Science of the Total Environment 703 (2020) 135557

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Synergy between Cu-NPs and fungicides against *Botrytis cinerea*

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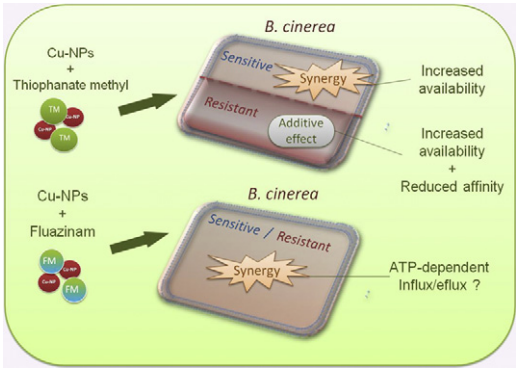
HIGHLIGHTS

- Cu-NPs are effective against both sensitive and fungicide resistant *B. cinerea* isolates.
- Cu-NPs exhibit synergistic action with thiophanate methyl and fluazinam.
- Copper ions and ATP metabolism are probably involved in Cu-NPs mode of action.
- Cu-NPs are potentially suitable for combating resistance and reducing fungicide residues in the environment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 October 2019
Received in revised form 14 November 2019
Accepted 14 November 2019
Available online 18 November 2019

Editor: Frederic Coulon

Keywords:
Benzimidazoles
QoIs
Nanofungicides
Plant pathogens
CuNPs
Synergism

ABSTRACT

Combating drug-resistance is a daunting task, especially due to the shortage of available drug alternatives with multisite modes of action. In this study, the potential of copper nanoparticles (Cu-NPs) to suppress 15 *Botrytis cinerea* isolates, which are sensitive or resistant to fungicides, alone or in combination with conventional fungicides, was tested *in vitro* and *in vivo*. Sensitivity screening *in vitro* revealed two fungicide resistance phenotypes, resulting from target site mutations. DNA sequencing revealed three *B. cinerea* isolates highly resistant to benzimidazoles (BEN-R), thiophanare methyl (TM), and carbendazim, bearing the E198A resistance mutation in the β-tubulin gene, and four isolates highly resistant to the QoI pyraclostrobin (PYR-R) with a G143A mutation in the *cytb* gene. Cu-NPs were equally effective against sensitive and resistant isolates. An additive/synergistic effect was observed between Cu-NPs and TM in the case of BEN-S isolates both *in vitro* and when applied in apple fruit. A positive correlation was observed between TM and TM + Cu-NPs treatments, suggesting that an increased TM availability in the target site could be related with the observed additive/synergistic action. No correlation between $Cu(OH)_2$ and Cu-NPs sensitivity was found, indicating that different mechanisms govern the fungitoxic activity between nano and bulk counterparts. A synergistic profile was observed between Cu-NPs and fluazinam (FM) - an oxidative phosphorylation inhibitor - in all isolates regardless of resistance phenotype, suggesting that ATP metabolism could be involved in the mode of action of Cu-NPs. Furthermore, the observed cross sensitivity and antagonistic action between Cu-NPs and NaCl also provided evidence for copper ions contribution to the fungitoxic action of Cu-NPs. The results suggested that Cu-NPs in

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https://doi.org/10.1016/j.scitotenv.2019.135557

combination with conventional fungicides can provide the means for an environmentally safe, sustainable resistance management strategy by reducing fungicide use and combating resistance against *B. cinerea*.

1. Introduction

Synthetic fungicides are essential for efficient disease management in modern agricultural production systems as they provide the most effective and cost-efficient means for combating plant parasites, accounting for at least 25% of yield losses worldwide (Pandey et al., 2018). Certain limitations of conventional fungicides, as well as risks concerning their fate in the food chain and environmental systems identify the necessity for the development of alternative control methods. Nanoparticles (NPs) constitute perfect candidates for answering this challenge, because they can be used as novel environmentally compatible nano-fungicides with unique properties, which include increased effectiveness in lower doses, enhanced drug delivery, and slower active ingredient release rates (Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). Nanoparticles containing copper (Cu-NPs) combine a protective action against bacterial and fungal diseases with low cost and reduced risk for resistance development due to the multi-site mode of action of [Cu^{+2}]. Cu-NPs exhibit a greater effectiveness against a number of fungal pathogens compared to fungicides containing copper (Baker et al., 2002; Winter and Davis, 2006; Park et al., 2016; Keller et al., 2017; Malandrakis et al., 2019).

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is the causal agent of the grey mould disease, typically described as "high resistance risk pathogen", and is known to have developed resistance to almost all available site-specific botrycides (Brent and Hollomon, 1998; Ma and Michailides, 2005; FRAC, 2013). Several studies have reported *B. cinerea* control failures due to resistance development to the intensively used benzimidazoles (Stehmann and de Waard, 1996; Malandrakis et al., 2011). In the majority of cases, benzimidazole resistance in *B. cinerea*, and to an increasing number of other plant pathogens, has been attributed to target site modifications resulting from the well characterized E198A, E198V, E198K and F200Y mutations in the β-tubulin gene (Leroux et al., 2002; Ziogas et al., 2009). Even the highly commercially successful fungicides, like the Qo inhibitors (QoI) of the cytochrome *bc1* complex of the respiratory chain, could not escape control failures, as indicated by a number of reports of rapid resistance development in a large number of plant pathogens including: *Blumeria* (Erysiphe) *graminis*, *Plasmopara viticola, Venturia inaequalis, Pyricularia (Magnaporthe) grisea, Mycosphaerella fijiensis, Alternaria alternata* and *B.cinerea* (FRAC, 2013; Malandrakis et al., 2011, 2018). The observed resistance to QoIs was in most cases attributed to target site changes resulting from mutations in the mitochondrial *cytb* gene, the most important being the substitution of Glycine with Alanine at posision 143 (G143A) in the respective gene (Avenot and Michailides, 2015; Malandrakis et al., 2018). It is evident that, despite the strict regulation limitations driven by environmental and health safety concerns, managing fungicide resistance is becoming a pressing matter, especially for high-risk pathogens such as *B. cinerea.*

A novel approach in the direction of integrated disease and resistance management is the use of alternative antifungal agents in rotation or combination with conventional fungicides, in order to both reduce the environmental impact caused by xenobiotics and retain effective control of diseases compromised by resistance development (Malandrakis et al., 2019). Metal nanoparticles including Ag-NPs, ZnO-NPs and FeO-NPs have demonstrated a promising potential when used as alternatives or in combination with antibiotics against sensitive or drug-resistant pathogenic bacteria such as *Eschericia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis*, *Klebsiella pneumonia*, and *Enterococcus faecalis* (Punjabi et al., 2018; Paralikar et al., 2019; Hamed et al., 2017; Nejabatdoust et al., 2019; Gabrielyan et al., 2019). Similarly, Ag-NPs and ZnO-NPs have proven to be effective against fungal strains both sensitive and resistant to fungicides and to exert a synergistic/additive effect when applied in combination with conventional fungicides, such as carbendazim, mancozeb, thiram, tebuconazole, fludioxonil, and propineb (Huang et al., 2018; Jamdagni et al., 2018; Xue et al., 2014).

This study focuses on the: (a) identification of fungicide resistant phenotypes among *Botrytis cinerea* isolates, (b) evaluation of the control efficacy of Cu-NPs and Cu-NPs/fungicide combinations against sensitive and fungicide resistant *B. cinerea* isolates *in vitro* and *in vivo*, and (c) investigation of possible mechanisms underlying the mode of action of Cu-NPs and the synergistic/additive interactions observed between Cu-NPs and selected fungicides. To the best of our knowledge, the potential of copper nanoparticles to control fungal isolates resistant to fungicides alone or in combination with conventional fungicides has not been previously explored.

