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# Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola*

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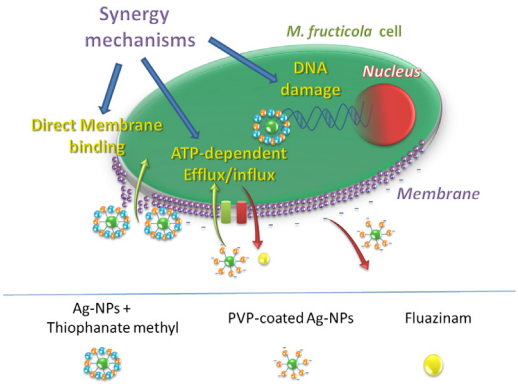
## HIGHLIGHTS

- Ag-NPs are effective against *M. fructicola* isolates both sensitive and resistant to benzimidazole fungicides.
- Ag-NPs exhibit synergistic action with thiophanate methyl and fluazinam.
- ATP metabolism rather than silver ion release is probably involved in Ag-NPs mode of action.
- Ag-NPs have great potential for combating resistance and reducing fungicide footprint in the environment.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The potential of Ag-NPs to suppress *Monilia fructicola* isolates and to broaden the effectiveness of fungicides to overcome resistance was tested *in vitro* and *in vivo*. Twenty-three *M. fructicola* isolates were subjected to fungitoxicity screening with a number of fungicides *in vitro*, which resulted in the detection of 18 isolates resistant to benzimidazoles (BEN-R) thiophanare methyl (TM) and carbendazim (CARB). DNA sequencing revealed the E198A resistance mutation in the $\beta$-tubulin gene, target site of the benzimidazole fungicides in all resistant isolates. Ag-NPs effectively suppressed mycelial growth in both sensitive (BEN—S) and resistant isolates. The combination of Ag-NPs with TM led to a significantly enhanced fungitoxic effect compared to the individual treatments regardless resistant phenotype (BEN-R/S) both *in vitro* and when applied on apple fruit. The above observed additive/synergistic action is probably associated with an enhanced Ag-NPs activity/availability as indicated by the positive correlation between Ag-NPs and TM + Ag-NPs treatments. No correlation was found between $AgNO_3$ and Ag-NPs suggesting that difference(s) exist in the fungitoxic mechanism of action between nanoparticles and their ionic counterparts. Synergy observed between Ag-NPs and the oxidative phosphorylation-uncoupler fluazinam (FM) against both resistance phenotypes indicates a possible role of energy (ATP) metabolism in the mode of action of Ag-NPs. Additionally, the role of released silver ions on the fungitoxic action of Ag-NPs against *M. fructicola* was found to be limited because the combination with NaCl revealed a synergistic rather than the antagonistic effect that would be expected from silver ion binding with chlorine ions. The results of this study suggested that Ag-NPs can be effectively used against *M. fructicola* and when used in combination with conventional fungicides they could provide the means for countering benzimidazole

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resistance and at the same time reduce the environmental impact of synthetic fungicides by reducing doses needed for the control of the pathogen.



## 1. Introduction

Chemical control of plant diseases utilizing systemic, highly effective fungicides has revolutionised modern agriculture by providing the means for efficient and economically feasible disease management especially in hard to manage plant pathogens (Pandey et al., 2016). Albeit of their undeniable performance benefits, conventional fungicides are being heavily criticized for the risk they pose for the environment and food safety. This was the reason for the dramatic increase of the number of fungicide active ingredients that are being withdrawn by implementation of the strict EU regulations for safety and environmental reasons (Malandrakis et al., 2020). Nanoparticles (NPs) have been proposed as perfect disease control alternatives because they present a number of unique properties such as increased effectiveness in lower doses, slower a.i. release and enhanced drug delivery while being considered environmentally compatible (Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). Silver containing nanoparticles are known to exert a "oligodynamic activity": effectiveness against a wide range of microorganisms causing infections including bacteria, fungi and viruses (Huang et al., 2018; Rudramurthy et al., 2016). Taking advantage of a number of different biochemical mechanisms of antimicrobial action such as DNA and membrane damages, interruption of electron transport and/or ATP synthesis, inhibition of protein synthesis and ROS generation/induction of oxidative stress, Ag-NPs have been successfully utilized against both human and plant pathogens (Rai et al., 2017; Khan et al., 2016; Malandrakis et al., 2019). Ag-NPs are increasingly considered as an effective alternative to $Ag^{+}$ because they exhibit a greater effectiveness and duration against microbes and have been demonstrated to act by mechanisms besides silver ion release in many cases (Rudramurthy et al., 2016; Franci et al., 2015; Huang et al., 2018; Malandrakis et al., 2019). Nevertheless, before silver NPs can be commercially introduced as antimicrobial agents, a number of challenges must be addressed. These challenges are: high costs of silver, and research and development of appropriate formulations that may ensure effectiveness and safety against non-target organisms (Hoseinzadeh et al., 2017).

Brown rot caused by the fungus *Monilia fructicola* (teleomorph *Monilinia fructicola*) is a destructive stone fruit disease attacking fruit both pre- and post-harvest in Greece and worldwide (Agrios, 2005). Control of the disease heavily depends on the use of fungicides belonging to benzimidazoles, triazoles, dicarboximides, hydroxyanilides, succinate dehydrogenase inhibitors (SDHI) and Quinone outside Inhibitors (QoIs) (Miessner and Stammler, 2010). Most of the above fungicides have suffered control failures due to the emergence of *M. fructicola* resistant isolates over the last decades (Chen et al., 2013; Penrose et al., 1985; Brent and Hollomon, 1998; Ma and Michailides, 2005; FRAC, 2018). Being the oldest systemic fungicides in brown rot control and due to their extensive use, benzimidazoles soon lost their control efficacy against *M. fructicola* because of resistance development (Luo et al., 2007; Ma et al., 2003; Stehmann and de Waard, 1996; Malandrakis et al., 2011). Benzimidazole resistance in *M. fructicola* resulting from the E198A amino acid substitution was found to be associated with high levels of benzimidazole resistance (Ma and Michailides, 2005; Luo et al., 2007; May-De Mio et al., 2011; Chen et al., 2014). To our knowledge, this study constitutes the first report of *M. fructicola* resistance to benzimidazoles. This fact, in conjunction with the increasing concerns for environmental and health safety, point out the necessity for combating fungicide resistance and at the same time reducing fungicide use especially for pathogens at-risk such as *M. fructicola*.

An appealing approach for combating fungicide resistance and reducing the environmental risk imposed by xenobiotics, has been proposed recently and concerns the use of novel antifungal agents in combination with conventional drugs (Malandrakis et al., 2019, 2020). Silver, zinc and ferric containing nanoparticles can be used as alternatives or in combination with antibiotics because they have shown a promising potential against sensitive or drug-resistant pathogenic bacteria including *Klebsiella pneumonia*, *Eschericia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Staphylococcus aureus* (Hamed et al., 2017; Punjabi et al., 2018; Gabrielyan et al., 2019; Nejabatdoust et al., 2019; Paralikar et al., 2019). In the case of plant pathogens, Cu-NPs, Ag-NPs and ZnO-NPs have demonstrated a marked effectiveness against fungicide resistant fungal pathogens alone or in combination with conventional antifungals, including thiophanate methyl, tebuconazole, mancozeb, fluazinam, fludioxonil, carbendazim, thiram, and propineb (Malandrakis et al., 2020; Huang et al., 2018; Jamdagni et al., 2018; Xue et al., 2014).

In light of this, we hypothesized that silver nanoparticles could aid to control sensitive and drug-resistant isolates of *M. fructicola* alone or in combination with conventional fungicides. Specifically, the purpose of this study was to: (a) identify fungicide resistant *M. fructicola* isolates, (b) evaluate the effectiveness of Ag-NPs alone and in combinations with fungicides against sensitive/resistant *M. fructicola* phenotypes both *in vitro* and on fruit, and (c) gain insight on mechanisms underlying the mode of fungitoxic action of Ag-NPs and the potential interaction between Ag-NPs and tested fungicides.

