs (Table 2), this could be explained due to high concentration of the element, more aggregation of the product occurs and; consequently, a decrease of the exposed reactive surface area of the NPs with the microorganism, reducing the bactericide action. The biocidal mechanism of ZnO:Au NPs (CUI et al., 2012) is due to alterations in the membrane permeability, which improve cell wall binding and penetration properties, providing a higher concentration of photoactive molecules (ALLAKER, 2010).

ZnO:Cu NPs had shown better effect biocidal in lower dilution (10 mg mL^{-1}), which can be explained by the high solubility of Cu ions in different concentrations, forming ROS that degrade microbial membranes (AHAMED et al., 2014). Cu-based products are usually applied for the control of bacteriosis because of their good performance, nevertheless when they are nanostructured, such as ZnO:Cu, the biocidal efficacy increases as described to *Escherichia coli* (KEZHEN et al., 2020). The antibacterial activity of ZnO:0.15Ni NPs was related to *Streptococcus mutans* and *Pseudomonas aeruginosa in vitro* with high effect to Gram negative bacteria (VIJAYAPRASATH et al., 2016).

Table 2 - *Pantoea ananatis* growth inhibition halo (cm) by nanoparticles (NP) at 100 and 10 mg mL^{-1}.

Doped NP	----------Concentrations of doping elements----------						Pure NP
ZnO:Ag	0.7	1	3	7	9	11	Ag_2O
100 mg mL^{-1}	0.8 Ac+*	0.95 Ab+*	1.0 Ab+*	1.0 Ab+*	1.0 Ab+*	1.1 Aa+*	0.63
10 mg mL^{-1}	0.0 Bc+	0.75 Bb+*	0.95 Aa+*	1.0 Aa+*	0.95 Aa+*	1.0 Ba+*	
ZnO:Au	0.1	0.5	1	5	10		Au
100 mg mL^{-1}	0.6 Ab+*	0.7 Aa+*	0.7 Ba+*	0.7 Aa+*	0.0 Ac+		0.41
10 mg mL^{-1}	0.6 Ac+*	0.7 Ab+*	0.8 Aa+*	0.7 Ab+*	0.0 Ad+		
ZnO:Cu	0.1	0.4	1	4	12		CuO
100 mg mL^{-1}	0.0 Bb+	0.9 Aa+*	0.0 Bb+	0.0 Bb+	0.0 Ab+		0.48
10 mg mL^{-1}	0.9 Aa+*	0.95 Aa+*	0.9 Aa+*	0.8 Ab+*	0.0 Ac+		
ZnO:Fe	0.05	0.5	3	7	11		FeO
100 mg mL^{-1}	0.0 Ab	0.85 Aa+*	0.0 Ab	0.0 Ab	0.0 Ab		0.0
10 mg mL^{-1}	0.0 Ab	0.7 Ba+*	0.0 Ab	0.0 Ab	0.0 Ab		
ZnO:Mn	0.2	0.4	0.8	4	8	12	MnO
100 mg mL^{-1}	0.0 Bc	0.9 Aa+*	0.7 Bb+*	0.7 Ab+*	0.0 Ac	0.0 Ac	0.0
10 mg mL^{-1}	0.7 Ab+*	0.7 Bb+*	0.8 Aa+*	0.65Bc+*	0.0 Ad	0.0 Ad	
ZnO:Ni	0.7	1	3	11			NiO
100 mg mL^{-1}	0.8 Aa+*	0.0 Ab+	0.0 Ab +	0.0 Ab+			0.23
10 mg mL^{-1}	0.8 Aa+*	0.0 Ab+	0.0 Ab+	0.0 Ab+			
ZnO							
100 mg mL^{-1}	0.0						
10 mg mL^{-1}	0.0						
H_2O							
0.0							
Streptomycin							
500 µg mL^{-1}	1.2						

Means followed by uppercase letters in the column and lowercase letters in the row differ by the Tukey test at 5%. + It differs at the 5% level of significance by the Dunnett test for pure nanoparticles. * Differs at the 5% level of significance by the Dunnett test for ZnO.