2. Materials and methods

2.1. Nanoparticles, reagents and fungicides

Copper nanoparticles [Cu-NPs] (particle size 25 nm), copper sulphate [$CuSO_4$], sodium chloride [NaCl] and Salicylhydroxamate (SHAM), used in this study, were purchased from Sigma Aldrich, MO, USA. Commercial fungicides $Cu(OH)_2$ (Copperblau-N 50 WP), fluazinam (Azzuro 50 SC), mancozeb (Trimanoc 75 WG), and thiophanate methyl (Neotopsin 70 WG) were purchased from their respective manufacturers. The remainder of the fungicides used were pure analytical grade: carbendazim and fenhexamid were supplied by Bayer CropScience AG (Leverkusen, Germany), zoxamide by Dow Agrosciences (Indianapolis, USA), fludioxonil and difenoconazole by Syngenta Crop Protection AG (Basle, Switzerland), and pyraclostrobin by BASF AG (Limburgerhof, Germany). All stock solutions of the antifungal compounds were prepared using ethanol as a solvent, except for zoxamide and fenhexamid, which were dispersed in acetone and isopropanol, respectively. Analytical grade active ingredients were added aseptically to sterilized growth medium prior to inoculation in appropriate quantities, making sure the solvent never exceeded 1% (v:v) of the total volume, in both treated and control samples. Stock solutions of commercial fungicides and nanoparticles were prepared in distilled-sterilized water. Nanoparticle suspensions were subjected to sonication for 30 min with Transonic 420 (Elma, Germany) sonicator, prior to their incorporation in growth media.

2.2. Fungal isolates and culture conditions

Fifteen fungal *B. cinerea* isolates from the fungal collection of the Pesticide Science Lab (Agricultural University of Athens), originating from various crop fields located in Greece (Table 1), were evaluated in terms of their sensitivity against Cu-NPs and selected botrycides. All isolates were grown on Potato Dextrose Agar (PDA) medium in order to obtain inoculum for the fungitoxicity assays and kept in growth chambers in the dark at 25 °C and 70% humidity. For each strain, for long-term storage purposes, four 5-mm mycelial plugs from the margin of rapidly growing fungal colonies were placed in 1.5 mL tubes containing 50% *v*/v of sterilized glycerol:water, and stored at −20 °C.

Table 1
Origin of *Botrytis cinerea* isolates used in this study and spray history of the fields from which the isolates were obtained.

Isolate	Host	Location	Year of collection	Spray history				
				Thiophanate methyl	Pyraclostrobin	Fenhexamid	Fludioxonil	Mancozeb
BC1	Apple	Veroia	2017	+[a]	+		+	+
BC2	Apple	Veroia	2017	+	+		+	+
BC3	Peach	Veroia	2017	+	+		+	+
BC4	Apple	Veroia	2017	+	+		+	+
BC5	Grape	Crete	2017	+	+		+	
BC6	Cherry	Crete	2017	+	+		+	
BC7	Strawberry	Pelloponisos	2017	+	+	+	+	+
BC8	Strawberry	Pelloponisos	2017	+	+	+	+	+
BC9	Strawberry	Pelloponisos	2017	+	+	+	+	+
BC10	Pepper	Pelloponisos	2017	+	+	+	+	+
BC11	Cucumber	Crete	2018	+	+	+	+	
BC12	Plum	Crete	2018	+	+		+	
BC13	Plum	Crete	2018	+	+		+	
BC14	Plum	Crete	2018	+	+		+	
BC15	Plum	Crete	2018	+	+		+	

[a] Plus sign indicates that isolates were exposed to the respective fungicide for at least 2 years prior to the year of collection.

2.3. In vitro fungitoxicity tests

2.3.1. Sensitivity of B. cinerea isolates to Cu-NPs and fungicides

In order to evaluate the potential of Cu-NPs to control fungicide-resistant *B. cinerea* isolates as well as sensitive ones, *in vitro* fungitoxicity tests were conducted. The sensitivity of fungal strains to NPs and fungicides was evaluated by measuring radial growth of isolates on PDA. The fungitoxicity was expressed as percent relative growth of isolates grown on PDA containing concentrations equal to mean EC_{50} values (effective concentration causing 50% inhibition of mycelial growth). Previously reported mean EC_{50} values of *B. cinerea* to tested botrycides and nanoparticles are: 500 μg/mL for $Cu(OH)_2$, 300 μg/mL for Cu-NPs, 0.25 μg/mL for thiophanate methyl, 0.1 μg/mL for carbendazim, 0.5 μg/mL for zoxamide, 0.02 μg/mL for fluazinam, 0.5 μg/mL for pyraclostrobin, 0.19 μg/mL for fenhexamid, 0.01 μg/mL for fludioxonil, 0.25 μg/mL for difenoconazole and 50 μg/mL for mancozeb (Kalamarakis et al., 2000; Malandrakis et al., 2011, 2019; Markoglou et al., 2006). Isolates with reduced sensitivity to thiophanate methyl, carbendazim, and pyraclostrobin were subjected to higher doses in order to determine the actual EC_{50} values and resistance factors, Rf, defined as the ratio EC_{50} of the resistant isolate to the mean EC_{50} of sensitive isolates. Specifically, the following concentrations of 0, 0.5, 1, 2.5, 5, 10, 25 μg/mL thiophanate methyl; 0, 0.5, 1, 2.5, 5, 10 μg/mL carbendazim; 0, 0.01, 0.025, 0.05, 1 μg/mL zoxamide; and 0, 0.25, 1, 5, 50 μg/mL pyraclostrobin were used to obtain fungitoxicity-curves for resistant isolates. Three replicate plates were used for each fungicide concentration-isolate combination. The inoculum consisted of a 5-mm mycelial plug cut from the edge of 4-day old colony of each isolate grown on PDA, and transferred to nanoparticle or fungicide-amended and non-amended (control) media. The cultures were incubated at 25 °C in the dark for 4 days. Percent inhibition rates were calculated by the formula: 100-(mean diameter of the colony on the fungicide-amended plates divided by the mean diameter of the untreated control) × 100. Tests for each isolate were repeated twice for each concentration and fungicide.

2.3.2. Synergistic activity of Cu-NPs with fungicides

Combined antifungal effects of Cu-NPs and $Cu(OH)_2$ when applied simultaneously with the selected fungicides thiophanate methyl, fluazinam and pyraclostrobin were evaluated *in vitro* by poison agar assays. Concentrations of 0.25 μg/mL thiophanate methyl, 0.1 μg/mL carbendazim, 0.02 μg/mL fluazinam, and 0.5 μg/mL pyraclostrobin were added aseptically in PDA growth medium individually or in combination with 300 μg/mL Cu-NPs or 500 μg/mL $Cu(OH)_2$. Following an incubation period of 4 days at 25 °C in the dark, mycelial growth percent inhibition rates were calculated for each treatment. Combined effects of Cu-NPs with the selected fungicides were evaluated according to the Abbortt method (Gisi, 1996). Specifically, the expected combined percent inhibition (% CI_{exp}) was calculated as: % $CI_{exp} = I_A + I_B - (I_A \times I_B / 100)$, where I_A and I_B are the percent inhibition of each antifungal agent. The synergy factor (SF) was determined by the formula $SF = I_{AB}/(\%\ CI_{exp})$, where I_{AB} represents the observed percent inhibition of antifungal agents when applied together. It should be noted that SF values close to 1 were considered to indicate additive, >1 synergistic, and <0.75 antagonistic interactions.