## 2. Materials and methods

### 2.1. Nanoparticles, reagents and fungicides

Polyvinylpyrrolidone (PVP)-coated silver nanoparticles [Ag-NPs] (<100 nm particle size) and reagents utilized in this study including silver nitrate [$AgNO_3$], sodium chloride [NaCl] and Salicylhydroxamate [SHAM], were purchased from Sigma Aldrich, MO, USA. Commercial fungicides fluazinam (Azzuro 50 SC), thiophanate methyl (Neotopsin 70 WG) and $Cu(OH)_2$ (Copperblau-N 50 WP), were purchased from their respective manufacturers. Active ingredients of remaining fungicides used were of pure analytical grade: carbendazim, tebuconazole and fenhexamid were supplied by Bayer CropScience AG (Leverkusen, Germany), fludioxonil and difenoconazole by Syngenta Crop Protection AG (Basle, Switzerland), pyraclostrobin and boscalid by BASF AG (Limburgerhof, Germany). Ethanol was used as a solvent in all analytical grade stock solutions except for boscalid, pyraclostrobin and fenhexamid, which were dispersed in methanol, methanol and 2-propanol, respectively. Active ingredients were added to sterilized growth medium prior to inoculation under aseptic conditions. Appropriate quantities of the antifungal agents, taking care that the solvent never exceeded 1% (v:v) of the total volume, were added in both treated and control samples. In the case of commercial fungicides and nanoparticles, stock solutions were prepared in distilled-sterilized water. Prior to incorporation in growth media, in order to deter particle aggregation, nanoparticle suspensions were sonicated for 30 min using a Transonic 420 (Elma, Germany) sonicator. Zeta potentials and hydrodynamic diameter measurements for the Ag nanoparticles, TM and mixtures (see Table S1) were measured in triplicate with a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA).

### 2.2. Fungal isolates and culture conditions

*Monilia* sp. isolates collected from stone- and apple fruit orchards of central and northern Greece were isolated from fruit with brown rot

symptoms. Infected fruit were scrapped under aseptic conditions and conidia were plated on acidified PDA medium in order to acquire single spore isolates. Twenty-three single spore isolates, subsequently used in fungitoxicity bioassays against Ag-NPs and selected fungicides, were positively identified to be *Monilia fructicola* according to morphological examination and sequencing of the *cytb* gene (Lane, 2002; Hily et al., 2011). For inoculum production purposes, isolates were kept on Potato Dextrose Agar (PDA) medium in growth chambers at 25 °C with 14 h $day^{-1}$ light and 70% RH. Long-term storage was ensured by transferring isolates once a month in PDA containing glass tubes stored at 4 °C in the dark.

### 2.3. In vitro bioassays

#### 2.3.1. Sensitivity of M. fructicola to Ag-NPs and fungicides

The potential of Ag-NPs to suppress the growth of sensitive and fungicide-resistant *M. fructicola* isolates, was evaluated using *in vitro* fungitoxicity tests. Sensitivity of fungal strains to NPs and fungicides was assessed utilizing the poison agar assay. Fungitoxicity was expressed as percent relative inhibition of isolates grown on PDA (or water agar in the case of boscalid) containing appropriate concentrations of the antifungal agents. In the case of fungicides, *M. fructicola* isolates were subjected to discriminatory concentrations equal to mean $EC_{50}$ values (effective concentration causing 50% inhibition of mycelial growth) reported in previous studies. Specifically, concentrations used were: 500 μg/mL for $Cu(OH)_2$, 0.5 μg/mL for thiophanate methyl, 0.01 μg/mL for carbendazim, 0.01 μg/mL for pyraclostrobin, 0.02 μg/mL for fluazinam, 0.1 μg/mL for fenhexamid, 0.01 μg/mL for fludioxonil, 0.01 μg/mL for tebuconazole and 2.5 μg/mL for boscalid (Avenot and Michailides, 2015; Kalamarakis et al., 2000; Malandrakis et al., 2011, 2019; Markoglou et al., 2006). In order to evaluate baseline sensitivity of *M. fructicola* to Ag-NPs, concentrations of 10, 25, 50, 100, 250, 500 and 1000 25 μg/mL Ag-NPs were used to obtain fungitoxicity-curves and calculate $EC_{50}$ values. *M. fructicola* isolates resistant to benzimidazoles thiophanate methyl and carbendazim were subjected to additional doses to determine the actual $EC_{50}$ values and calculate resistance factors (Rf: $EC_{50}$ of the resistant isolate over mean $EC_{50}$ of sensitive isolates). Specifically, the following concentrations of 0, 0.1, 1, 2.5, 5, 10, 50 μg/mL thiophanate methyl and 0, 0.01, 0.05, 1, 2.5, 5, 10 μg/mL carbendazim were used to obtain fungitoxicity-curves for *M. fructicola* resistant isolates. Each fungicide concentration was applied in triplicate. After solidification of the NP or fungicide-amended and non-amended (control) growth media, a 5-mm mycelial plug cut from the edge of a 4-day old colony of each isolate was transferred to the center of each plate. Subsequently, cultures were incubated in a growth chamber at 25 °C with 70% RH in the dark for 4 days. Percent inhibition was calculated according to the formula:

$$\textbf{Percent Inibition} = 100 - \frac{\text{mean diameter of the colony on the fungicide} - \text{treated plates}}{\text{mean diameter of the untreated control}} \times 100 \quad (1)$$

Tests for each isolate were repeated twice for each concentration and antifungal agent.

#### 2.3.2. Potential interaction between Ag-NPs and fungicides

Potential interaction of Ag-NPs when applied in combination with the fungicides thiophanate methyl, carbendazim and fluazinam was assessed *in vitro* by poison agar assays. Selected concentrations of 0.5 μg/mL thiophanate methyl, 0.15 μg/mL carbendazim and 0.2 μg/mL fluazinam individually or in combination with 50 μg/mL Ag-NPs were achieved by aseptically adding appropriate volumes from stock solutions to PDA medium. Plates inoculated with each *M. fructicola* isolate were incubated for 4 days at 25 °C in the dark and then, mycelial growth percent inhibition rates were calculated. Synergistic interaction of Ag-NPs with tested fungicides were evaluated according to the method described by Abbortt (Gisi, 1996). Briefly, the expected combined percent inhibition (% $CI_{exp}$) was calculated as:

$$\%CI_{\text{exp}} = I_A + I_B - \frac{I_A}{100} \times I_B \quad (2)$$

where $I_A$ and $I_B$ are the percent inhibition of each antifungal agent. Synergy factors (SFs) were determined according to the formula:

$$\boldsymbol{SF} = \frac{I_{AB}}{\%CIexp} \quad (3)$$

where $I_{AB}$ stands for the observed combined percent inhibition of 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. In vivo fungitoxicity tests

Wound-free apple fruit (*Malus sylvestris cv firiki*) selected for their uniform maturity, size, and shape were used to test the efficacy of Ag-NPs to control sensitive and fungicide-resistant *M. fructicola* isolates, alone or in combination with thiophanate methyl, carbendazim and fluazinam *in vivo*. Four apple fruits treated with fungicides, Ag-NPs and their combinations were inoculated with two sensitive (MF1, MF5) and two benzimidazole-resistant (MF18, MF25) isolates while the control treatment comprised of distilled water-sprayed apple fruits. Apple fruits were surface-disinfected by immersion in a 1% sodium hypochlorite solution for 10 min, and immediately after, rinsing the fruit three times with distilled-sterilized water. Fruit were left to dry before treatment with fungicides/Ag-NPs. Consequently, fruit were sprayed with solutions of 100 μg/mL Ag-NPs; 20 and 1000 μg/mL thiophanate methyl (1/50, 1× of the maximum recommended dose) and 500 μg/mL fluazinam (1/2 of the maximum recommended dose), and their combinations. After a period of 2 h in which fruit were left to air-dry, the front face of each apple fruit was wounded using a lancet, creating a 2 × 2 mm [length×width] cross-shaped scar. A 5-mm mycelial plug, from the edge of a 4-day old colony from each *M. fructicola* isolate was immediately placed on top of each wound. Inoculated fruit were placed on top of a wet sterilized paper inside plastic boxes 24 × 34 × 10 cm [length×width×height] and covered by a lid before incubation at 25 °C in the dark for 4 days. Percent symptom severity was calculated by dividing lesion diameter around each wound of treated fruit by the respective lesion diameter of the water-treated control. All experiments were conducted in triplicate.

