Pesticide Biochemistry and Physiology 173 (2021) 104796

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates

Anastasios A. Malandrakis [a,b,*], Nektarios Kavroulakis [c], Constantinos V. Chrysikopoulos [a]

[a] School of Environmental Engineering, Technical University of Crete, 73100 Chania, Greece
[b] Pesticide Science Laboratory, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos, 118 55 Athens, Greece
[c] Hellenic Agricultural Organization "Demeter", Institute for Olive Tree, Subtropical Plants and Viticulture, Agrokipio-Souda, 73164 Chania, Greece

ARTICLE INFO

Keywords:
Benzimidazoles
Nano fungicides
Plant pathogens
Cu-NPs
CuO-NPs
Synergism

ABSTRACT

Nano-fungicides are expected to play an important role in future plant disease management. Their unique properties include a broad antimicrobial action, increased effectiveness in lower doses, slower a.i. release and/or enhanced drug delivery and an ability to control drug-resistant pathogens, which makes them appealing candidates for use as eco-friendly antifungal alternatives to counter fungicides resistance.

Copper nanoparticles (Cu-NPs) could suppress mycelial growth in both sensitive (BEN—S) and resistant (BEN-R) *Monilinia fructicola* isolates harboring the E198A benzimidazole resistance mutation, more effectively than copper oxide NPs (CuO-NPs) and $Cu(OH)_2$. A significant synergy of Cu-NPs with thiophanate methyl (TM) was observed against BEN-S isolates both *in vitro* and when applied on plum fruit suggesting enhanced availability or nanoparticle induced transformation of TM to carbendazim. ATP-dependent metabolism is probably involved in the mode of fungitoxic action of Cu-NPs as indicated by the synergy observed between Cu-NPs and the oxidative phosphorylation-uncoupler fluazinam (FM). Copper ion release contributed in the toxic action of Cu-NPs against *M. fructicola,* as indicated by synergism experiments with ethylenediaminetetraacetic acid (EDTA), although the lack of correlation between nano and bulk/ionic copper forms indicate an additional nano-property mediated mechanism of fungitoxic action. Results suggested that Cu-NPs can be effectively used in future plant disease management as eco-friendly antifungal alternatives to counter fungicides resistance and reduce the environmental footprint of synthetic fungicides.

1. Introduction

In modern crop protection systems, both conventional and integrated pest management (IPM) heavily depends on chemical control of plant pathogens as a means for efficient and economically feasible disease management. Systemic, highly effective modern fungicides constitute an invaluable arsenal for farmers especially in the cases of hard to manage plant pathogens (Pandey et al., 2018). Despite of their undeniable performance benefits -although increasingly compromised by resistance development- conventional fungicides are subject to negative criticism concerning the role of their residues in hazard issues related to food safety and the environment. As a result, an increasingly high number of fungicide active ingredients are being withdrawn by implementation of strict EU regulations concerning environmental safety – especially water pollution by pesticide leakage (Malandrakis et al., 2020a). Nanoparticle (NP) compounds are demonstrating a tremendous potential in various aspects of the agricultural sector including plant protection. Their small size (typically less than 100 nm) coupled with a set of unique properties that result in a wide range of antimicrobial action, increased effectiveness in lower doses, slower active ingredient release and/or enhanced drug delivery when formulated with fungicides, makes them promising, environmentally compatible alternatives to synthetic fungicides(Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). However, before NPs can be commercially introduced as antimicrobial agents, a number of challenges such as compatibility with plants and safety against humans and generally non-target organisms (Hoseinzadeh et al., 2017). Micro- or nano-sized copper containing compounds (Cu-NPs) add an enhanced effectiveness against a number of fungal pathogens to the already well established properties of bulk sized copper fungicides such as low cost, wide range of action -including bacterial diseases- and multi-site protective action that minimizes the risk for resistance development (Keller et al., 2017; Malandrakis et al., 2019). Effectiveness of Cu-NPs and CuO-

* Corresponding author at: School of Environmental Engineering, Technical University of Crete, 73100 Chania, Greece.
E-mail address: tasmal@aua.gr (A.A. Malandrakis).

https://doi.org/10.1016/j.pestbp.2021.104796
Received 5 December 2020; Received in revised form 22 January 2021; Accepted 24 January 2021
Available online 5 February 2021

NPs against various plant pathogens has been demonstrated in toxicity studies against *Fusarium oxysporum, F. oxysporum* fsp *radicis lycorersici, F. solani, Aspergillus flavus, A. fumigatus, A. niger, Phytophthora parasitica, Botrytis cinerea, Alternaria alternata, Vericillium dahliae, Colletotrichum gloiosporioides, Monilinia fructicola* and *Penicillium digitatum* (El-Abeid et al., 2020; Muthuchamy et al., 2020; Ammar et al., 2019; Malandrakis et al., 2019; Khamis et al., 2017). Several biochemical mechanisms explaining the antimicrobial action of copper nanoparticles have been proposed including damages affecting membrane integrity or DNA replication/transcription, interruption of electron transport and/or ATP synthesis, protein inactivation and oxidative stress induction (Nisar et al., 2019; Rai et al., 2017; Khan et al., 2016; Malandrakis et al., 2019). An ongoing debate regarding the role of ion release *versus* the nano-properties of the metal is fuelled by the typically greater effectiveness of copper nanoparticles compared with its bulk copper containing counterparts (Rudramurthy et al., 2016; Franci et al., 2015; Huang et al., 2018; Malandrakis et al., 2019).

Monilia fructicola (teleomorph *Monilinia fructicola*), is the causal agent of brown rot, one of the most important stone-fruit pre- and post-harvest diseases in Greece and worldwide (Agrios, 2005). Fungicides belonging to the chemical classes of benzimidazoles, dicarboximides, triazoles, hydroxyanilides and more recently QoIs and succinate dehydrogenase inhibitors (SDHI) consist the main arsenal for controlling the disease (Miessner and Stammler, 2010). Regardless of their initial effectiveness, most of the above chemical compounds have suffered from the emergence of *M. fructicola* isolates with reduced sensitivity over the last decades (Chen et al., 2013; Penrose et al., 1985; Brent and Hollomon, 1998; Ma and Michailides, 2005; FRAC, n.d.). Benzimidazoles, being the oldest systemic fungicides used against brown rot, soon lost their efficacy against the pathogen due to their extensive and exclusive use that led to resistance development (Ma et al., 2003; Stehmann and de Waard, 1996; Malandrakis et al., 2011). The most important mechanism leading to benzimidazole resistance in *M. fructicola* has been identified to be target site modification in most cases resulting from the E198A amino acid substitution of the β-tubulin gene that is associated with high levels of benzimidazole resistance (Ma and Michailides, 2005; Chen et al., 2014; Malandrakis et al., 2020a). Benzimidazoles consist a paradigm demonstrating the risk of resistance development compromising fungicide control efficacy worldwide and at the same time the need of alternative means for combating resistance in order to safeguard the increasingly diminishing arsenal against fungal diseases.