2.4. In vivo fungitoxicity tests

The efficacy of Cu-NPs to control sensitive and fungicide-resistant *B. cinerea* isolates, alone or in combination with thiophanate methyl and fluazinam *in vivo*, was tested on apple fruit (*Malus sylvestris cv firiki*), which were selected for their uniform maturity, size, shape, and absence of any wound. Two sensitive (BC1, BC2) and two fungicide resistant (BC4, BC11) isolates were used in four apple fruits per treatment with fungicide, Cu-NPs and combinations of the two, while the control treatment comprised of distilled water. The surfaces of the apple fruits were disinfected by dipping the fruit in a 1% sodium hypochlorite solution for 10 min, rinsing the fruit three times with distilled-sterilized water, and drying the fruit before fungicide/Cu-NPs treatments. Subsequently, the fruit were sprayed with solutions of 500 μg/mL Cu-NPs; 20 and 2000 μg/mL thiophanate methyl (1/50, 2× of the maximum recommended dose); and 40 μg/mL fluazinam (1/20 of the maximum recommended dose), individually and in combinations. The fruit were air-dried for 2 h and then, a 2 × 2 mm [length × width] cross-shaped wound was created at the front face of each apple fruit using a sterile needle. The inoculation of the fruit was carried out by placing a 5-mm mycelial plug, from a 4-day old colony of each *B. cinerea* isolate, on top of each wound. Plastic boxes 24 × 34 × 10 cm [length × width × height] containing the inoculated fruit were placed on top of a wet sterilized paper covered by a lid and incubated in a growth chamber at 25 °C for 4 days. The lesion diameter around each wound of treated fruit was measured and recorded, then divided by the respective lesion diameter of the water-treated control, and next the percent symptom severity was calculated. All experiments were repeated at least twice.

2.5. DNA extraction and sequence analysis of β-tubulin and cytb genes from B. cinerea isolates

Mycelium of selected *B. cinerea* isolates was peeled from the surface of 4-day old cultures grown on fungicide-free PDA at 25 °C, and ground in liquid nitrogen using a mortar and pestle. TRI reagent (Sigma) was

used to isolate the total DNA. A 1.6-kb fragment of the *B. cinerea* β-tubulin gene was amplified using the primers BCTubF (5' CTTGAGCGT ATGAACGTCTAC 3′) and BCTubR (5′ TGTACCAATGCAAGAAAGCCTT 3′) from the template gDNA. The PCR reactions involved 0.2 mM from each of the primers, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1.25 units of HotStar Taq DNA polymerase (Qiagen) in 20 mM TrisHCl and 50 mM KCl. The PCR conditions were as follows: 95 °C for 15 min followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min with a final 10 min extension at 72 °C. A 463-bp fragment of the *B. cinerea cytb* gene was amplified using the primers Bccytb-F1 (5′ CGTCGGCCA TATAAAAGGTC 3′) and Bccytb-R1 (5′ CTCCATCCACCATACCTACA 3′) from template gDNA under PCR conditions of 95 °C for 15 min followed by 35 cycles of 94 °C for 1 min, 65 °C for 25 s, and 72 °C for 1 min with a final 10 min extension at 72 °C. The QIAquick gel extraction kit (Qiagen) was used to purify the PCR products, which were subsequently ligated to pGEM-Teasy (Promega) vectors and transformed into *Escherichia coli* (DH5a Library Efficiency® Competent Cells, Invitrogen) competent cells. Recombinant plasmids were purified using QIAprep spin miniprep kit plasmid (Qiagen) and then sequenced in both directions. Ten independent clones from each *B. cinerea* isolate were analyzed. Sequence data analysis was performed by use of the Lasergene software (DNAstar, Madison, USA).

3. Statistical analysis

The EC_{50} values for each strain and antifungal compound were calculated by regressing the relative inhibition of mycelial growth against the Log_{10} compound concentrations. Pearson correlation coefficients were used to correlate isolate sensitivities between the various antifungal treatments. The estimated Cu-NPs and fungicide inhibition rates were subjected to analysis of variance and the resulting means were separated according to Tukey's HSD test ($\alpha = 0.05$). The SPSS v20 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

4. Results

4.1. Sensitivity of B. cinerea isolates to antifungal agents in vitro

The sensitivity of *B. cinerea* isolates to selected fungicides against the pathogen was evaluated using discriminatory doses, based on mean EC_{50} values reported in previous studies (Kalamarakis et al., 2000; Malandrakis et al., 2011; Markoglou et al., 2006). The observed mean percent inhibition caused by Cu-NPs and each of the fungicides used in this study are shown in Table 2. Although there was significant variability between the various isolates, in most cases, wild type (baseline) sensitivity to the antifungal agents used was observed. A reduced sensitivity to the benzimidazoles thiophanate-methyl and carbendazim was detected for the isolates BC4, BC5 and BC11 (mean percent inhibition values ranging from 0 to 1.53). Additionally, the isolates BC4, BC5, BC11, and BC13 showed a reduced sensitivity response to the QoI fungicide pyraclostrobin (see Table 2). Consequent fungitoxicity tests were conducted to determine the EC_{50} values and to calculate the resistance factors (Rfs) of the resistant phenotypes (see Table 3). Based on calculated Rf values, isolates BC4, BC5 and BC11 were classified as highly benzimidazole resistant (BEN-R phenotype), because they were highly resistant to both carbendazim and thiophanate methyl. The calculated Rf values were > 100 for thiophanate methyl and >200 for carbendazim in all BEN-R isolates. All BEN-R isolates were more sensitive (Rf values 0.06–0.1) to the benzimide zoxamide than the

Table 2
Sensitivity of *Botrytis cinerea* isolates to Cu-NPs and selected fungicides.

Isolate	Percent Inhibition[a] (mean ± SD[b])										
	Cu-NPs (300)[c]	$Cu(OH)_2$ (500)	thiophanate methyl (0.25)	Carbendazim (0.1)	Zoxamide (0.5)	Fuazinam (0.02)	Pyraclostrobin (0.15)	Fenhexamid (0.19)	Fludioxonil (0.01)	Difenoconazole (0.25)	Mancozeb (50)
BC1	49.27 ± 0.30	51.75 ± 1.15	85.00 ± 0.10	83.64 ± 1.15	56.15 ± 2.01	51.22 ± 1.25	52.26 ± 0.35	50.10 ± 0.20	41.67 ± 6.52	45.65 ± 5.02	53.33 ± 2.22
BC2	50.70 ± 0.42	50.00 ± 0.12	100	95.56 ± 0.85	57.48 ± 1.05	53.33 ± 2.12	30.19 ± 1.25	47.04 ± 0.12	50.04 ± 1.12	37.14 ± 8.04	43.57 ± 2.75
BC3	39.28 ± 2.34	48.32 ± 0.41	95.24 ± 0.05	61.70 ± 2.12	50.32 ± 0.38	53.33 ± 2.65	50.10 ± 2.45	50.00 ± 2.02	49.98 ± 1.10	35.53 ± 0.35	38.82 ± 1.19
BC4	54.66 ± 0.05	47.13 ± 0.05	1.11 ± 0.28	1.53 ± 0.05	100	64.29 ± 4.34	0.00	48.93 ± 1.43	49.55 ± 0.21	50.00 ± 0.12	56.94 ± 0.66
BC5	46.67 ± 3.19	49.88 ± 0.23	0	0	100	52.50 ± 0.89	4.52 ± 0.12	40.23 ± 1.88	44.57 ± 3.77	14.73 ± 2.72	20.95 ± 2.05
BC6	45.92 ± 2.85	52.18 ± 1.25	67.11 ± 0.16	35.62 ± 0.51	48.72 ± 0.12	45.90 ± 0.23	49.39 ± 0.97	51.62 ± 0.32	52.09 ± 2.26	50.25 ± 1.17	45.83 ± 1.01
BC7	56.07 ± 0.19	45.89 ± 0.80	42.86 ± 0.12	50.75 ± 1.25	39.28 ± 1.08	36.51 ± 3.12	65.00 ± 0.55	42.21 ± 5.35	49.99 ± 1.10	60.09 ± 5.10	41.25 ± 4.02
BC8	33.84 ± 1.32	60.00 ± 4.39	54.22 ± 0.34	25.00 ± 1.11	49.24 ± 2.32	44.00 ± 1.90	42.32 ± 1.70	45.52 ± 3.26	54.68 ± 4.32	46.23 ± 4.45	27.22 ± 2.05
BC9	38.66 ± 2.44	39.62 ± 1.40	62.5 ± 0.16	27.53 ± 2.22	49.94 ± 3.01	52.56 ± 0.04	47.50 ± 0.64	49.27 ± 2.05	52.35 ± 0.75	48.99 ± 3.39	24.68 ± 5.14
BC10	40.00 ± 5.30	51.09 ± 1.84	100	65.00 ± 1.07	52.36 ± 0.21	47.06 ± 4.00	51.52 ± 2.03	52.65 ± 1.14	49.95 ± 0.60	41.64 ± 5.34	50.31 ± 2.27
BC11	35.87 ± 1.15	45.22 ± 1.01	0	0	100	38.10 ± 1.12	2.90 ± 1.83	57.84 ± 4.33	49.69 ± 1.55	52.25 ± 2.00	35.79 ± 1.32
BC12	60.00 ± 2.12	42.34 ± 2.12	100	64.86 ± 2.05	55.62 ± 0.11	54.79 ± 0.62	47.04 ± 4.00	30.59 ± 5.20	54.07 ± 3.25	55.00 ± 0.85	29.49 ± 1.15
BC13	45.16 ± 0.98	47.92 ± 2.08	32.26 ± 0.02	75.03 ± 1.43	54.34 ± 1.67	55.79 ± 1.31	0.00	58.85 ± 3.12	51.63 ± 0.17	48.08 ± 1.72	58.06 ± 023
BC14	56.92 ± 3.07	57.55 ± 3.02	100	100	50.03 ± 1.22	35.71 ± 3.02	48.18 ± 2.70	65.72 ± 4.78	50.45 ± 2.08	48.06 ± 2.16	45.16 ± 4.01
BC15	68.00 ± 2.65	36.09 ± 2.67	65.22 ± 0.85	48.46 ± 0.15	60.28 ± 2.53	41.18 ± 0.08	50.00 ± 0.04	50.49 ± 1.09	32.63 ± 6.05	65.76 ± 5.64	45.63 ± 2.15