### 2.5. DNA extraction and sequence analysis of β-tubulin gene from M. fructicola isolates

In order to identify benzimidazole resistance mutations in *M. fructicola* isolates with reduced sensitivity to thiophanate methyl and carbendazim, a β-tubulin gene fragment from selected resistant strains was isolated and sequenced. Mycelia from fungal cultures grown on fungicide-free PDA at 25 °C for seven days were harvested by scraping and ground in liquid nitrogen using a mortar and pestle. TRI reagent (Sigma) was used to isolate total DNA from all isolates following the manufacturer's instructions. Using gDNA from each *M. fructicola* isolate as template, a 1.6-kb fragment of the β-tubulin gene was amplified with the aid of the primers TubA (5′ AAATGCGTGAGATTGTA 3′) and TubR1 (5′ TGTACCAATGCAAGAAAGCCTT 3′) adopted from Ma et al. (2003). The PCR reactions included 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: 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. PCR products were purified using the QIAquick gel extraction kit (Qiagen), ligated to

pGEM-Teasy (Promega) vectors and transformed into *E. coli* competent cells (DH5a Library Efficiency® Competent Cells, Invitrogen). Plasmids containing the β-tubulin gene fragment were purified using QIAprep spin miniprep kit plasmid (Qiagen) and then sequenced in both directions. Ten independent clones from each *M. fructicola* isolate were analyzed while analysis of sequence data was performed using the Lasergene (DNAstar, Madison, USA) software.

## 3. Statistical analysis

The $EC_{50}$ values for each isolate and antifungal agent were estimated by regression analysis of the relative inhibition of mycelial growth against the $Log_{10}$ of the compound concentrations. Correlation of isolate sensitivities to tested NPs/fungicides was evaluated using Pearson correlation coefficients. Ag-NPs and fungicide inhibition rates were subjected to analysis of variance while 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).

## 4. Results

### 4.1. Sensitivity screening of M. fructicola isolates in vitro

The sensitivity distribution of *M. fructicola* isolates to Ag-NPs based on $EC_{50}$ values *in vitro* is shown in Fig. 1. Sensitivity to Ag-NPs was widely distributed with $EC_{50}$ values ranging between 7 and 870 μg/mL with a median value of 50 μg/mL. This is typical for protective antifungal agents and an indication for a probable multisite mode of action of Ag-NPs against *M. fructicola*. Fungitoxicity tests *in vitro* showed that Ag-NPs were significantly ($P<0.01$) more effective against *M. fructicola* than the protective fungicide containing $Cu(OH)_2$ used as a reference (see Table 1). In order to investigate the existence of fungicide resistant isolates, discriminatory doses of selected fungicides were used based on previously reported mean $EC_{50}$ values (Kalamarakis et al., 2000; Malandrakis et al., 2019; Markoglou et al., 2006). Despite the observed variability, in all but one fungicide cases, *M. fructicola* isolates exhibited wild type (baseline) sensitivity (see Table 1). Most of the isolates tested had a reduced sensitivity to the benzimidazole fungicides thiophanate-methyl and carbendazim (mean percent inhibition values ranging from 0 to 3.95), a fact that was expected since this class of fungicides has been extensively used for decades against the pathogen (see Table 1). Resistance factors (Rfs) of the resistant phenotypes were calculated based on $EC_{50}$ values determined by additional fungitoxicity tests (see Table 2). A highly benzimidazole resistant (BEN-R) phenotype was revealed for 16 out of 23 isolates tested with Rf values >100 and >200 for thiophanate methyl and carbendazim respectively (see Table 2).

### 4.2. Detection of target-site resistance mutations

In order to validate the hypothesis that the resistance observed in the BEN-R isolates was due to mutations in the gene encoding the target sites of benzimidazole fungicides, a gene fragment coding β-tubulin was isolated from sensitive and resistant isolates and sequenced.

Sequencing results and subsequent comparison of the deduced amino-acid sequence between BEN-R and BEN-S isolates revealed a glutamic acid (E: GAG) substitution by alanine (A: GCG) at position 198 of the β-tubulin protein leading to the E198A resistance mutation. This well documented E198A benzimidazole resistance mutation, known to confer high resistance levels in many pathogens (Ma and Michailides, 2005), was detected in all BEN-R isolates (see Table 2). These results confirmed the hypothesis that target-site modification reducing the affinity between benzimidazoles and their β-tubulin target was the mechanism responsible for the observed resistant phenotypes.

### 4.3. Synergy between Ag-NPs and fungicides

#### 4.3.1. In vitro bioassays

The effectiveness of Ag-NPs against sensitive and resistant *M. fructicola* isolates when applied in combination with thiophanate methyl, carbendazim, tebuconazole and fluazinam was tested *in vitro*. Synergistic factors (SF) were calculated for Ag-NPs and combinations and respective values are listed in Table 3. Addition of fluazinam significantly enhanced the fungitoxic effect of Ag-NPs in almost all cases of both BEN-S and BEN-R isolates, resulting in complete inhibition of mycelial growth (see Fig. 2c). Estimated SF values between Ag-NPs and fluazinam ranged between 1.11 and 1.53 (see Table 3). A similar synergistic profile (SF: 0.99–5.20) was observed between Ag-NPs and thiophanate methyl (see Table 3). The above combination completely inhibited BEN-S isolates while it significantly enhanced Ag-NPs effectiveness in most of the BEN-R isolates (Fig. 2a, Fig. 3). On the contrary, synergistic relationships between Ag-NPs and carbendazim were inconsistent and seemed to be isolate dependent. In most of the isolate cases, the addition of carbendazim to Ag-NPS resulted in little or no additional statistically significant fungitoxic effect against the pathogen (Fig. 2b).