A promising approach to counter fungicide resistance and reduce the environmental footprint of chemicals, concerns the use of metal nanoparticles alone or in combination with conventional fungicides against fungal pathogens (Malandrakis et al., 2019, 2020a). In the public health sector, strong evidence of a marked efficacy of NPs containing copper, silver, zinc and ferric when used instead or in combination with antibiotics has been reported especially against multi-drug (MDR) resistant pathogenic bacteria such as *Eschericia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis* (Assadi et al., 2018; Hamed et al., 2017; Punjabi et al., 2018; Gabrielyan et al., 2019; Nejabatdoust et al., 2019; Paralikar et al., 2019). As far as plant health is concerned, silver and zinc oxide NPs have shown a significant toxic action against fungal pathogens resistant to conventional fungicides applied alone or in mixtures with fungicides such as thiram, tebuconazole, propineb, carbendazim, fludioxonil, and mancozeb (Malandrakis et al., 2020b; Huang et al., 2018; Jamdagni et al., 2018; Xue et al., 2014). Apart from a recent study by Malandrakis et al. (2020a) where Cu-NPs (alone or in combination with conventional fungicides) were studied against *B. cinerea* fungicide-resistant isolates, no data is available regarding the potential of copper nanoparticles to control fungal isolates resistant to fungicides.

In this study, the potential of Cu-NPs to control *M. fructicola* isolates sensitive or resistant to benzimidazoles alone or in combination with fungicides was evaluated. Specifically, the aim of this study was to: (a) evaluate the potential of Cu-NPs to be used against sensitive/resistant *M. fructicola* phenotypes both *in vitro* and *in vivo,* (b) to investigate any potential synergism between copper NPs and conventional fungicides when applied against the above phenotypes, and (c) to elucidate mechanisms underlying the mode of fungitoxic action of Cu-NPs and/or any synergistic relationships existing between Cu-NPs and tested fungicides.

2. Materials and methods

2.1. Nanoparticles, reagents and fungicides

Copper (Cu-NPs) (particle size 25 nm) and copper oxide (CuO-NPs) (particle size <50 nm) nanoparticles, salicylhydroxamate (SHAM), $CuSO_4$ and ethylenediaminetetraacetic acid (EDTA) used in this study were purchased from Sigma Aldrich, MO, USA. Commercial fungicides containing $Cu(OH)_2$ (Copperblau-N 50 WP), thiophanate methyl (Neotopsin 70 WG) and fluazinam (Azzuro 50 SC), were purchased from their respective manufacturers. Other fungicides used in this study were of pure analytical grade: tebuconazole and carbendazim were kindly supplied by Bayer CropScience AG (Leverkusen, Germany). All analytical grade stock solutions were prepared using ethanol as a solvent. Antifungal agents were added under aseptic conditions to sterilized growth medium prior to inoculation taking care that the solvent never exceeded 1% (v:v) of the total volume. Distilled-sterilized water was used for the preparation of commercial fungicide and nanoparticle stock solutions. In order to prevent particle aggregation, nanoparticle suspensions were sonicated for 30 min using a Transonic 420 (Elma, Germany) sonicator prior to incorporation in growth media.

2.2. Fungal isolates and culture conditions

Monilinia fructicola isolates used in this study originated from stone-fruit orchards of southern Greece, collected during a monitoring survey in 2019. Positive identification at the species level as well as sensitivity characterization of fungal isolates was conducted in a previous study by Malandrakis et al. (2020b). Twenty-three single spore isolates were positively identified to be *Monilia fructicola*, according to morphological examination and sequencing of the *cytb* gene and subsequently used in fungitoxicity bioassays against Cu-NPs and fungicides (Malandrakis et al., 2020b). Benzimidazole resistant isolates carried the E198A resistance mutation resulting from the substitution of glutamic acid (E: GAG) by alanine (A: GCG) at the respective position of the β-tubulin protein as revealed by sequencing of the above gene (Malandrakis et al., 2020b).

Fungal cultures grown on Potato Dextrose Agar (PDA), used for inoculum production were kept in growth chambers at 25 °C with 14 h day^{-1} light and 70% RH. For long-term storage, isolates were transferred once a month in PDA containing glass tubes and stored at 4 °C in the dark.

2.3. In vitro bioassays

2.3.1. Sensitivity of M. fructicola to copper-NPs and fungicides

The potential fungitoxic activity of Cu-NPs and CuO-NPs against sensitive and benzimidazole-resistant *M. fructicola* isolates, was tested *in vitro* by poison agar assays. Fungitoxicity of NPs and fungicides was assessed by inoculating isolates on growth medium containing appropriate concentrations of the antifungal agents compared to the untreated control and expressed as percent inhibition. Baseline sensitivity of *M. fructicola* to Cu-NPs and $Cu(OH)_2$ was evaluated by obtaining fungitoxicity-curves and subsequently calculating EC_{50} values based on concentrations of 10, 25, 50, 100, 250, 500 and 1000 μg/mL of each antifungal agent. Sensitivity of *M. fructicola* to EDTA was determined by preliminary fungitoxicity tests utilizing, concentrations of 0, 1, 100, and 1000 μg/mL. Each antifungal agent treatment was applied trice.

Specifically, following solidification of the treated and non-treated (control) growth media, a 5-mm mycelial plug from the edge of a 5-day old colony of each isolate was transferred to the center of each plate. Cultures were then incubated in a growth chamber at 25 °C with 70% RH for 4 days in the dark. Percent inhibition rates were calculated according to the formula: 100-(mean diameter of the colony on the fungicide-treated plates divided by the mean diameter of the untreated control) × 100. Fungitoxicity tests conducted for each isolate were repeated twice for each concentration and antifungal agent.

2.3.2. Synergy between copper nanoparticles and antifungal agents

The potential of Cu-NPs, CuO-NPs and $Cu(OH)_2$ to act synergistically in combination with selected fungicides was assessed *in vitro*. Concentrations of 0.5 μg/mL thiophanate methyl, 0.15 μg/mL carbendazim, 0.01 μg/mL tebuconazole, 0.2 μg/mL fluazinam and 100 μg/mL EDTA and their combinations with 250 μg/mL Cu-NPs were applied aseptically from stock solutions to PDA medium consequently poured on Petri plates. 500 μg/mL of CuO-NPs or $Cu(OH)_2$ were used in respective synergy bioassays with the above concentrations of thiophanate methyl and fluazinam. Following inoculation with each *M. fructicola* isolate, plates were incubated at 25 °C for 4 days in the dark and then the mycelial growth percent inhibition was calculated. The Abbortt method was adopted in order to evaluate the potential synergistic interactions of nanoparticles with antifungal agents (Gisi, 1996). Briefly, the expected combined percent inhibition (% CI_{exp}) was calculated according to the formula: % $CI_{exp} = I_A + I_B – (I_A \times I_B/100)$, where I_A and I_B represent the percent inhibition of each treatment.