[a] Calculated as percent inhibition of mycelial growth compared to the untreated control after 4 days incubation at 22 °C ($n = 3$).

[b] Standard deviation of the means (n = 3).

[c] Numbers in parenthesis indicate fungicide concentrations in μg/mL of active ingredient.

Table 3
Cross-resistance profiles of *B. cinerea* isolates sensitive and benzimidazole/pyraclostrobin resistant and respective resistance mutations.

Isolate	Fungicides								Resistance mutations	
	Thiophanate methyl		Carbendazim		Zoxamide		Pyraclostrobin		Cytb gene	β-Tubulin gene
	EC_{50}[a] (mean ± SD[b])	Rf[c]	EC_{50} (mean ± SD)	Rf	EC_{50} (mean ± SD)	Rf	EC_{50} (mean ± SD)	Rf	Amino acid substitution	
BC1	0.16	0.65	0.05 ± 0.00	0.92	0.40 ± 0.05	1.00	0.09 ± 0.01	1.01	G143	E198
BC2	0.13 ± 0.05	0.55	0.05 ± 0.00	0.90	0.41 ± 0.04	1.12	0.74 ± 0.14	8.22	G143	E198
BC4	>25	>100	>10	>200	0.03 ± 0.01	0.09	>10	>100	G143A	E198A
BC5	>25	>100	>10	>200	0.03 ± 0.01	0.10	>10	>100	G143A	E198A
BC11	>25	>100	>10	>200	0.02 ± 0.00	0.06	>10	>100	G143A	E198A
BC13	0.49 ± 0.11	1.98	0.05 ± 0.00	1.00	0.37 ± 0.05	1.05	>10	>100	G143A	E198

[a] Effective concentration causing 50% reduction in mycelial growth rate after 4 days incubation at 22 °C (n = 3).
[b] Standard deviation of the means ($n = 3$).
[c] Resistance factor (EC_{50} of the resistant isolate/mean EC_{50} of sensitive isolates).

wild type isolates BC1 and BC2. Pyraclostrobin resistant isolates (PYR-R phenotype) included the BEN-R isolates BC4, BC5, BC11 and the isolate BC13, which was specifically pyraclostrobin-resistant. All PYR-R isolates were highly resistant to pyraclostrobin with Rf values exceeding 100 (see Table 3).

4.2. Detection of target-site resistance mutations

The high resistance levels we observed in the BEN-R and PYR-R phenotypes lead to the hypothesis that resistance was observed due to mutations in the genes encoding the target sites of benzimidazole and pyraclostrobin fungicides. In order to confirm this hypothesis, gene fragments coding β-tubulin and cytochrome *b*, target sites of benzimidazole and pyraclostrobin fungicides, respectively, were isolated from sensitive and resistant isolates and sequenced. Sequence comparisons of the β-tubulin gene between sensitive and resistant isolates revealed the E198A resistance mutation in all BEN-R isolates, which is known to confer high resistance levels to benzimidazoles (Ma and Michailides, 2005) (see Table 3). Also, a well-known resistance mutation (G143A) was detected in the cytb gene in all PYR-R isolates. These results confirmed the hypothesis that the resistant phenotypes observed resulted from target-site modifications that reduce the affinity between fungicides and their target, and in turn require very high concentrations to inhibit the resistant isolates.

4.3. Synergistic activity

4.3.1. Synergistic activity in vitro

The ability of Cu-NPs to control sensitive and resistant isolates in combination with thiophanate methyl, pyraclostrobin, and fluazinam was tested *in vitro*. The calculated synergistic factor (SF) values for Cu-NPs are listed in Table 4. An enhanced synergistic effect for Cu-NPs with fluazinam was observed in most cases of both fungicide sensitive and resistant isolates, with estimated SF values ranging from 1.01 to 1.50 (see Table 4, Fig. 1c). On the contrary, an antagonistic effect was observed (SF: 0.41–0.97) for the combined use of Cu-NPs and pyraclostrobin in most isolate cases. For the BC4 isolate, synergy was observed (SF = 1.22), for the combined use of Cu-NPs and pyraclostrobin mixture; however, inhibition rates were barely statistically significant different from those corresponding to individual use of Cu-NPs (see Fig. 1b). The combined use of Cu-NPs with thiophanate methyl resulted in inhibition rates, which were significantly greater than those for the individual antifungal agents in all BEN-S isolates (see Fig. 1a). In BEN-R isolates, no significant differences were found between Cu-NPs sensitivity and the combined use of Cu-NPs and thiophanate methyl (see Fig. 1a). SF values between Cu-NPs and thiophanate methyl for the BEN-S isolates ranged between 1.03 and 1.59, indicating synergism, while the respective values for BEN-R isolates were close to 1 (0.85 to 0.99), indicating a slight additive effect (see Table 4, Fig. 2).

Table 4
In vitro synergistic activity of Cu-NPs or $Cu(OH)_2$ with selected fungicides against fungicide sensitive and resistant *Botrytis cinerea* isolates. TM: thiophanate methyl, FM: fluazinam.