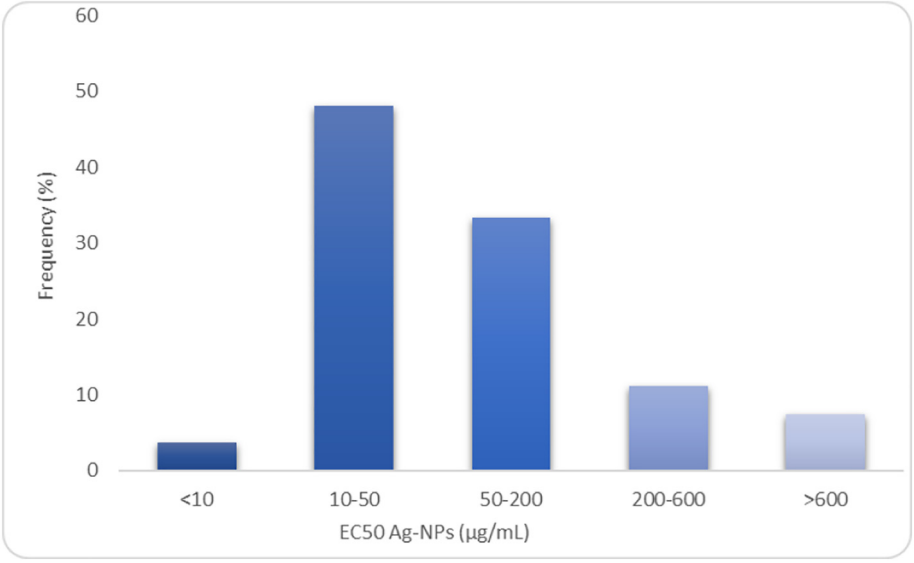


**Fig. 1.** Sensitivity distribution of *Monilia fructicola* field isolates based on EC50 values to Ag-NPs.

**Table 1**
Sensitivity of *Monilia fructicola* isolates to Ag-NPs and selected fungicides.

| Isolate | Percent inhibition[a] (mean ± SD[b]) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ag-NPs (50)[c] | $AgNO_3$ (3) | Thiophanate methyl (0.5) | Carbendazim (0.05) | Boscalid (2.5) | Tebuconazole (0.01) | Fluazinam (0.2) | Pyraclos-trobin (0.05) | Fenhexamid (0.1) | Fludioxonil (0.01) | $Cu(OH)_2$ (500) |
| MF1 | 27.14 ± 2.01 | 39.13 ± 3.13 | 50.00 ± 0.00 | 65.33 ± 2.65 | 41.18 ± 1.28 | 41.51 ± 2.30 | 65.38 ± 3.38 | 100.00 | 65.38 ± 5.60 | 65.38 ± 0.05 | 100.00 |
| MF3 | 52.63 ± 0.25 | 56.25 ± 2.78 | 36.84 ± 1.64 | 49.90 ± 1.58 | 48.21 ± 2.89 | 48.00 ± 6.44 | 66.10 ± 0.40 | 100.00 | 61.02 ± 2.77 | 66.10 ± 0.02 | 100.00 |
| MF4 | 46.77 ± 1.30 | 42.03 ± 1.13 | 0.00 | 0.00 | 65.31 ± 5.00 | 56.60 ± 4.10 | 57.97 ± 0.09 | 100.00 | 65.22 ± 6.11 | 57.97 ± 1.07 | 60.53 ± 0.10 |
| MF5 | 39.39 ± 0.02 | 38.33 ± 0.82 | 30.30 ± 3.33 | 51.69 ± 0.67 | 31.82 ± 2.63 | 12.24 ± 2.03 | 62.50 ± 0.50 | 100.00 | 68.75 ± 0.99 | 62.50 ± 0.60 | 36.84 ± 4.99 |
| MF6 | 23.53 ± 4.40 | 39.22 ± 0.65 | 31.76 ± 2.66 | 52.23 ± 2.50 | 41.46 ± 4.27 | 18.75 ± 1.61 | 48.00 ± 3.80 | 100.00 | 36.00 ± 7.41 | 48.00 ± 3.30 | 55.26 ± 2.90 |
| MF7 | 57.69 ± 2.15 | 36.00 ± 1.00 | 0.00 | 1.31 ± 0.52 | 51.16 ± 0.15 | 44.07 ± 2.33 | 63.75 ± 2.00 | 100.00 | 70.00 ± 3.05 | 63.75 ± 0.11 | 54.00 ± 0.05 |
| MF8 | 31.75 ± 0.07 | 17.57 ± 6.24 | 0.00 | 0.00 | 27.50 ± 0.70 | 34.29 ± 2.86 | 64.71 ± 0.35 | 100.00 | 76.47 ± 3.97 | 64.71 ± 2.00 | 55.32 ± 2.12 |
| MF9 | 67.86 ± 5.63 | 41.30 ± 0.28 | 0.00 | 0.00 | 60.53 ± 1.55 | 50.00 ± 3.72 | 48.57 ± 6.56 | 100.00 | 74.29 ± 9.00 | 48.57 ± 1.00 | 100.00 |
| MF10 | 26.15 ± 3.00 | 27.27 ± 0.16 | 3.38 ± 1.07 | 1.77 ± 0.60 | 50.00 ± 5.30 | 20.00 ± 2.20 | 58.11 ± 5.00 | 100.00 | 66.22 ± 2.90 | 58.11 ± 0.89 | 87.10 ± 2.50 |
| MF12 | 36.36 ± 0.39 | 100.00 | 77.27 ± 2.27 | 51.99 ± 0.19 | 100.00 | 57.58 ± 3.00 | 55.56 ± 5.51 | 100.00 | 75.56 ± 4.52 | 55.56 ± 2.01 | 46.15 ± 1.85 |
| MF14 | 63.16 ± 0.85 | 54.35 ± 3.85 | 78.95 ± 8.31 | 66.17 ± 4.81 | 36.36 ± 0.14 | 5.66 ± 1.11 | 63.93 ± 4.00 | 100.00 | 67.21 ± 4.28 | 63.93 ± 1.50 | 37.50 ± 1.51 |
| MF15 | 22.00 ± 5.11 | 30.65 ± 0.12 | 0.00 | 0.00 | 47.37 ± 4.40 | 47.06 ± 3.38 | 60.81 ± 1.88 | 100.00 | 68.92 ± 3.30 | 60.81 ± 1.38 | 51.35 ± 0.01 |
| MF16 | 32.14 ± 0.98 | 13.79 ± 0.17 | 1.79 ± 1.13 | 0.0 | 54.05 ± 2.31 | 49.25 ± 1.80 | 54.93 ± 8.02 | 100.00 | 70.42 ± 2.69 | 54.93 ± 0.07 | 46.00 ± 4.03 |
| MF17 | 40.00 ± 0.83 | 23.61 ± 0.91 | 0.00 | 0.00 | 62.16 ± 6.00 | 28.85 ± 5.62 | 51.28 ± 0.32 | 100.00 | 74.36 ± 3.33 | 51.28 ± 0.51 | 100.00 |
| MF18 | 60.00 ± 3.33 | 28.71 ± 6.33 | 0.00 | 0.00 | 40.91 ± 4.25 | 43.30 ± 4.07 | 60.19 ± 5.00 | 100.00 | 74.07 ± 3.84 | 60.19 ± 1.70 | 42.22 ± 3.22 |
| MF20 | 10.00 ± 2.32 | 20.00 ± 2.05 | 0.00 | 0.00 | 50.00 ± 8.02 | 44.44 ± 2.22 | 60.98 ± 4.07 | 100.00 | 69.51 ± 2.65 | 60.98 ± 2.18 | 68.18 ± 4.80 |
| MF21 | 50.00 ± 5.45 | 27.54 ± 0.17 | 0.00 | 0.00 | 59.18 ± 2.99 | 46.58 ± 4.50 | 67.02 ± 5.53 | 100.00 | 76.60 ± 5.05 | 67.02 ± 1.67 | 64.00 ± 6.00 |
| MF22 | 9.09 ± 1.33 | 30.00 ± 5.06 | 2.45 ± 0.25 | 0.00 | 44.19 ± 7.68 | 30.56 ± 6.61 | 61.54 ± 2.90 | 100.00 | 64.10 ± 7.19 | 61.54 ± 2.20 | 70.00 ± 0.00 |
| MF23 | 24.44 ± 2.00 | 24.62 ± 2.57 | 4.44 ± 0.03 | 0.00 | 48.39 ± 2.82 | 50.77 ± 3.88 | 58.90 ± 2.99 | 100.00 | 65.75 ± 5.63 | 58.90 ± 0.99 | 40.91 ± 5.44 |
| MF25 | 16.67 ± 1.74 | 31.43 ± 1.61 | 0.00 | 0.00 | 59.52 ± 4.01 | 27.12 ± 2.67 | 62.16 ± 6.02 | 100.00 | 71.62 ± 1.44 | 62.16 ± 0.60 | 60.00 ± 3.49 |
| MF27 | 34.21 ± 1.00 | 39.29 ± 3.02 | 3.95 ± 1.01 | 0.84 ± 0.40 | 40.63 ± 2.10 | 36.84 ± 5.55 | 71.25 ± 4.99 | 100.00 | 75.00 ± 2.81 | 71.25 ± 4.40 | 68.00 ± 1.58 |
| MF28 | 56.00 ± 2.62 | 21.31 ± 2.75 | 0.00 | 0.00 | 70.27 ± 1.09 | 42.86 ± 0.07 | 54.30 ± 0.00 | 100.00 | 72.05 ± 0.02 | 65.77 ± 1.00 | 77.78 ± 1.15 |
| MF29 | 68.00 ± 4.24 | 60.00 ± 7.00 | 72.00 ± 0.30 | 71.14 ± 2.20 | 8.82 ± 5.05 | 22.64 ± 3.64 | 68.52 ± 4.82 | 100.00 | 77.78 ± 1.88 | 68.52 ± 0.02 | 50.00 ± 5.63 |

[a] Calculated as percent inhibition of mycelial growth compared to the untreated control after a 4-day incubation period 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.