Despite of pure ZnO NPs (100 and 10 mg mL^{-1}), and FeO, MnO (100 mg mL^{-1}) did not inhibit bacterial growth, ZnO NPs doped with Fe and Mn have shown antibacterial activity. This can be explained due to the increase of interaction between oxygen and dehydrogenase enzyme when ZnO NPs was doped with Fe and Mn, which improved the antibacterial activity by the generation of ROS (SHARMA et al. 2016).

The area under the disease progress curve (AUDPC), for MWS, in the curative or preventive application of NPs reduced disease severity compared with the control (Table 3), in both assays. In the first assay (5 and 10 mg mL^{-1}), a similar AUDPC was observed between the NPs and the control (Table 3). In the second assay, the NPs of ZnO:0.1Cu at 2.5 and 5 mg mL^{-1} (3.1 and 2.3), and ZnO:0.2Mn at 2.5 and 5 mg mL^{-1} (4.1 and 4.9) reduced the severity of MWS, in both applications. Otherwise the ZnO:0.05Fe and ZnO;07Ni did not reduce the disease severity, in the second assay; also they did not control the MWS.

The ZnO:0.1Cu and ZnO:0.2Mn NPs significantly reduced MWS, in preventive and curative applications, showing a similar result with the *in vitro* experiment. The concentrations of the NPs – 2.5 or 5 mg mL^{-1} – did not show signs of phytotoxicity to maize plants.

The concentration and particle size used must be considered when spraying NPs. In a previous test in a greenhouse, high concentrations of NPs (20

Table 3 - Area under the disease-progress curve (AUDPC) of the maize white spot, in preventive and curative application of different nanoparticles at different concentrations, in different assays.

NPs	----------1st Assay----------				----------2nd Assay----------			
	Concentration [mg mL^{-1}]	----------AUDPC----------			Concentration [mgmL^{-1}]	----------AUDPC----------		
		C	P	Mean		C	P	Mean
ZnO:0.1Cu	5	3.8	3.0	3.4 A	2.5	3.3*	2.9*	3.1 A
ZnO:0.1Cu	10	6,9	5,0	5,9 A	5	3.0*	1.5*	2.3 A
ZnO:0.05Fe	5	5.9	4.6	5.3 A	2.5	7.8	8.8	8.3 B
ZnO:0.05Fe	10	6.5	4,4	5.4 A	5	7.9	8.5	8.2 B
ZnO:0.2Mn	5	4.0	2.3	3.1 A	2.5	3.0*	5.3	4.1 A
ZnO:0.2Mn	10	4.3	5.9	5.3 A	5	3.8	6.0	4.9 A
ZnO:0.7Ni	5	4.4	6.4	5.4 A	2.5	6.5	7.9	7.2 B
ZnO:0.7Ni	10	4.6	5.8	5.1 A	5	7.3	5.8	6.5 B
Control*		--------10.50------				-------10.00-----		
Means		5.0a	4.7a			5.3a	5.8a	

NPs: nanoparticles; C: Curative application; P: Preventive application; Control: with water application.
Means followed by uppercase letters in the column and lowercase letters in the row differ by the Scott-Knott test at 5%. * Differs at the 5% level of significance by Dunnett's test.

mg mL^{-1}) caused phytotoxicity to maize plants. The size of the NPs, around 20 nm, its rod shape, and the lower concentrations of the doping element leads to an increase in the contact surface (SANTANA et al., 2015), facilitating its penetration (ELMER & WHITE, 2016) and; consequently, favoring bacterial death.

Copper hydroxide and copper oxychloride used for the control of *Pantoea ananatis* had a phytotoxic effect on maize plants (BOMFETI et al., 2007). Thus, in the management of MWS, ZnO:0.1Cu NPs can be a good alternative to copper-based bactericides, also ZnO:0.2Mn NPs, since they reduced the severity of the disease without causing phytotoxicity.

This is the first report of the use of ZnO NPs doped with Cu, Fe, Mn and Ni for the control of MWS. Other essays should be carried out using another concentrations to assess their efficacy in controlling the disease.

The study reported out that ZnO NPs doped with Ag, Au, Cu, Fe, Mn, Ni in different concentrations, and pure NPs Ag_2O, CuO and NiO inhibited growth of *P. ananatis in vitro*. ZnO:0.1Cu and ZnO:0.2Mn reduced the severity of MWS in preventive and curative applications, being a promising technology for the management of MWS.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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