Isolate	Resistance Phenotype	SF[a]					
		Cu-NPs (300)				$Cu(OH)_2$ (500)	
		TM (0.25)[c]	FM (0.02)	pyraclostrobin (0.15)	NaCl (10,000)	TM (0.25)	FM (0.02)
BC1	BEN-S/PYR-S[b]	1.08	1.50	0.43	0.08	1.39	0.79
BC2	BEN-S/PYR-S	1.07	1.35	0.56	0.00	3.97	0.87
BC3	BEN-S/PYR-S	1.03	1.33	0.71	0.00	1.48	0.79
BC6	BEN-S/PYR-S	1.13	1.08	0.81	0.63	1.00	0.90
BC7	BEN-S/PYR-S	1.12	1.07	0.66	0.97	0.99	0.67
BC8	BEN-S/PYR-S	1.39	1.01	0.66	0.90	1.78	0.61
BC9	BEN-S/PYR-S	1.17	1.02	0.97	0.00	3.12	0.88
BC10	BEN-S/PYR-S	1.07	1.08	0.84	0.04	1.28	1.01
BC12	BEN-S/PYR-S	1.10	1.21	0.55	0.70	1.34	0.84
BC14	BEN-S/PYR-S	1.29	1.41	0.65	0.00	1.10	0.89
BC15	BEN-S/PYR-S	1.13	1.24	0.43	0.58	1.03	0.73
BC13	BEN-S/PYR-R	1.59	1.08	0.53	0.30	0.52	0.83
BC4	BEN-R/PYR-R	0.85	1.08	1.22	0.78	0.59	0.75
BC5	BEN-R/PYR-R	0.99	1.28	0.44	0.41	0.23	0.14
BC11	BEN-R/PYR-R	0.95	1.36	0.76	0.98	0.71	0.76

[a] Synergy factor.
[b] BEN-S/R: Benzimidazole Sensitive/Resistant and PYR-S/R: Pyraclostrobin Sensitive/Resistant isolate.
[c] Numbers in parenthesis indicate antifungal agent concentrations in μg/mL of active ingredient.

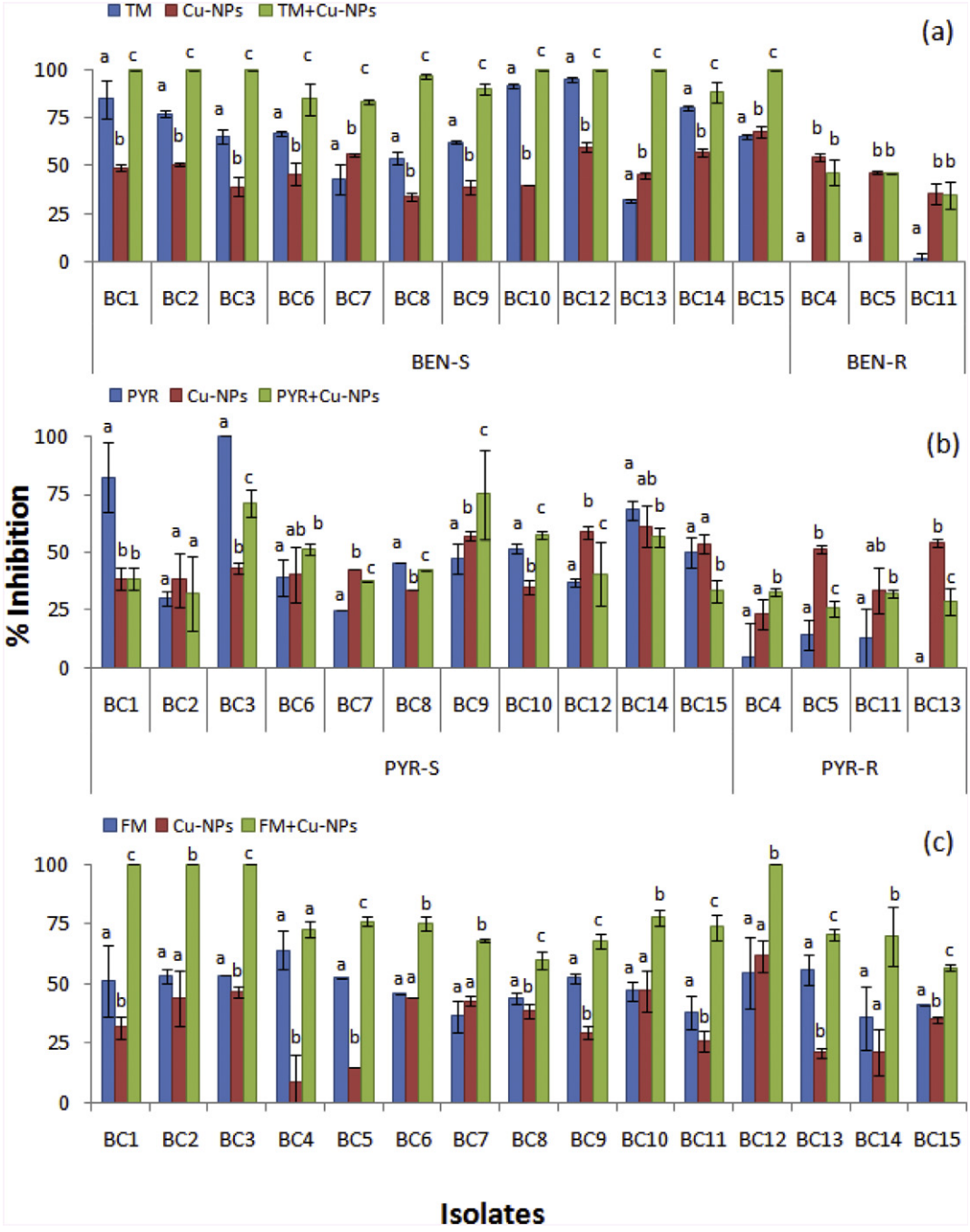

Fig. 1. Sensitivity of fungicide-sensitive/resistant *B. cinerea* isolates to Cu-NPs (300 μg/mL) in comparison with (a) thiophanate methyl (0.25 μg/mL) (b) pyraclostrobin (0.15 μg/mL) and (c) fluazinam (0.02 μg/mL) and combinations. BEN-S/R: benzimidazole- Sensitive/Resistant and PYR-S/R: pyraclostrobin-Sensitive/Resistant isolates. TM: thiophanate methyl, FM: fluazinam). Error lines represent the standard deviation of means. Between treatments, bars marked by the same letter do not differ significantly according to Tukey's HSD test ($\alpha = 0.05$).

In an attempt to evaluate the role of nanoparticle nature *versus* the release of copper ions in the synergistic effect observed between Cu-NPs and fluazinam or thiophanate methyl, a commercial fungicide containing $Cu(OH)_2$ was used in a synergism test *in vitro* with the above fungicides. In contrast to Cu-NPs, the combined use of $Cu(OH)_2$ with fluazinam resulted in an antagonistic effect in almost all the isolate cases with SF values ranging between 0.14 and 1.01 (see Table 4), indicating a possible contribution of nanoparticle properties in the observed synergism between Cu-NPs and fluazinam. A strong antagonistic effect was observed between $Cu(OH)_2$ and thiophanate methyl in BEN-R isolates (SF: 0.23–0.72), contrary to BEN-S isolates where a strong synergistic effect (Rf: 1.00–3.97) was observed in most cases (see Table 4). These results indicate a possible role of the copper ions released in the observed synergism between Cu-NPs and thiophanate methyl. A possible role of copper ion release in the fungitoxic activity of Cu-NPs against *B. cinerea* isolates was revealed by the synergy tests conducted between Cu-NPs and NaCl. These synergy tests involved the addition of 1% NaCl in PDA containing 300 μg/mL Cu-NPs, which neutralized the fungitoxic effect of Cu-NPs, and yielded very low SF values in the majority of isolate cases (see Table 4).