**Table 2**
Cross-resistance profiles of representative *M. fructicola* isolates sensitive (BEN—S) and benzimidazole resistant (BEN-R) and respective resistance mutations.

| | Fungicides | | | | Resistance mutations in β-tubulin gene |
|---|---|---|---|---|---|
| | Thiophanate methyl | | Carbendazim | | |
| Isolate | $EC_{50}$[a] (mean ± SD[b]) | Rf[c] | $EC_{50}$ (mean ± SD) | Rf | Amino acid substitution |
| MF1 | 0.50 ± 0.00 | 1.00 | 0.04 ± 0.00 | 0.80 | E198 |
| MF3 | 0.75 ± 0.10 | 1.50 | 0.05 ± 0.00 | 1.00 | E198 |
| MF5 | 0.75 ± 0.05 | 1.50 | 0.05 ± 0.00 | 1.00 | E198 |
| MF6 | 0.70 ± 0.02 | 1.40 | 0.05 ± 0.00 | 1.00 | E198 |
| MF16 | >50 | >100 | >10 | >200 | E198A |
| MF18 | >50 | >100 | >10 | >200 | E198A |
| MF25 | >50 | >100 | >10 | >200 | E198A |
| MF28 | >50 | >100 | >10 | >200 | E198A |

Here, r is the Pearson correlation.
[a] Effective concentration causing 50% reduction in mycelial growth rate after a 4-day incubation period at 25 °C (n = 3).
[b] Standard deviation of the means (n = 3).
[c] Resistance factor ($EC_{50}$ of each isolate/mean $EC_{50}$ of sensitive isolates).

Besides this slight additive effect, in a few cases, antagonism or even synergism (SF: 0.54–1.22) was observed between the above antifungals (see Table 3). The synergistic relationship between Ag-NPs and tebuconazole was additive (see Table 3) in most isolate cases, while in two BEN-S isolates (MF14 and MF29) a synergistic effect was observed (SF: 1.51, 1.30).

The possible role of silver ions release on the observed synergistic effect observed between Ag-NPs and thiophanate methyl or fluazinam was evaluated using $AgNO_3$, a silver ion releasing reagent, in synergism bioassays *in vitro*. Contrary to the synergistic profile exhibited by Ag-NPs, the combined use of $AgNO_3$ with fluazinam resulted in an antagonistic/additive effect in most of the cases with SF values ranging between 0.40 and 1.01 (see Table 3), probably indicating that nanoparticle properties contribute more to the observed synergism between Ag-NPs and fluazinam than silver ion release. An additive effect was observed between $AgNO_3$ and thiophanate methyl in BEN-S isolates (SF: 1.00–1.09), whereas in BEN-R isolates a strong antagonistic effect (SF: 0.00–0.68) was observed in most cases (see Table 3). No evidence on the involvement of silver ion release in the fungitoxic activity of Ag-NPs against *M. fructicola* isolates was revealed by the combination of Ag-NPs and NaCl. The addition of 1% NaCl in PDA containing 50 μg/mL Ag-NPs resulted in a strong synergism enhancing the fungitoxic effect of Ag-NPs with high SF values instead of neutralizing it (see Table 3). In a similar approach, aiming to investigate the involvement of the ergosterol biosynthesis pathway on the fungitoxic mode of action of Ag-NPs, the ergosterol biosynthesis inhibitor (EBI) fungicide tebuconazole was employed in synergy tests with Ag-NPs. In most isolate cases, the Ag-NPs – tebuconazole combination resulted in an additive effect indicating a possible role of Ag-NPs on inhibition of ergosterol biosynthesis and the subsequent potential disruption of the fungal membrane's integrity. Synergy factors ranged between 0.95 and 1.51 (see Table 3).

**Table 3**
*In vitro* interaction of Ag-NPs or $AgNO_3$ with selected fungicides against fungicide sensitive and resistant *Monilia fructicola* isolates (TM: thiophanate methyl, FM: fluazinam, TEB: tebuconazole).

| | | | | SF[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Ag-NPs (50) | | | $AgNO_3$ (3) | |
| Isolate | Resistance phenotype | TM (0.5)[c] | FM (0.2) | CARB (0.05) | TEB (0.01) | NaCl (10,000) | TM (0.5) | FM (0.2) |
| MF1 | BEN-S[b] | 1.27 | 1.19 | 1.01 | 1.06 | 27.00 | 1.09 | 1.01 |
| MF3 | BEN-S | 1.43 | 1.17 | 0.99 | 0.86 | 1.88 | 1.00 | 0.99 |
| MF5 | BEN-S | 1.73 | 1.13 | 1.14 | 1.31 | 1.04 | 1.08 | 1.00 |
| MF6 | BEN-S | 3.07 | 1.33 | 1.01 | 1.17 | 2.55 | 1.02 | 0.88 |
| MF12 | BEN-S | 1.17 | 1.17 | 0.54 | 1.05 | 2.35 | 1.00 | 1.06 |
| MF14 | BEN-S | 1.08 | 1.19 | 1.02 | 1.51 | 1.77 | 1.06 | 1.03 |
| MF29 | BEN-S | 1.14 | 1.29 | 0.81 | 1.30 | 3.06 | 1.07 | 0.98 |
| MF4 | BEN-R | 1.45 | 1.24 | 0.98 | 1.00 | 1.58 | 0.17 | 1.01 |
| MF7 | BEN-R | 0.99 | 1.19 | 0.65 | 0.88 | 1.20 | 0.00 | 0.89 |
| MF8 | BEN-R | 2.15 | 1.30 | 0.99 | 0.96 | 8.43 | 0.25 | 1.02 |
| MF9 | BEN-R | 1.21 | 1.18 | 0.65 | 0.95 | 1.85 | 0.35 | 1.05 |
| MF10 | BEN-R | 1.35 | 1.28 | 1.04 | 1.02 | 4.29 | 0.28 | 1.05 |
| MF15 | BEN-R | 2.27 | 1.44 | 1.04 | 0.92 | 3.29 | 0.22 | 1.00 |
| MF16 | BEN-R | 1.18 | 1.53 | 1.00 | 0.92 | 2.03 | 0.68 | 0.91 |
| MF17 | BEN-R | 1.13 | 1.22 | 1.06 | 1.06 | 25.50 | 0.29 | 1.07 |
| MF18 | BEN-R | 1.07 | 1.22 | 0.96 | 1.03 | 1.99 | 0.36 | 0.99 |
| MF20 | BEN-R | 5.20 | 1.21 | 0.67 | 0.88 | 1.95 | 0.49 | 0.88 |
| MF21 | BEN-R | 1.20 | 1.16 | 1.13 | 0.90 | 2.94 | 0.00 | 1.01 |
| MF22 | BEN-R | 1.42 | 1.40 | 1.05 | 1.02 | 2.51 | 0.11 | 1.02 |
| MF23 | BEN-R | 1.36 | 1.11 | 0.80 | 0.78 | 4.03 | 0.00 | 0.90 |
| MF25 | BEN-R | 3.00 | 1.13 | 1.05 | 0.99 | 2.88 | 0.63 | 0.95 |
| MF27 | BEN-R | 1.44 | 1.21 | 1.17 | 0.94 | 9.12 | 0.53 | 1.00 |

[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.

### 4.4. In vivo bioassays

The demonstrated *in vitro* synergistic activity between Ag-NPs, thiophanate methyl and fluazinam was tested on apple fruit for selected BEN-S and BEN-R *M. fructicola* isolates. When applied on apple fruit inoculated with BEN-R isolates MF1 and MF5, 100 μg/mL Ag-NPs resulted in a 16.67 and 6.75% inhibition while 50 μg/mL thiophanate methyl resulted in 18.54 and 22.33% inhibition. The combination of the above antifungal agents caused a decrease in disease symptoms of the BEN-S isolates demonstrating an additive effect (SF:1.03 and 1.07, respectively, see Table 4, Fig. 4a). Interestingly, a profound synergistic effect was observed in the case of BEN-R isolates MF18 and MF25, when 1000 μg/mL thiophanate methyl was co-applied with 100 μg/mL Ag-NPs (SF: 1.38 to 2.53, respectively, see Table 5, Fig. 4b). The synergy between Ag-NPs and fluazinam demonstrated *in vitro* was also observed in the *in vivo* experiments (see Fig. 4c). This synergistic effect was observed regardless resistant phenotype with SF values ranging from 1.31 to 1.51, indicating the potential of FM to enhance Ag-NPs effectiveness against both sensitive and resistant isolates (see Table 4).