% CI_{exp} values were subsequently used to calculate Synergy factors (SFs) according to the formula: $SF = I_{AB}/(\%\ CI_{exp})$, where I_{AB} is the observed combined percent inhibition caused by the antifungal agents. SF values close to 1 were considered to indicate additive, greater than 1 synergistic, and less than 0.75 antagonistic interactions.

2.4. Fungitoxicity tests in vivo

Plum fruit (*Prunus domestica*) without any visible wound, selected based on their uniformity of size, shape and maturity were used to assess the *in vivo* efficacy of Cu-NPs to suppress sensitive/benzimidazole-resistant *M. fructicola* isolates, alone or in combination with thiophanate methyl and fluazinam. Four representative −2 sensitive (MF1, MF5) and 2 benzimidazole-resistant (MF18, MF28)- *M. fructicola* isolates were used to inoculate 4 fruits treated with Cu-NPs, fungicides and their respective combinations. Plum fruits sprayed with sterilized distilled water were used as control treatments. Before treatment, fruits were surface-disinfected by immersion in a 1% sodium hypochlorite solution for 10 min, rinsed 3 times with distilled-sterilized water and then left to dry for 1 h. Fruit were then sprayed with solutions containing 250 μg/mL Cu-NPs; 50 and 1000 μg/mL thiophanate methyl (1/20, 1× of the maximum recommended dose) and 500 μg/mL fluazinam (1/2 of the maximum recommended dose), and their combinations. Fruit were air-dried for 2 h and then, the front face of each fruit was wounded using a needle, creating a 2 × 2 mm [length×width] cross-shaped scar. Inoculum consisting of a 5-mm mycelial plug cut from the edge of a 4-day old colony from each *M. fructicola* isolate was placed in each wound. Inoculated fruit were placed on top of a wet sterilized paper inside plastic boxes 24 × 34 × 10 cm [length×width×height], covered by a lid and were incubated at 25 °C for 4 days in the dark. Symptom severity was scored by the following formula:

% Symprom severity = (lesion diameter around each wound of treated fruit) / (lesion diameter of the water-treated control) × 100. All experiments were repeated two times.

2.5. Carbendazim detection

In order to investigate any possible contribution of copper-mediated TM transformation to carbendazim in the observed synergism profile between Cu-NPs and TM, UV-measurements were conducted using a UVICON 922 UV spectrophotometer. Water suspensions containing 250 μg/mL Cu-NPs, 500 μg/mL CuO-NPs, 500 μg/mL $Cu(OH)_2$, 0.5 μg/mL TM and their respective mixtures were prepared. Standard curves of thiophanate methyl and carbendazim were obtained using concentrations of 0.01, 0.05, 0.075, 0.1, 0.35, 0.5, 1.0,1.25 μg/mL from stock solutions. In order to remove background absorbance caused by nano/$Cu(OH)_2$ particles, mixtures were agitated for 10 min, then centrifuged at 2000 rpm for 10 min and then carbendazim concentration of the supernatant was measured. The concentrations of carbendazim and TM were calculated by measuring the absorbance of samples at 285 and 265 nm, respectively, and using equations of the standard curves. For each treatment, three replicates were used.

2.6. Statistical analysis

EC_{50} values for each isolate and antifungal compound were estimated by regressing the relative inhibition of mycelial growth against the Log_{10} of the compound concentrations. Sensitivity correlation to tested NPs/fungicides in all isolates was evaluated using Pearson correlation coefficients. Inhibition rates of Cu-NPs and fungicides were subjected to analysis of variance and means were separated according to Tukey's HSD test ($\alpha = 0.05$). All statistical analyses were conducted using the SPSS v20 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. In vitro sensitivity of M. fructicola isolates to Cu-NPs and fungicides

The sensitivity distribution of *M. fructicola* isolates to Cu-NPs and $Cu(OH)_2$ based on EC_{50} values revealed by *in vitro* fungitoxicity assays is shown in Fig. 1. Sensitivity to Cu-NPs ranged between 104 and 395 μg/mL and a median value of 252 μg/mL, while respective values for $Cu(OH)_2$ were more widely distributed with EC_{50} values ranging between 200 and 792 μg/mL with a median value of 498 μg/mL. Note that Cu-NPs were significantly more effective against *M. fructicola* ($P < 0.01$) than CuO-NPs, $CuSO_4$ and the protective fungicide containing $Cu(OH)_2$ *in vitro* (see Table 1). Despite the wide range of variation in the sensitivity observed, Cu-NPs were generally equally effective against both BEN-S and BEN-R isolates (see Tables 1, S1).

3.2. Synergy between copper NPs and fungicides

3.2.1. In vitro bioassays

Synergy factors (SF) calculated between copper nanoparticles and combinations with fungicides in *in vitro* fungitoxicity tests are listed in Table 2. A significant synergistic effect was observed between Cu-NPs and fluazinam with the respective mixture completely inhibiting mycelial growth in almost all cases of BEN-S and BEN-R isolates (see Fig. 2c). Respective SF values ranged between 0.99 and 1.61 (see Table 2). An additive effect was observed in the combination of CuO-NPs with fluazinam with SF values ranging from 0.92 to 1.06 (see Table 3). The addition of thiophanate methyl significantly enhanced the Cu-NPs fungitoxic action only in the case of BEN-S isolates, which were completely inhibited by the combination (see Fig. 2b). On the contrary, in most BEN-R isolates an antagonistic effect was observed although in more than half of those isolates the mixture toxicity did not differ statistically from the most toxic individual treatment (see Table 2, Fig. 2b, Fig. 3). A similar synergistic profile, although to a lesser extend, was observed between CuO-NPs and thiophanate methyl (see Table 2, Fig. 2a). The above combination significantly enhanced the inhibition of BEN-S isolates while it did not increase the fungitoxic action of the CuO-NPs in most of the BEN-R isolates (see Fig. 2a). In most isolate cases the synergistic relationship between Cu-NPs and tebuconazole ranged between additive (SF:0.89) and synergistic (SF:2.50) regardless the benzimidazole resistance phenotype (see Table 3, Fig. 2d).