4.3.2. Synergistic activity in vivo

The synergistic activity between Cu-NPs, thiophanate methyl and fluazinam demonstrated *in vitro* was tested on apple fruit for selected BEN-S and BEN-R/PYR-R *B. cinerea* isolates. Treatment of apple fruit inoculated with fungicide sensitive isolates BC1 and BC2 with 500 μg/mL

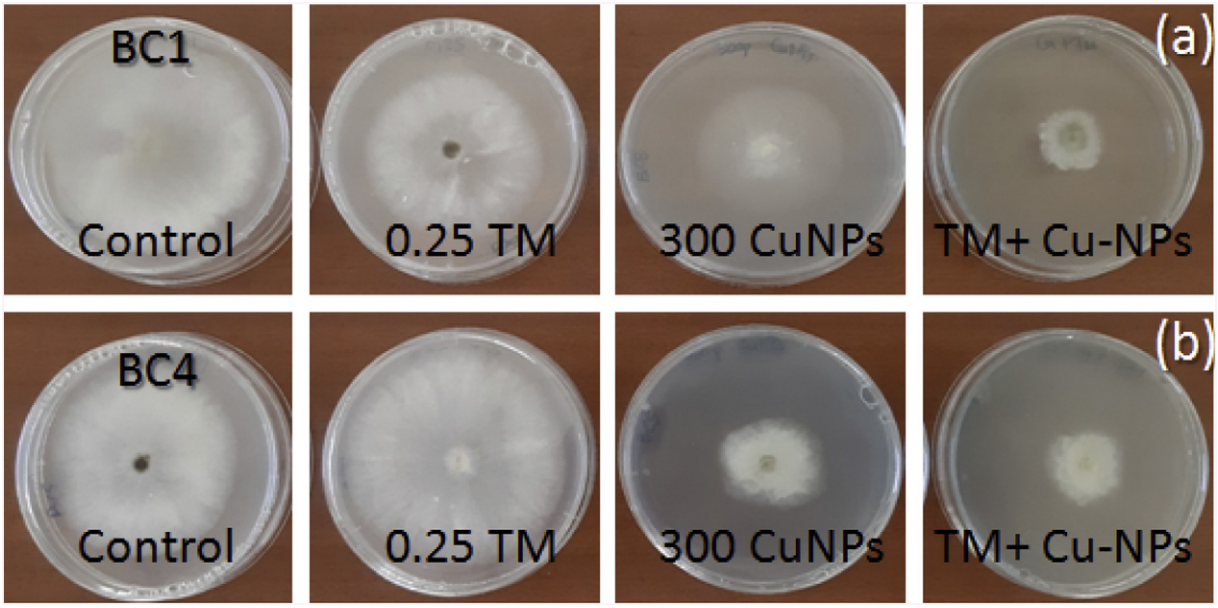

Fig. 2. Sensitivity of a (a) sensitive (BC1) and a (b) TM-resistant (BC4) *B. cinerea* isolate to Cu-NPs, TM and their combination. TM:thiophanate methyl.

Cu-NPs resulted in inhibition rates of 30.95 and 27.27%, while 20 μg/mL thiophanate methyl resulted in 54.76 and 43.18% inhibition (SF:1.45 and 1.70, respectively, see Table 5). When the two antifungal agents were applied in combination, disease symptoms caused by BC1 and BC2 BEN-S isolates were almost completely suppressed, demonstrating a strong synergistic effect (see Fig. 3). In the case of BEN-R isolates BC4 and BC11, thiophanate methyl could not suppress lesion development even at 2000 μg/mL, while combination with Cu-NPs had a slight additive effect (SF: 0.93 to 0.96, respectively, see Table 5). Combination of Cu-NPs with fluazinam resulted in a synergistic effect *in vivo* similar to that observed in the *in vitro* experiments (see Fig. 3). A clear synergistic effect was observed in all *B. cinerea* phenotypes with SF values ranging from 1.31 to 1.51, indicating a potential of the above nanoparticles to control both sensitive and resistant isolates in mixtures with FM (see Table 5).

4.4. B. cinerea sensitivity correlations

In an attempt to investigate the contribution of Cu-NPs, thiophanate methyl (TM), fluazinam (FM) and $Cu(OH)_2$ in the observed synergistic effect of the respective mixtrures, pearson correlation coefficient values were calculated (see Table 6). No significant correlation was found between Cu-NPs and TM, FM or any of their combinations (see Table 6). On the contrary, a significant correlation was found between TM, Cu-NPs + TM and $Cu(OH)_2$ + TM treatments (see Fig. 4a,b). This result indicates that the observed enhanced inhibitory effect of the above mixtures is probably related with the action of TM rather than that of either Cu-NPs or $Cu(OH)_2$. This was also evident by the positive correlation observed between Cu-NPs + TM and $Cu(OH)_2$ + TM treatments (see Table 6). An interesting positive correlation was found between Cu-NPs sensitivity and NaCl, which possibly indicates a common mechanism of fungitoxic action between the two compounds (see Fig. 4c).

5. Discussion

In order to evaluate Cu-NPs potential to control fungicide sensitive and resistant isolates, a sensitivity screening of 15 *B. cinerea* isolates to 9 fungicides was undertaken utilizing *in vitro* bioassays. Three of the isolates tested were highly resistant to benzimidazoles thiophanate methyl and carbendazim (BEN-R) due to a well-known target site mutation (E198A) in the *B. cinerea* β-tubulin gene, as revealed by DNA sequencing. The above mutation has been related to high levels of benzimidazole resistance in many plant pathogenic fungi, including *B. cinerea* (Leroux et al., 2002; Ziogas et al., 2009; Ma and Michailides, 2005; FRAC, 2013). The above isolates as well as an additional isolate (BC13) were also highly resistant to the QoI fungicide pyraclostrobin. All pyraclostrobin resistant isolates (PYR-R) harbored the G143A resistance mutation in their mitochondrial *cytb* gene. An extensive list of plant

Table 5
Synergistic activity of Cu-NPs co-applied with thiophanate mehyl or fluazinam on apple fruit against *Botrytis cinerea* isolates sensitive and resistant to selected fungicides. TM: thiophanate methyl, FM: fluazinam.

Isolate	Phenotype	Percent inhibition[a] (mean ± SD[b])				Percent inhibition (mean ± SD)			SF
		Cu-NP (500)[d]	TM (20/2000)[e]	Cu-NPs + TM	SF[f]	Cu-NP (500)	FM (40)	Cu-NPs + FM	
BC1	BEN-S/PYR-S[c]	30.95 ± 0.15	54.76 ± 3.56	100	1.45	18.46 ± 1.96	12.30 ± 1.30	43.07 ± 4.11	1.51
BC2	BEN-S/PYR-S	27.27 ± 1.10	43.18 ± 4.34	95.45 ± 2.82	1.70	28.32 ± 2.25	15.45 ± 2.01	41.25 ± 2.15	1.51
BC4	BEN-R/PYR-R	5.75 ± 0.26	0	5.35 ± 0.48	0.93	8.25 ± 1.32	22.24 ± 2.81	40.76 ± 3.98	1.42
BC11	BEN-R/PYR-R	21.27 ± 2.34	0	20.53 ± 2.65	0.96	25.97 ± 0.14	9.09 ± 1.67	42.85 ± 1.88	1.31

[a] Calculated as percent inhibition of lesion development on apple fruit sprayed with Cu-NPs/fungicides and their combinations compared to the untreated control after 7 days incubation at 22 °C (n = 3).
[b] Standard deviation of the means (n = 3).
[c] BEN-S/R: Benzimidazole Sensitive/Resistant and PYR-S/R: Pyraclostrobin Sensitive/Resistant isolate.
[d] Numbers in parenthesis indicate fungicide concentrations in μg/mL of active ingredient.
[e] Apple fruit inoculated with BEN-S isolates were sprayed with 20 μg/mL while those with BEN-R isolates with 2000 μg/mL TM.
[f] Synergy factor.