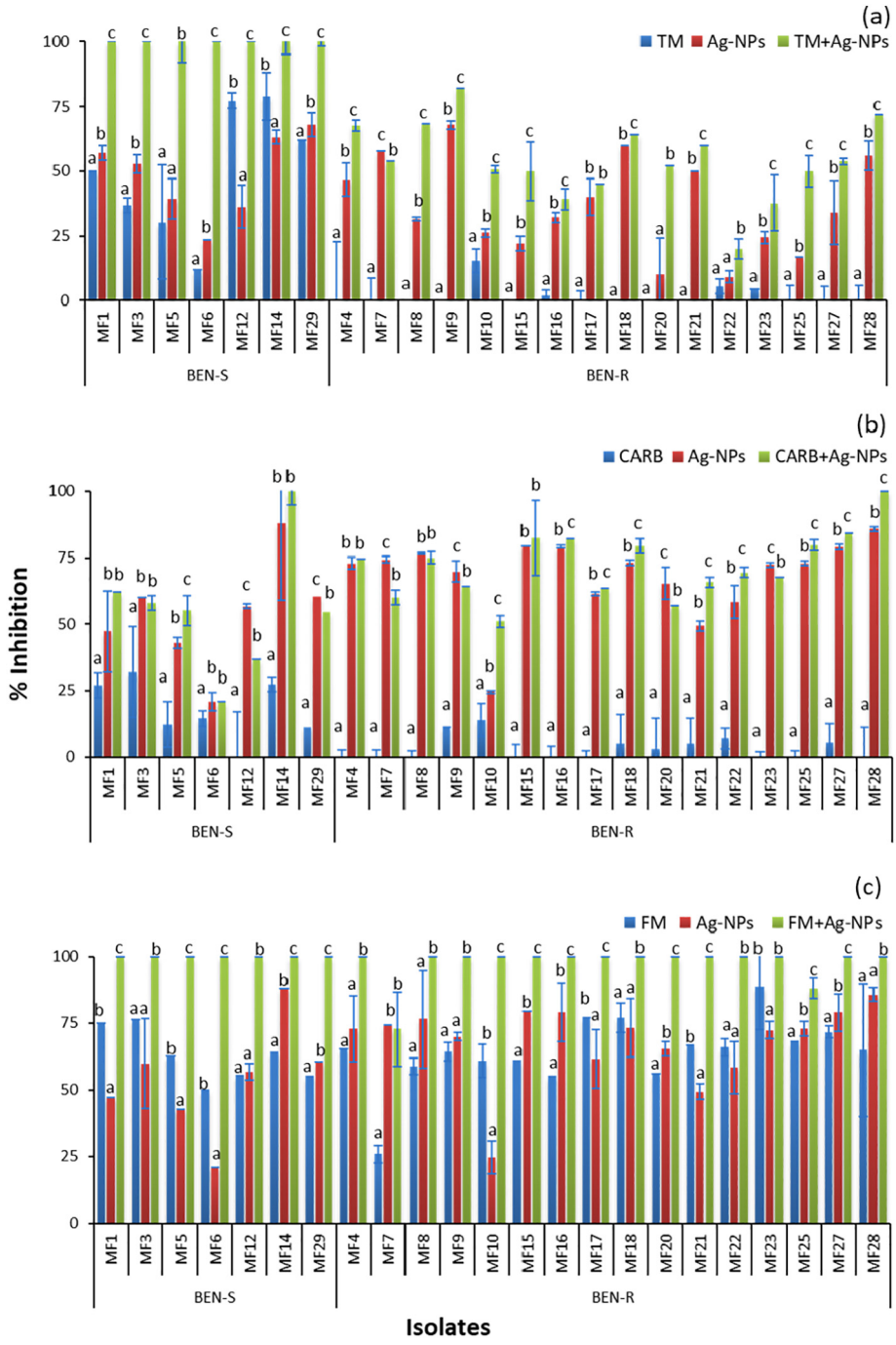


**Fig. 2.** Sensitivity of fungicide-sensitive/resistant *M. fructicola* isolates to Ag-NPs (50 μg/mL) in comparison with: (a) thiophanate methyl (0.5 μg/mL), (b) carbendazim (0.15 μg/mL), and (c) fluazinam (0.2 μg/mL), and combinations. BEN-S/R: benzimidazole- Sensitive/Resistant isolates (TM: thiophanate methyl, CARB: carbendazim, 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 ($\alpha = 0.05$).

### *4.5. Sensitivity correlations between Ag-NPs, $AgNO_3$ and fungicide combinations*

In order to evaluate any possible contribution of Ag-NPs, thiophanate methyl (TM), fluazinam (FM) and $AgNO_3$ in the observed synergistic relations between them, correlations were calculated using Pearson correlation coefficients (see Table 5). No significant correlation was found between TM and Ag-NPs, FM and their respective combinations (see Table 5). On the contrary, a significant correlation was found between Ag-NP and Ag-NPs + TM treatments (see Fig. 5a) indicating that the enhanced fungitoxic effect of the above combination is probably associated with the action of Ag-NPs rather than that of TM. No correlation was found between $AgNO_3$, FM or their combination. A significant correlation was found between TM, $AgNO_3$ and their combination indicating that both antifungals contribute in the fungitixic effect of their mixture (see Table 5, Fig. 5b). No correlation was found between Ag-NPs and NaCl (see Table 5).

## 5. Discussion

A total of 23 *M. fructicola* isolates collected from Greek orchards were screened for resistance against 9 registered fungicides by *in vitro* bioassays in order to investigate Ag-NPs potential to combat both sensitive and resistant isolates. Most of the isolates tested were sensitive to all

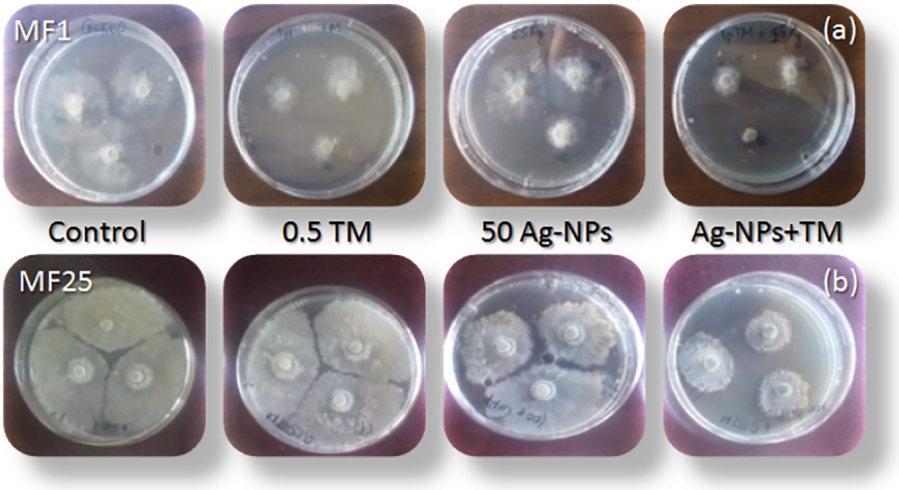


**Fig. 3.** Fungitoxic activity of Ag-NPs, TM and their combination in (a) sensitive (MF1), and (b) TM-resistant (MF25) *M. fructicola* isolates (TM: thiophanate methyl).

fungicides except for 18, which were highly resistant only to benzimidazoles (BEN-R) thiophanate methyl and carbendazim. DNA sequencing and comparison between resistant and sensitive isolates revealed a well-documented resistance mutation in the *M. fructicola* β-tubulin gene, target site of benzimidazoles. The specific mutation (E198A) results in a structural modification in the β-tubulin protein leading to a reduced affinity of the fungicide with its target and has been associated with high resistance levels to benzimidazoles in plenty plant pathogens including *M. fructicola* in many countries worldwide, however, it is reported for the first time in Greece for this species (Luo et al., 2007; Ma et al., 2003; Stehmann and de Waard, 1996; Malandrakis et al., 2011; Ziogas et al., 2009; Ma and Michailides, 2005; FRAC, 2018). The effectiveness of Ag-NPs alone and in combination with selected fungicides against resistant and sensitive phenotypes was investigated.