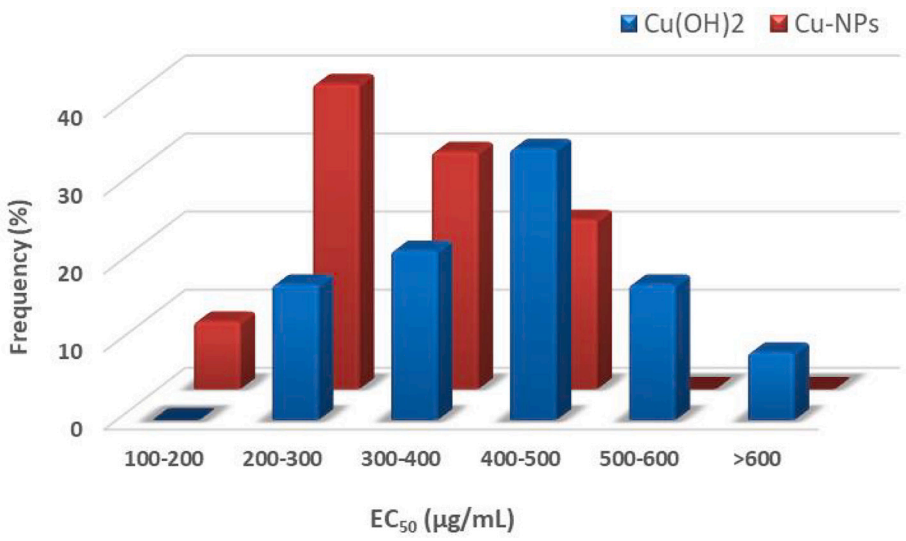

Fig. 1. Sensitivity distribution of *Monilia fructicola* field isolates to Cu-NPs and $Cu(OH)_2$ based on EC_{50} values.

Table 1
Sensitivity of *Monilia fructicola* isolates to copper nanoparticles, their bulk counterparts and benzimidazole-resistance phenotypes.

Resistance phenotypes/mutations in the β-tubulin gene		Percent Inhibition [a] (mean ± SD[b])				
Phenotype	Amino acid substitution	Cu-NPs (250)[c]	CuO-NPs (500)	$Cu(OH)_2$ (500)	$CuSO_4$ (250)	EDTA (100)
BEN-S[d]	E198	44.62 ± 16.06 (b/A)[e]	10.93 ± 9.35 (a/A)	44.01 ± 16.59 (b/A)	16.53 ± 17.34 a/A (a/A)	13.26 ± 1.08 (a/A)
BEN-R	E198A	32.55 ± 18.72 (b/A)	7.66 ± 7.90 (a/A)	57.18 ± 11.47 (b/A)	10.08 ± 7.99 (a/A)	24.90 ± 12.74 (ab/A)

[a] Calculated as percent inhibition of mycelial growth compared to the untreated control after 4 days incubation at 25 °C ($n = 3$).
[b] Standard deviation of the means (n = 3).
[c] Numbers in parenthesis indicate fungicide concentrations in μg/mL of active ingredient.
[d] BEN-S/R: Benzimidazole Sensitive/ Resistant isolate.
[e] Means followed by the same letter do not differ significantly according to Tukey's HSD test (α = 0.05). Small letters correspond to statistical differences between rows (treatments) while capitals to columns (phenotypic groups).

In an attempt to evaluate the potential involvement of copper ions release on the synergistic patterns observed between Cu-NPs/CuO-NPs and thiophanate methyl or fluazinam, a bulk counterpart fungicide containing $Cu(OH)_2$ was used instead of the above copper nanoparticles in synergism bioassays. Combination of $Cu(OH)_2$ with thiophanate methyl resulted in mixed synergy patterns that were isolate dependent and could not be associated with the resistance phenotypes. In most isolates, an additive effect was recorded, although antagonistic as well as synergistic effects were also observed (see Table 2). In the case of the fluazinam/$Cu(OH)_2$ combination, most isolates responded in an additive manner while the fungitoxic activity was decreased in a few isolates (see Table 2), probably indicating that copper ions release contributes less than nanoparticles to the observed synergistic effect between Cu-NPs and fluazinam.

Evidence of the involvement of copper ion release in the fungitoxic activity of Cu-NPs against *M. fructicola* isolates was found in the combination of Cu-NPs with the strong chelating agent EDTA. Addition of 100 μg/mL EDTA in growth medium containing 250 μg/mL Cu-NPs resulted in the neutralizing of the fungitoxic action of the copper nanoparticles. This was evident by the synergy factors values between Cu-NPs and EDTA that ranged between 0.02 and 0.45 (see Table 2).

3.2.2. *In vivo bioassays*

The potential synergistic activity of Cu-NPs used in combination with thiophanate methyl and fluazinam was evaluated on accordingly treated plum fruit artificially inoculated with selected BEN-S and BEN-R *M. fructicola* isolates. Treatment of plum fruit inoculated with BEN-S isolates MF1 and MF5, with 250 μg/mL Cu-NPs resulted in a 32.05 and 16.67% inhibition while 50 μg/mL thiophanate methyl resulted in 52.56 and 30.00% inhibition. Combination of the above treatments caused a complete inhibition of disease symptoms of the BEN-S isolates demonstrating an strong synergistic effect (SF:1.48 and 2.40, respectively, Table 3, Fig. 4a). A slight additive effect was observed in the case of BEN-R isolates MF18 and MF28, when 1000 μg/mL thiophanate methyl was combined with 250 μg/mL Ag-NPs (SF: 0.81 and 0.88, respectively, Table 3, Fig. 4b). The synergy profiles observed *in vitro* between Cu-NPs and fluazinam were consistent with that observed *in vivo* (see Fig. 4c). This strong synergistic effect was observed in both resistance phenotypes with SF values ranging from 1.36 to 2.08, pointing out a promising potential of FM to enhance Cu-NPs effectiveness against sensitive and resistant isolates (see Table 3).

3.3. *Sensitivity correlations between copper nanoparticles their bulk counterparts and fungicide combinations*

Pearson correlation coefficients were calculated in an attempt to evaluate any possible contribution of Cu-NPs, thiophanate methyl (TM), fluazinam (FM) and $Cu(OH)_2$ in the observed synergistic relations between them, and the potential involvement of copper ions in the fungitoxic activity of Cu-NPs (see Table 4). No significant correlation was

Table 2
In vitro synergistic activity of Cu-NPs or $Cu(OH)_2$ with selected fungicides against fungicide sensitive and resistant *Monilia fructicola* isolates (TM: thiophanate methyl. FM: fluazinam. TEB: tebuconazole).