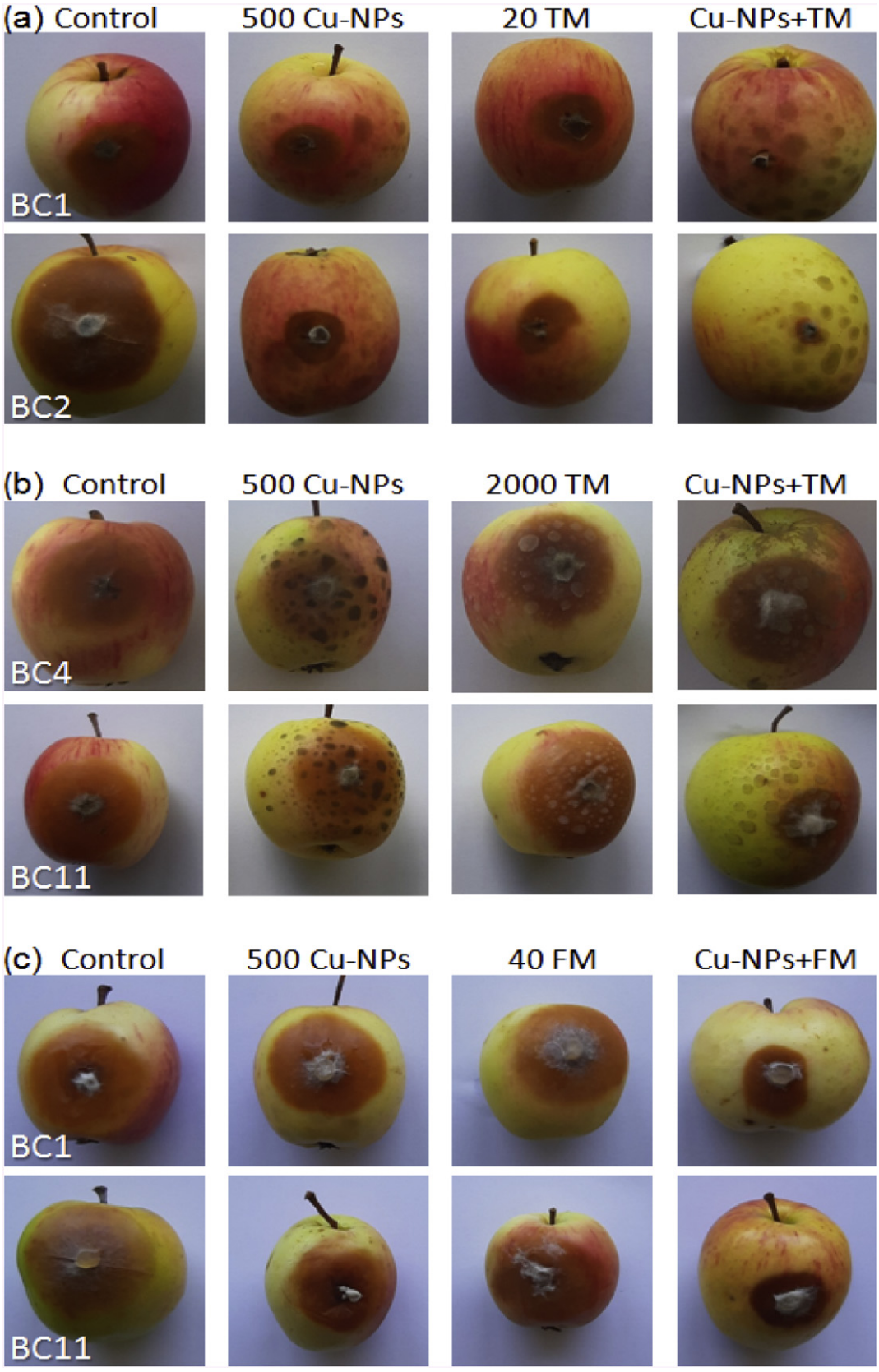

Fig. 3. Synergistic activity of Cu-NPs (500 μg/mL) in combination with thiophanate methyl (20, 2000 μg/mL) or fluazinam (40 μg/mL) on apple fruit against selected *Botrytis cinerea* isolates sensitive (BC1, BC2) and resistant (BC4, BC11) to thiophanate methyl. TM: thiophanate methyl, FM: fluazinam.

pathogens that developed high resistance to QoI fungicides due to the G143A mutation have been explored over the last decade by a large number of studies (FRAC, 2013; Malandrakis et al., 2011; Avenot and Michailides, 2015; Malandrakis et al., 2018). The effect of Cu-NPs, selected fungicides and their combinations against the above resistant phenotypes were investigated.

Table 6
Correlation between sensitivity of *B. cinerea* isolates to Cu-NPs, $Cu(OH)_2$, selected fungicides and their combinations.

	Cu-NPs	TM	Cu-NPs + TM	FM	Cu-NPs + FM	$Cu(OH)_2$	$Cu(OH)_2$ + TM	$Cu(OH)_2$ + FM
Cu-NPs	1.0	−0.02[a]	−0.23	−0.26	0.01	0.07	−0.04	0.12
TM	–	1.0	0.76**	0.06	0.48	0.08	0.67**	0.24
Cu-NPs + TM	–	–	1.0	0.03	0.16	0.24	0.76**	0.33
FM	–	–	–	1.0	0.23	0.24	−0.44	0.24
Cu-NPs + FM	–	–	–	–	1.0	−0.32	0.33	−0.06
$Cu(OH)_2$	–	–	–	–	–	1.0	0.22	0.39
$Cu(OH)_2$ + TM	–	–	–	–	–	–	1.0	0.29
$Cu(OH)_2$ + FM	–	–	–	–	–	–	–	1.0

[a] Pearson correlation coefficient values.
** Corresponds to a significance lever of $P = 0.01$.