An increasing number of studies are focusing on the ability of metal NPs to combat clinical drug-resistant pathogens, especially against life-threatening multi drug resistant bacteria (MDR) and their potential as alternatives or antibiotics partners (Jampilek, 2016; Punjabi et al., 2018). Most of these studies concern the broadening of antibiotic efficacy against bacterial MDR-strains when used in combination with metal NPs, while very few reports are available on the respectful interaction of nanoparticles with drugs/fungicides against sensitive or fungicide resistant fungal strains. Sun et al. (2016) have reported an enhanced toxicity of PVR-coated Ag-NPs when combined with azole fungicides against *Candida albicans* drug-resistant strains. Ag-NPs and ZnO-NPs combined with fungicides carbendazim, mancozeb and thiram, showed a significant synergistic effect against plant pathogens *B. cinerea*, *Aspergilus niger*, *Alternaria alternata*, *P. expansum and F. oxysporum*, an effect which was more profound when green synthesized NPs were utilized (Jamdagni et al., 2018). A similar synergistic effect was reported by Huang et al. (2018) in *Bipolaria maydis*, when Ag-NPs were used in mixtures with tebuconazole, fludioxonil or propineb. ZnO-NPs used in combination with thiram have been reported to exhibit both synergistic and photo-degradation properties against *Phytophthora capsici* (Xue et al., 2014). A recent study by Malandrakis et al. (2020) reported a synergistic effect of Cu-NPs used in combination with thiophanate methyl and fluazinam against both sensitive and fungicide resistant strains of *B. cinerea*.

Silver nanoparticles tested in this study, were effective against both BEN-S and BEN-R *M. fructicola* isolates and significantly more effective compared to the reference protective fungicide containing $Cu(OH)_2$. Ag-NPs used in combination with TM *in vitro* were significantly more fungitoxic to *M. fructicola* than any of the individual treatments. A similar synergistic interaction was more profound in the case of BEN-R isolates in *in vivo* experiments where the Ag-NPs-TM mixture significantly increased the suppression of disease symptoms compared to the individual treatments. This synergy between Ag-NPs and TM, regardless resistance phenotype and the positive correlation found between Ag-NPs and TM + Ag-NPs but not between TM and TM + Ag-NPs treatments indicate that synergism observed is probably related to an enhancement in Ag-NPs fungitoxicity. Contrary to this, in our previous study synergy observed between Cu-NPs and TM was mainly attributed to a higher TM bioavailability in *B. cinerea* (Malandrakis et al., 2020). In the present study, it is probable that the Ag-NPs + TM combination facilitates an

**Table 4**
Synergistic activity of Cu-NPs co-applied with thiophanate methyl or fluazinam on apple fruit against *Monilia fructicola* isolates sensitive and resistant to benzimidazole fungicides (TM: thiophanate methyl, FM: fluazinam).

| Isolate | Phenotype[c] | Percent inhibition[a] (mean±SD[b]) | | | | Percent inhibition (mean ± SD) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ag-NPs (100)[d] | TM(50/1000)[e] | Ag-NPs + TM | SF[f] | Ag-NPs (100) | FM (500) | Ag-NPs + FM | SF |
| MF1 | BEN-S | 16.67 ± 2.17 | 18.54 ± 1.85 | 33.33 ± 1.99 | 1.03 | 20.30 ± 2.12 | 19.39 ± 1.30 | 47.96 ± 1.65 | 1.34 |
| MF5 | BEN-S | 6.75 ± 0.56 | 22.33 ± 0.66 | 29.60 ± 2.62 | 1.07 | 14.43 ± 1.00 | 21.41 ± 2.00 | 43.91 ± 0.18 | 1.31 |
| MF18 | BEN-R | 12.53 ± 0.12 | 37.50 ± 2.38 | 62.29 ± 0.34 | 1.38 | 6.76 ± 0.56 | 24.32 ± 1.80 | 44.32 ± 0.97 | 1.51 |
| MF25 | BEN-R | 10.27 ± 1.08 | 0 | 25.37 ± 1.45 | 2.53 | 13.21 ± 1.35 | 24.90 ± 1.67 | 51.56 ± 1.78 | 1.48 |

[a] Calculated as percent inhibition of lesion development on apple fruit sprayed with Ag-NPs/fungicides and their combinations compared to the untreated control after 4-day incubation period 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] Apple 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.

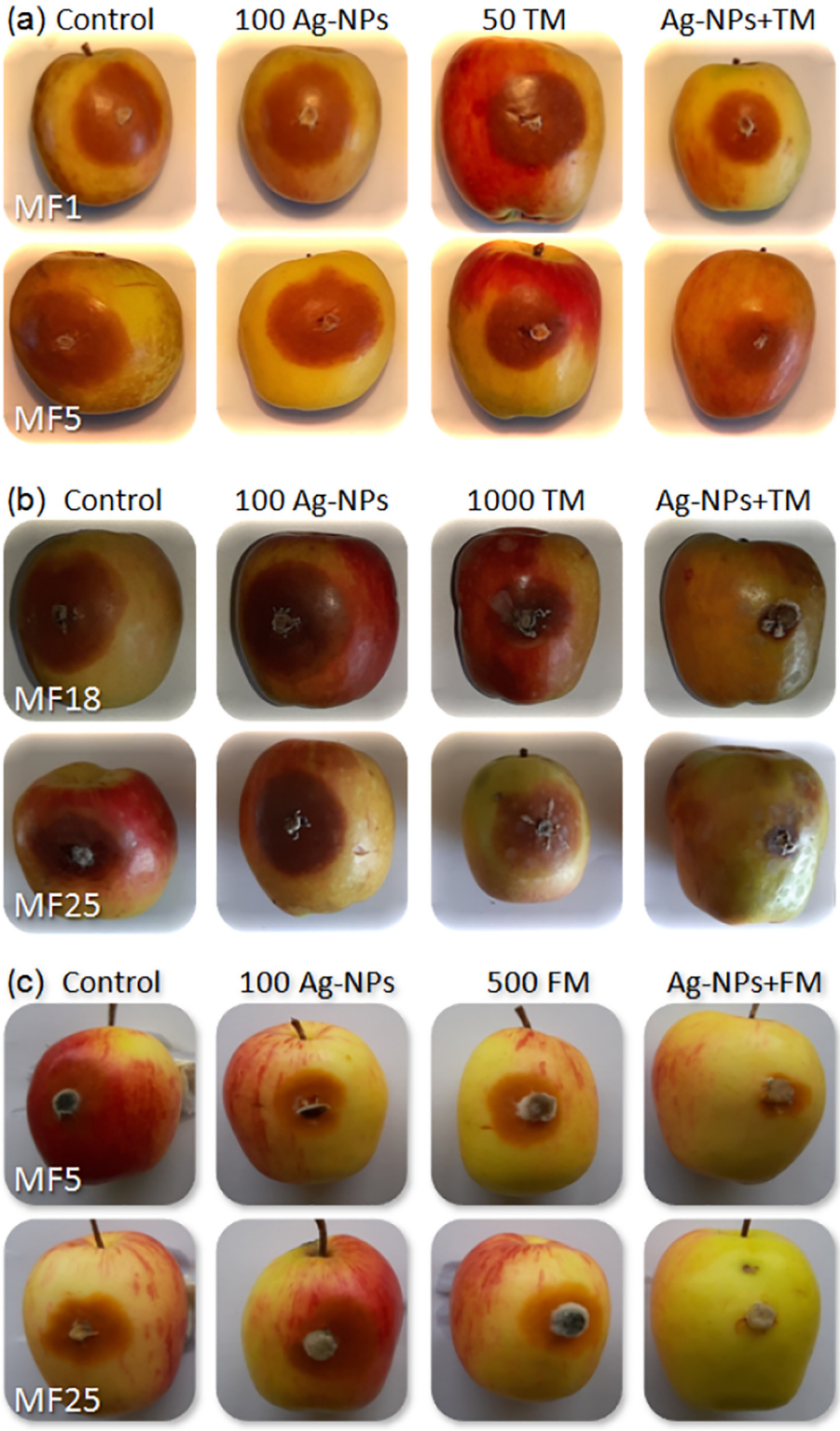


**Fig. 4.** Synergistic activity of Ag-NPs (100 μg/mL) in combination with a, b) thiophanate methyl (50,) and c) fluazinam (500 μg/mL) on apple fruit against selected *Botrytis cinerea* isolates sensitive (MF1, MF5) and resistant (MF18, MF25) to thiophanate methyl (TM: thiophanate methyl, FM: fluazinam).