Isolate		SF[a]								
			Cu-NPs (250)				$Cu(OH)_2$ (500)		CuO-NPs (500)	
	Resistance Phenotype	TM (0.5)[c]	FM (0.2)	CARB (0.05)	TEB (0.01)	EDTA (100)	TM (0.5)	FM (0.2)	TM (0.5)	FM (0.2)
MF1	BEN-S[b]	1.29	1.16	0.39	1.49	0.02	1.16	0.67	1.03	1.06
MF3	BEN-S	1.20	1.10	0.96	0.96	0.04	0.84	0.94	1.09	1.00
MF5	BEN-S	1.21	1.03	1.04	1.01	0.03	0.89	1.02	1.46	1.00
MF6	BEN-S	1.10	1.61	0.56	1.53	0.14	0.69	0.63	0.98	0.92
MF12	BEN-S	1.18	1.24	1.02	1.12	0.23	0.81	1.00	0.96	0.94
MF14	BEN-S	1.70	0.99	1.03	0.96	0.13	0.90	0.98	1.15	0.95
MF29	BEN-S	1.13	1.16	0.70	2.50	0.07	1.08	0.99	1.01	1.00
Group Mean		**1.26 d/B**[d]	**1.18 c/A**	**0.81 b/A**	**1.37 d/B**	**0.09 a/A**	**0.91 a/A**	**0.89 a/A**	**1.10 a/B**	**0.98 a/A**
MF4	BEN-R	0.76	1.10	0.95	1.09	0.05	0.92	1.00	0.72	0.90
MF7	BEN-R	0.66	1.11	0.96	0.95	0.13	0.64	0.59	0.65	0.94
MF8	BEN-R	0.56	1.10	0.56	1.41	0.42	0.43	1.00	0.63	0.89
MF9	BEN-R	0.75	1.12	0.86	0.96	0.06	1.68	0.85	0.37	1.02
MF10	BEN-R	0.95	1.42	0.35	0.93	0.09	0.93	1.00	0.94	1.01
MF15	BEN-R	0.58	1.13	0.91	1.02	0.43	0.86	0.90	0.78	1.03
MF16	BEN-R	0.74	1.10	1.04	0.89	0.71	0.58	0.96	0.79	0.95
MF17	BEN-R	0.14	1.02	0.56	0.91	0.34	0.89	0.97	0.68	0.99
MF18	BEN-R	0.71	1.07	0.61	1.13	0.21	0.95	0.80	0.84	0.97
MF20	BEN-R	0.54	1.18	1.01	0.97	0.17	0.78	1.00	0.46	0.98
MF21	BEN-R	0.64	1.20	0.70	0.86	0.17	0.98	0.71	0.65	1.00
MF22	BEN-R	0.71	1.23	0.92	1.46	0.45	0.79	0.92	0.42	0.93
MF23	BEN-R	0.13	1.03	0.90	0.93	0.24	1.02	0.99	0.63	0.97
MF25	BEN-R	0.53	0.96	0.46	0.89	0.34	1.08	0.99	0.76	0.92
MF27	BEN-R	0.67	1.07	0.87	0.99	0.05	0.83	1.00	0.21	1.01
MF28	BEN-R	0.44	1.16	0.67	0.86	0.29	0.90	1.03	0.47	1.01
Group Mean		**0.59 b/A**	**1.13 c/A**	**0.77 b/A**	**1.02 c/A**	**0.26 a/B**	**0.89 b/A**	**0.92 b/A**	**0.63 a/A**	**0.97 b/A**

[a] Synergy Factor.
[b] BEN-S/R: Benzimidazole Sensitive/ Resistant isolate.
[c] Numbers in parenthesis indicate antifungal agent concentrations in μg/mL of active ingredient.
[d] Means followed by the same letter do not differ significantly according to Tukey's HSD test ($\alpha = 0.05$). Small letters correspond to statistical differences between rows (treatments) while capitals to columns (phenotypic groups).

found between TM and Cu-NPs, FM or their respective combinations while, the correlation found between TM and Cu-NPs + TM treatments (see Fig. 5a) probably indicate that TM contributed more to the enhanced fungitoxic effect of the above combination than Cu-NPs. A similar significant correlation was found between TM and the TM + $Cu(OH)_2$ and TM + CuO-NPs treatments also indicating a possible contribution of TM on the fungitoxic effect of the combination (see Fig. 5b,c). In the cases of $Cu(OH)_2$, FM and Cu-NPs and the respective combinations, no significant correlation was found (see Table 4). No correlation was found between the inhibition caused by Cu-NPs and CuO-NPs or its bulk-ionic counterparts $Cu(OH)_2$ and CuSO4 (see Table 4) while CuO-NPs toxicity was positively ($r = 0.43$, $P = 0.038$) correlated with the toxicity exerted by the commercial fungicide containing $Cu(OH)_2$.

3.4. Copper mediated TM tansformation to carbendazim

After 10 min incubation of 0.5 μg/mL TM with 250 μg/mL Cu-NPs, 500 CuO-NPs μg/mL, and 500 μg/mL $Cu(OH)_2$, the carbendazim concentration was found to be 0.25, 0.05 and 0.15 μg/mL, respectively, while no detectable carbendazim amount was found in the sample containing only TM. These results indicate a possible copper-mediated acceleration of the known natural process of TM transformation to carbendazim.

4. Discussion

The potential of Cu-NPs to combat sensitive or fungicide resistant *M. fructicola* isolates collected from Greek orchards was tested. *In vitro* screening for resistance among a number registered fungicides revealed 16 isolates with high resistant levels to the benzimidazole (BEN-R) fungicides carbendazim and thiophanate methyl. All BEN-R isolates harbored the E198A resistance mutation in the β-tubulin gene, as revealed by DNA sequencing analysis. The above well-documented target site mutation is one of the major benzimidazole resistance mutations associated with high resistance levels in many plant fungal pathogens including *M. fructicola* worldwide worldwide (Ma et al., 2003; Stehmann and de Waard, 1996; Malandrakis et al., 2011; Ziogas et al., 2009; Ma and Michailides, 2005; FRAC, n.d.). The Cu-NPs toxicity, individually or in combination with selected fungicides, was subsequently evaluated against both BEN-S and BEN-R phenotypes.

The ability of metal NPs alone or in combination with drugs to control clinical drug-resistant pathogens, has recently become subject of scientific focus in an increasing number of studies (Jampilek, 2016; Punjabi et al., 2018). Jankauskaitė et al. (2016) reported that combination of Cu and Ag-NPs, as well as GO-Cu-Ag nanocomposite materials exhibit enhanced antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and Methicillin-resistant *S. aureus* strains through a possible synergy of multiple toxicity mechanisms. Also, CuO-Nps have been reported to act as a tetracycline carrier resulting in a synergistic effect against *P.aeruginosa* and *S.aureus* due to an enhanced accumulation of the antibiotic (Assadi et al., 2018). Similar synergistic effects of copper nanoparticles with antibiotics, tetracycline, and kanamycin against *Bacillus subtilis* and *Pseudomonas fluorescens* have been reported in the literature (Khurana et al., 2016).