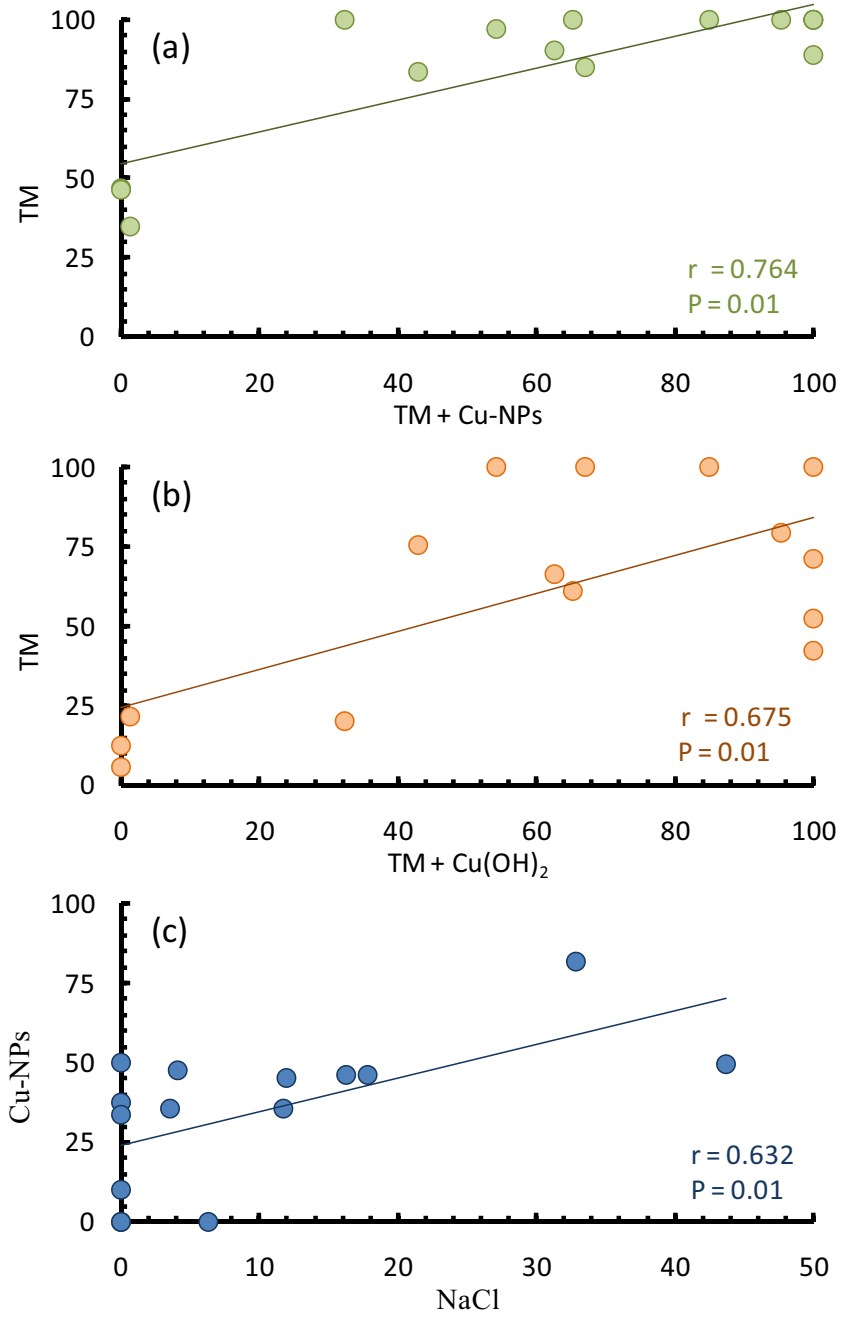

Fig. 4. Correlation between sensitivities of *B. cinerea* isolates to thiophanate methyl (TM) (0.25 μg/mL) and (a) Cu-NPs (300 μg/mL), (b) $Cu(OH)_2$ (500 μg/mL), and between (c) Cu-NPs (300 μg/mL) and NaCl (10^4 μg/mL) in terms of percent inhibition. Here, r is the Pearson correlation coefficient, and P the significance level.

The use of metal nanoparticles against drug-resistant pathogens is gaining ground especially in the case of multi drug resistant (MDR) clinical bacteria as a promising alternative/partner to antibiotics (Jampilek, 2016; Punjabi et al., 2018). Although a number of studies have demonstrated an enhanced antibacterial efficacy of antibiotics when used with metal NPs against MDR-strains, very few reports have focused on the synergistic action of nanoparticles with drugs against fungal pathogens, and even fewer reports have focused on fungicides and/or fungicide resistant strains. Jamdagni et al. (2018) tested both silver and zinc oxide nanoparticles used in combination with fungicides carbendazim, thiram, and mancozeb against plant pathogens *A. alternata, A.niger, B. cinerea, F. oxysporum* and *P. expansum*, and reported a significant synergistic effect, especially where green synthesized nanoparticles were used. A prominent synergistic effect was also reported in the literature for the case of *Bipolaria maydis*, when Ag-NPs were applied in combination with fungicides tebuconazole, propineb or fludioxonil (Huang et al., 2018). Xue et al. (2014) have reported synergistic and photodegradation properties of ZnO-NPs, when used in combination with thiram against *Phytophthora capsici*. Sun et al. (2016) have observed synergistic effects between PVR-coated Ag-NPs and azole fungicides against drug-resistant strains of *Candida albicans*.

In the present study, copper nanoparticles were equally effective against sensitive and BEN-R or PYR-R *B. cinerea* isolates. The combination of Cu-NPs with TM *in vitro* resulted in an enhanced inhibition of BEN-S *B. cinerea* isolates, compared to individual treatments. This observation indicates a synergistic interaction, which was more profound in *in vivo* experiments where even 50-fold decrease in the recommended TM dose in combination with Cu-NPs could fully suppress disease symptoms. Inhibition of BEN-R isolates by the Cu-NPs/TM mixture was not statistically different from that of the individual Cu-NPs treatment *in vitro*. This difference in synergistic interaction of Cu-NPs and TM between BEN-S and BEN-R isolates and the positive correlation found between TM and TM + Cu-NPs treatments indicate that sensitivity to TM is a key factor behind the observed synergism. These results lead to the hypothesis that in Cu-NPs + TM treatments a higher dose of the active ingredient of TM is available, in contact with its target site inside the fungal cell. A number of mechanisms could accomplish such an increased availability of the fungicide to its target site, including increased uptake, decreased efflux activity or faster transformation of the fungicide to a more toxic form inside the fungal cell (Jampilek, 2016). Even though the role of Cu-NPs in the above mechanisms is not certain, we speculate that a potential involvement of fungal influx/efflux pumps could contribute to a higher accumulation of TM inside the fungal cell. The involvement of the ABC transporter BcmfsM2 in efflux regulation of carbendazim leading to resistance in *B. cinerea* has been demonstrated by Leroux and Walker (2013). Increased availability of the fungicide is consistent with the lack of synergism in the case of BEN-R isolates where additional TM concentration would not lead to enhanced toxicity, because of the reduced affinity of β-tubulin with TM resulting from the target site mutation E198A. An indication that the fungitoxic activity of Cu-NPs is associated with ATP-dependent metabolism is the enhanced inhibition rates observed in all isolate cases when the Cu-NPs/fluazinam mixture was used, compared to the inhibition caused by the individual antifungal agents. Fluazinam is a known ATP-synthetase inhibitor preventing oxidative phosphorylation and thus resulting in energy starvation and subsequent impairment of energy-dependent efflux pumps (Kalamarakis et al., 2000; Leroux and Walker, 2013). A decreased efflux pump activity regulating metal ion homeostasis caused by fluazinam could result in an increased accumulation of Cu-NPs inside the fungal cell and account for the observed increased fungitoxic effect of the Cu-NPs/FM mixture. Certainly, further studies are needed to validate such a hypothesis. A decrease in multidrug resistance transporter activity of the marine organism *Mytilus galloprovincialis* following treatment with CuO-NPs or copper ions, as well as dysregulation of efflux pumps associated with the ability of Ag-NPs to control *Candida albicans* MDR strains in combination with antibiotics has been reported in the literature (Torres-Duarte et al., 2019; Sun et al., 2016).

The majority of the proposed mechanisms for the antibacterial/antifungal action of NPs implicate antimicrobial ions liberated by nanoparticle surfaces (Sun et al., 2018; Hoseinzadeh et al., 2017; Król et al., 2017). Several studies have used NaCl or KCl in an attempt to elucidate the role of metal cations in the fungitoxic effect of metal nanoparticles by reducing the concentration of available ions due to binding with chlorine anions (Jo et al., 2009). In this study, a positive cross sensitivity was found between Cu-NPs and NaCl as well as a profound antagonism between the two compounds when applied in combination. This result indicates that [Cu^{+2}] ions could be at least partly responsible for the observed fungitoxic action of the copper nanoparticles. Copper cations could also be implicated in the observed synergistic interaction between Cu-NPs or $Cu(OH)_2$ and TM in the BEN-S isolates, as indicated by the positive correlation between TM and any of the two Cu-NPs/TM or $Cu(OH)_2$/TM mixtures. However, Cu-NPs sensitivity was not significantly correlated with that of $Cu(OH)_2$, indicating differences in the mode of action between Cu-NPs and their bulk counterpart fungicide, a result which is in accordance with our previous study (Malandrakis et al., 2019).

Concluding, Cu-NPs were effective against *B. cinerea* isolates, sensitive and resistant to benzimidazoles and pyraclostrobin while their antifungal activity was in most cases enhanced when applied in combination with fluazinam or thiophanate methyl both *in vitro* and *in vivo*. Indications that [Cu^{+2}] cations and ATP-dependent metabolism are involved in the fungitoxic action and the synergistic interactions of Cu-NPs with tested fungicides were found. The results of this study suggest that Cu-NPs are promising antifungal alternatives, suitable both for effective anti-resistance strategies and a means for reducing environmental pollution caused by synthetic fungicides.

Conflict of interest

All three authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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.

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