The majority of cases studied involve bacterial infections caused by MDR-strains, while limited are the reports on the effect of nanoparticles with fungicides on sensitive or fungicide resistant fungal pathogens. Jamdagni et al. (2018) reported a significant synergy of Ag-NPs and ZnO-NPs when combined with mancozeb, carbendazim and thiram

Fig. 2. Sensitivity of fungicide-sensitive/resistant *M. fructicola* isolates to (a) CuO-NP and (500 μg/mL) in comparison with TM (0.5 μg/mL) and Cu-NPs (250 μg/mL) in comparison with: (b) TM (0.5 μg/mL). (c) fluazinam (0.2 μg/mL). d) TEB (0.01 μg/mL) and combinations. BEN-S/R: benzimidazole- Sensitive/Resistant isolates (TM: thiophanate methyl. FM: fluazinam. TEB: tebuconazole). 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 ($\alpha = 0.05$) .

Table 3
Synergistic activity of Cu-NPs co-applied with thiophanate mehyl or fluazinam on plum fruit against *Monilia fructicola* isolates sensitive and resistant to benzimidazole fungicides (TM: thiophanate methyl. FM: fluazinam).

Isolate		Percent inhibition[a] (mean±SD)				Percent inhibition (mean ± SD)[b]			
	Phenotype	Cu-NPs (250)[d]	TM (50/1000)[e]	Cu-NPs + TM	SF[f]	Cu-NPs (250)	FM (500)	Cu-NPs + FM	SF
MF1	BEN-S[c]	32.05 ± 1.00	52.56 ± 3.45	100.00	1.48	32.05 ± 1.00	47.44 ± 3.70	87.18 ± 4.65	1.36
MF5	BEN-S	16.67 ± 0.59	30.00 ± 2.15	100.00	2.40	16.67 ± 0.59	31.67 ± 1.67	83.33 ± 3.18	1.94
MF18	BEN-R	26.87 ± 1.15	20.90 ± 4.07	34.33 ± 2.94	0.81	26.87 ± 1.15	22.39 ± 2.00	85.07 ± 2.55	1.97
MF28	BEN-R	6.15 ± 0.04	4.62 ± 2.00	9.23 ± 0.45	0.88	6.15 ± 0.04	38.46 ± 1.86	87.69 ± 0.84	2.08

[a] Calculated as percent inhibition of lesion development on plum fruit sprayed with Ag-NPs/fungicides and their combinations compared to the untreated control after 4 days incubation at 25 °C (n = 3).
[b] Standard deviation of the means (n = 3).
[c] BEN-S/R: Benzimidazole Sensitive/ Resistant isolate.
[d] Numbers in parenthesis indicate fungicide concentrations in μg/mL of active ingredient.
[e] Plum fruit inoculated with BEN-S isolates were sprayed with 50 μg/mL while those with BEN-R isolates with 1000 μg/mL TM.
[f] Synergy Factor.

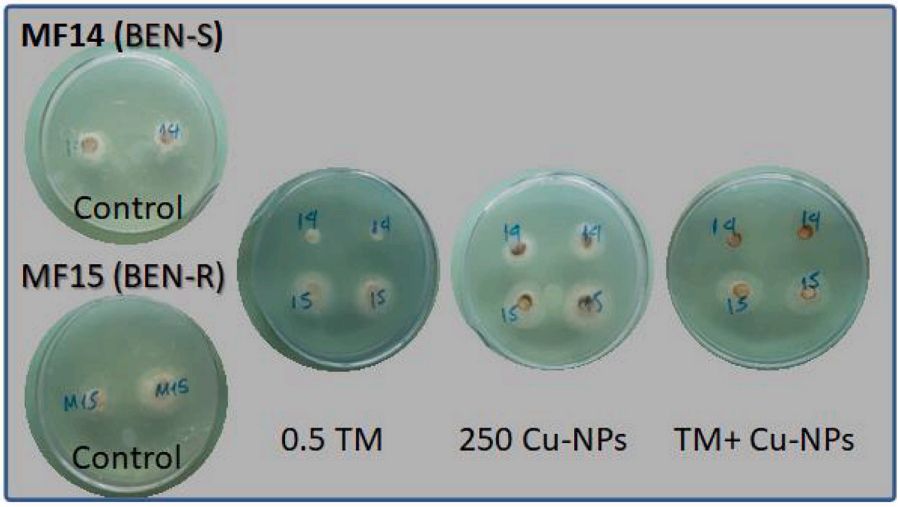

Fig. 3. Fungitoxic activity of Cu-NPs. TM and their combination in sensitive (MF14). and TM-resistant (MF15) *M. fructicola* isolates (TM: thiophanate methyl. BEN-S/R: Benzimidazole Sensitive/ Resistant).

against plant pathogens *Alternaria alternata, Aspergilus niger, B. cinerea, P. expansum and F. oxysporum*. Similar results were reported by Huang et al. (2018) concerning the synergy observed between Ag-NPs and fungicides propineb, tebuconazole and fludioxonil in *Bipolaria maydis*. Zinc oxide NPs combined with thiram resulted in an enhanced fungitoxicity against *Phytophthora capsici* and at the same time led to a quicker thiram degradation due to ZnO-NPs photo-catalytic properties (Xue et al., 2014). Recently, a study on fungicide resistant strains of *B. cinerea* showed a strong synergy of Cu-NPs when used with fungicides fluazinam and thiophanate methyl (Malandrakis et al., 2020a).

Cu-NPs evaluated in the present study, were significantly more effective against both BEN-S and BEN-R *M. fructicola* isolates compared to CuO-NPs, $CuSO_4$ and the protective fungicide containing $Cu(OH)_2$. A significant synergy between Cu-NPs and TM was observed both *in vitro* and *in vivo* in all the BEN-S *M. fructicola* isolates whereas an antagonistic/additive effect was observed in the case of BEN-R isolates. Similar -but less profound- synergy patterns were observed when CuO-NPs were co-applied with TM *in vitro*. On the contrary, in the case of $Cu(OH)_2$ + TM treatment, no conclusive synergy pattern could be attributed to the different resistance phenotypes. This differential response between benzimidazole phenotypes to the Cu-NPs + TM combination, backed by the positive correlation found between TM and TM + Cu-NPs sensitivities, indicates that the major factor underlying the observed synergism is probably an enhancement of TM toxicity. This is in accordance with a previous study with *B. cinerea* resistant isolates, where Cu-NPs/TM synergy was mainly correlated with a higher TM bioavailability (Malandrakis et al., 2020a). Another possible explanation about the enhanced toxicity of TM in the Cu/CuO-NPs + TM combination could be an accelerated transformation of TM to the more toxic carbendazim. This could explain why BEN-S isolates were affected by the mixtures while the BEN-R, harboring the E198A target site resistance mutation, were not. The fact that bulk sized $Cu(OH)_2$ did not facilitate a similar synergy probably indicates that nanoparticle properties rather than $[Cu]^{+2}$ ion release are responsible for such an accelerated transformation.

The synergy observed between Cu-NPs and fluazinam is indicative for an involvement of ATP-dependent metabolism in the fungitoxic action of Cu-NPs against *M. fructicola*. Fluazinam, being a well-known ATP-synthetase inhibitor, is associated with the function of energy-dependent efflux pumps (Leroux and Walker, 2013). One major copper detoxification mechanism relies on extrusion pumps which are in fact ATP-dependent heavy metal translocators (Antsotegi-Uskola et al., 2020). A possible inhibition of the activity of such pumps by fluazinam disturbing metal ion homeostasis, could be responsible for an increased accumulation of Cu-NPs inside the fungal cell enhancing its fungitoxic effect. This synergistic effect between Cu-NPs and fluazinam was also observed in *B. cinerea* probably indicating the existence of an energy dependent mechanism affecting nano metal fungitoxicity common in

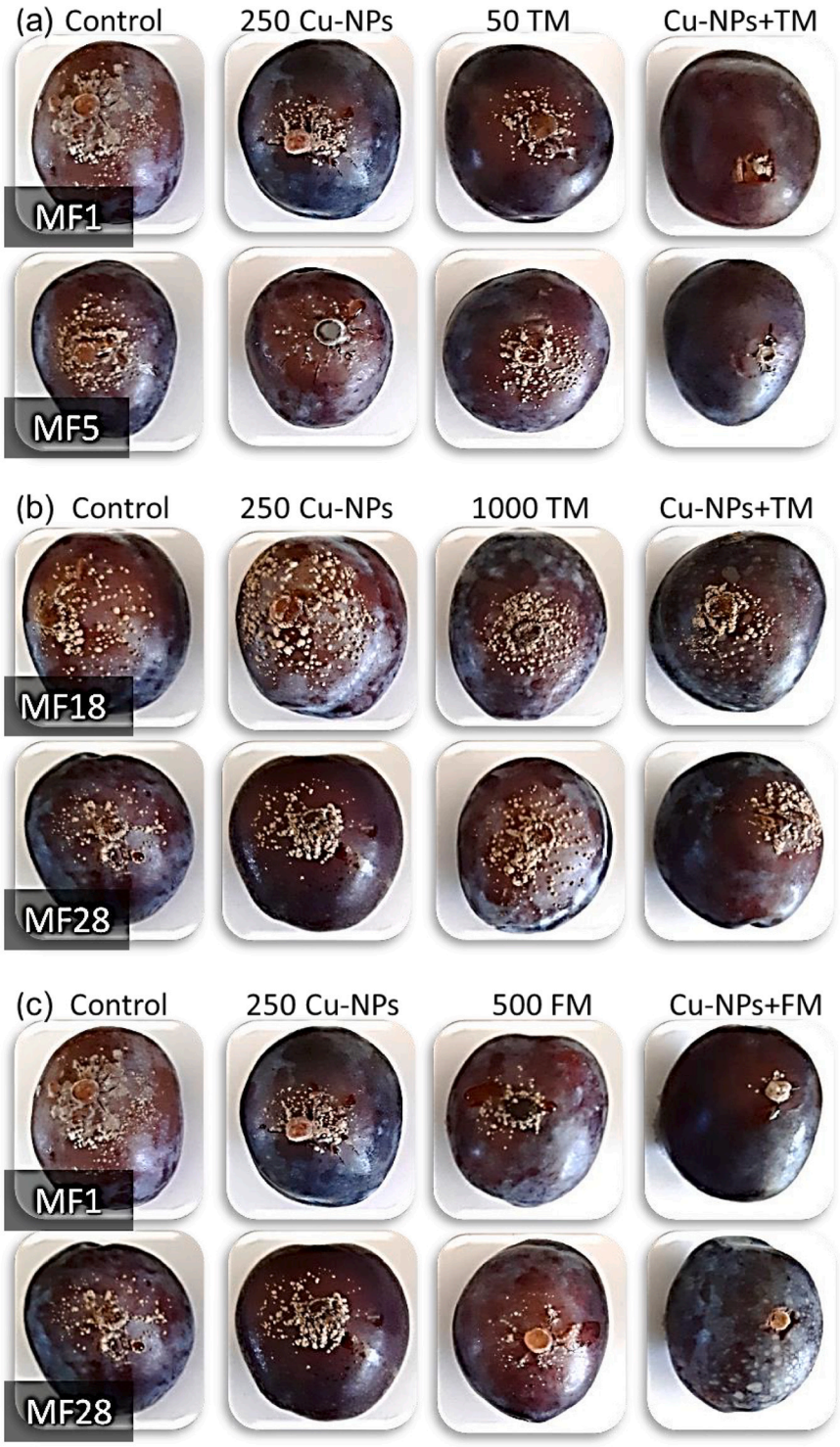

Fig. 4. Synergistic activity of Cu-NPs (250 μg/mL) in combination with a.b) thiophanate methyl (50.1000 μg/mL) and c) fluazinam (500 μg/mL) on plum fruit against selected *Monilia fructicola* isolates sensitive (MF1. MF5) and resistant (MF18. MF28) to thiophanate methyl (TM: thiophanate methyl. FM: fluazinam).

fungal species (Malandrakis et al., 2020a). A similar implication of CuO-NPs associated with multi-drug resistance transporter activity was observed in *Mytilus galloprovincialis* (Torres-Duarte et al., 2019). The additive rather than synergistic effect of fluazinam when applied with CuO-NPs or $Cu(OH)_2$ observed in the present study could be due to the significantly decreased effectiveness of the two compounds compared to Cu-NPs.

The mechanisms underlying the anti-microbial action of metal NPs are being scrutinized by scientists and include cell walls/membrane disruption, production of reactive oxygen species (ROS), interference with/or enzyme inactivation, DNA damage and disruption of electron transport during the respiration process (Rudramurthy et al., 2016; Ruddaraju et al., 2020). Whether the exhibited antimicrobial action is due to metal ions released from nanoparticle surfaces or other

Table 4
Correlation between sensitivity of *M. fructicola* isolates to copper nanoparticles. Selected fungicides and their combinations.

	Cu-NPs	CuO-NPs	$Cu(OH)_2$	$CuSO_4$	TM	FM	Cu-NPs + TM	CuO-NPs + TM	$Cu(OH)_2$ + TM	Cu-NPs + FM	CuO-NPs + FM	$Cu(OH)_2$ + FM
Cu-NPs	1.0[a]	−0.29	−0.28	0.13	−0.06	−0.04	0.17	−0.22	−0.09	−0.23	0.24	0.15
CuO-NPs	–	1.0	0.46*	−0.24	0.34	0.33	0.36	0.23	0.41	0.12	0.02	0.16
$Cu(OH)_2$	–	–	1.0	−0.12	0.06	0.20	−0.16	−0.04	0.33	0.22	0.12	0.11
$CuSO_4$	–	–	–	1.0	−0.17	0.04	−0.23	−0.16	−0.20	−0.35	0.05	−0.17
TM	–	–	–	–	1.0	0.14	0.87**	0.49*	0.53**	0.22	0.14	0.10
FM	–	–	–	–	–	1.0	0.18	0.26	−0.28	−0.04	−0.14	0.20
Cu-NPs + TM	–	–	–	–	–	–	1.0	0.32	0.05	0.02	−0.03	0.16
CuO-NPs + TM	–	–	–	–	–	–	–	1.0	0.38	0.04	0.36	0.12
$Cu(OH)_2$ + TM	–	–	–	–	–	–	–	–	1.0	0.05	0.15	−0.10
Cu-NPs + FM	–	–	–	–	–	–	–	–	–	−1.0	0.14	0.17
CuO-NPs + FM	–	–	–	–	–	–	–	–	–	–	1.0	−0.21
$Cu(OH)_2$ + FM	–	–	–	–	–	–	–	–	–	–	–	1.0

TM: thiophanate methyl. FM:fluazinam.
[a] Pearson correlation coefficient values.
* corresponds to a significance lever of $P = 0.05$.
** corresponds to a significance lever of $P = 0.01$.

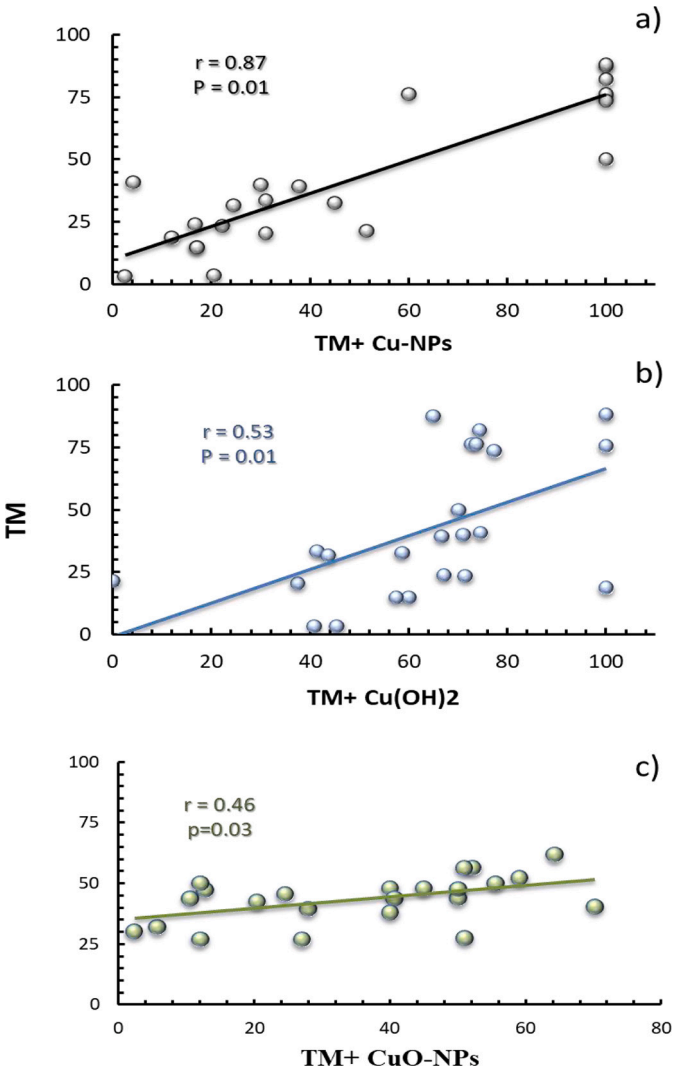

Fig. 5. Correlation between sensitivities of *M. fructicola* isolates to TM (0.5 μg/mL) and its combination with (a) Cu-NPs (250 μg/mL). (b) $Cu(OH)_2$ (500 μg/mL) and (c) and CuO-NPs (500 μg/mL). r is the Pearson correlation coefficient. and p the significance level.

nanoparticle properties is under debate (Sun et al., 2018; Hoseinzadeh et al., 2017; Król et al., 2017). In order to shed light about the mechanism of fungitoxic action of Cu-NPs against *M. fructicola,* a strong chelating agent (EDTA) was mixed with Cu-NPs and their synergistic effect was studied. The addition of EDTA resulted in an almost complete inactivation of the Cu-NPs toxicity indicating a significant role of $[Cu]^{+2}$ in the mechanism of fungitoxic action of the above NPs. Similar results were obtained when NaCl was used instead of EDTA in combination with Cu-NPs resulting in the negation of the fungitoxic effect of the NPs against *B. cinerea* (Malandrakis et al., 2020b). Nevertheless, an additional mechanism related with nanoparticle properties could be partly responsible for Cu-NPs fungitoxic action since no significant correlation was found between *M. fructicola* sensitivity to Cu-NPs and either Cu $(OH)_2$ or $CuSO_4$. The lack of correlation between NPs and their respective bulk/ionic counterparts has also been reported in previous studies (Malandrakis et al., 2019, 2020a, 2020b). Metal nanoparticles have been reported to interfere with/inhibit ergosterol biosynthesis which leads to the disruption of the fungal membrane integrity and function in a way resembling ergosterol biosynthesis inhibitor (EBI) fungicides (Prasher et al., 2018). Marathe et al. (2020) reported a reduction of ergosterol biosynthesis in *Fusarium verticillioides* following treatment with Ag-NPs, while Abed-Alwahed and Abed Al-Baqi (2020) observed that silver nanoparticles reduced the ergosterol biosynthesis gene (*erg11)* expression levels in *C. albicans*. To investigate a possible involvement of Cu-NPs in ergosterol biosynthesis of *M. fructicola,* the synergistic interaction of Cu-NPs with the EBI fungicide tebuconazole was studied. The additive/synergistic effect observed could be an indication for such a possible role, although further studies should be performed before any definite claim could be expressed.

Concluding, Cu-NPs were more effective against *M. fructicola* sensitive and benzimidazole-resistant isolates compared to CuO-NPs and the reference fungicide containing $Cu(OH)_2$. An enhanced antifungal activity of Cu-NPs was observed when applied in mixtures with fluazinam both *in vitro* and in plum fruit while in the case of TM, a strong synergy was observed mostly against BEN-S isolates. A potential increase in TM bioavailability or NP-mediated TM transformation to carbendazim could be responsible for the observed Cu-NPs/TM synergy, while indications that ATP-dependent metabolism and copper ion release are involved in the Cu-NPs fungitoxic mechanism were found. The demonstrated effectiveness of Cu-NPs against sensitive and benzimidazole-resistant isolates both *in vitro* and *in vivo* and synergistic profiles with fluazinam and TM render copper NPs promising candidates for use as fungicide alternatives/partners, both for effective anti-resistance strategies and for reducing fungicide doses/residues and thus, the environmental footprint of synthetic fungicides.

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.pestbp.2021.104796.

Ethical approval

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

Declaration of Competing Interest

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

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