enhancement of Ag-NPs bioavailability either by increasing the quantity of silver nanoparticles in direct contact with the fungal membrane or by influencing mechanisms that modulate delivery of the nanoparticle inside the fungal cell. Possible interaction mechanisms between of nanoparticles with pathogen surfaces involve forces of electrostatic or van der Waals nature and other interactions deriving from hydrophilic-hydrophobic or ligand-receptor relations (Ruddaraju et al., 2020). Direct contact of positive charged NPs with the microorganisms membrane is mediated by electrostatic attraction with the highly-negative charged cell surface, especially in gram-negative bacteria (Ruddaraju et al., 2020). PVP-coated Ag-NPs used in this study were negatively charged and as a consequence are expected to be repelled by the also negatively

**Table 5**
Correlation between sensitivity of *M. fructicola* isolates to Ag-NPs, $AgNO_3$, NaCl, selected fungicides and their combinations.

| | Ag-NPs | TM | Ag-NPs + TM | FM | Ag-NPs + FM | $AgNO_3$ | $AgNO_3$ + TM | $AgNO_3$ + FM | NaCl | TEB |
|---|---|---|---|---|---|---|---|---|---|---|
| Ag-NPs | 1.0[a] | 0.35 | 0.66** | 0.00 | −0.07 | 0.32 | 0.32 | 0.02 | 0.17 | 0.04 |
| TM | – | 1.0 | −0.32 | 0.20 | 0.19 | 0.80** | 0.80** | 0.05 | −0.37 | −0.23 |
| Ag-NPs + TM | – | – | 1.0 | −0.38 | −0.01 | 0.10 | 0.13 | 0.14 | 0.45 | 0.33 |
| FM | – | – | – | 1.0 | 0.18 | 0.00 | −0.07 | 0.00 | −0.08 | 0.50* |
| Ag-NPs + FM | – | – | – | – | 1.0 | 0.03 | 0.01 | 0.22 | 0.03 | 0.32 |
| $AgNO_3$ | – | – | – | – | – | 1.0 | 0.71** | 0.10 | −0.12 | 0.04 |
| $AgNO_3$ + TM | – | – | – | – | – | – | 1.0 | 0.34 | −0.40 | −0.35 |
| $AgNO_3$ + FM | – | – | – | – | – | – | – | 1.0 | 0.23 | 0.05 |
| NaCl | – | – | – | – | – | – | – | – | 1.0 | 0.40 |
| TEB | | | | | | | | | | 1.0 |

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

charged fungal cell. Interaction of Ag-NPs with TM through thiol-group or other lingands could be responsible for reducing/negating the NPs negative charge promoting attachment of these particles to the fungal cell surface. A similar negation effect between positively charged Na ions and negative charged PVP-coated Ag-NPs could explain the synergy observed between Ag-NPs and NaCl observed in the present study. Zheng et al. (2018) have reported such an interaction of citrate-capped Ag-NPs when combined with TM through hydrogen bonding between the citrate capping agent and a number of ligands of TM. PVP-coated Ag-NPs have exhibited enhanced binding to the fungal cell membrane of *C. albicans* in the presence of the fungicide fluconazole (Sun et al., 2016).

Another plausible explanation of the enhanced Ag-NPs toxicity when combined with TM is a potential 'protected' transport of the NPs-fungicide complex to the fungal nucleus where it reacts with DNA and prevent its unwinding, a phenomenon demonstrated by Batarseh (2004) in the case of chelated silver in *P. aeruginosa*. This way, Ag-NPs are transferred intracellularly as a "package" preventing Ag from binding with a large number of substances such as thiol groups or enzymes present inside or in the cell membrane, explaining the increase in toxicity observed in the case of the Ag-NPs + TM combination.

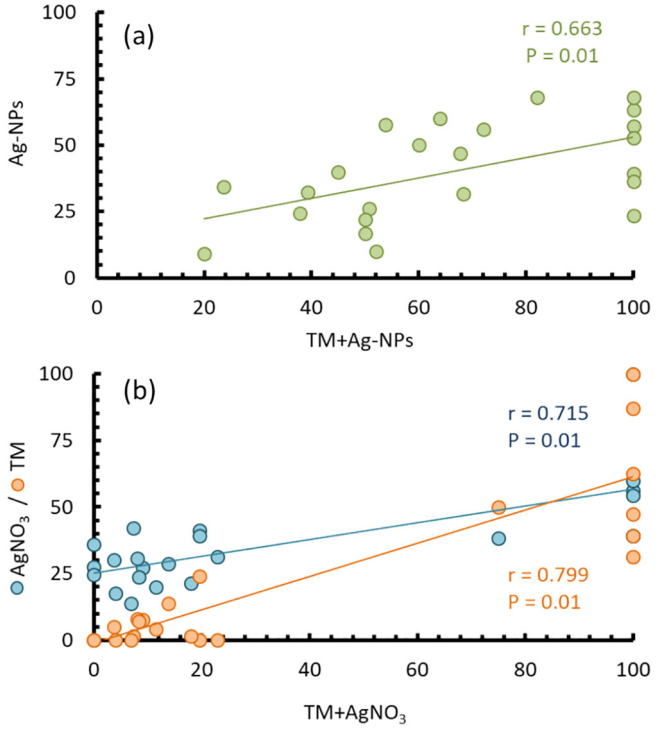


**Fig. 5.** Correlation between sensitivities of *M. fructicola* isolates to: (a) Ag-NPs (50 μg/mL) and its combination with thiophanate methyl (TM) (0.5 μg/mL) and, (b) $AgNO_3$ (3 μg/mL), TM (0. 5 μg/mL) and their combination. Here, r is the Pearson correlation coefficient, and P the significance level.

The enhanced inhibition rates the Ag-NPs/fluazinam combination compared to the individual treatments indicate an involvement of ATP-dependent metabolism in the fungitoxic activity of Ag-NPs. The biochemical mode of action of fluazinam as an ATP-synthetase inhibitor is well established and directly influences the function of energy-dependent efflux pumps (Kalamarakis et al., 2000; Leroux and Walker, 2013). The observed Ag-NPs/FM synergy could be attributed to a potential decrease/inhibition of efflux pump activity by fluazinam resulting an abnormal metal ion homeostasis and subsequent increased entry and accumulation of Ag-NPs inside the fungal cell enhancing its fungitoxic effect. Although additional studies would be required to validate this hypothesis, a similar synergy was found between fluazinam and Cu-NPs against *B. cinerea* indicating the existence of an energy dependent mechanism affecting nano metal fungitoxicity (Malandrakis et al., 2020). An efflux pumps dysregulation associated with Ag-NPs effectiveness against MDR *Candida albicans* strains when used in mixtures with antibiotics has been reported (Sun et al., 2016). A similar effect of CuO-NPs or copper ions against *Mytilus galloprovincialis* associated with multi-drug resistance transporter activity was also observed (Torres-Duarte et al., 2019).

A number of mechanisms for the antibacterial/antifungal action of NPs have been proposed including disruption of cell walls/membrane integrity, ROS production, enzyme inactivation, intervention in electron transport and DNA damage (Rudramurthy et al., 2016; Ruddaraju et al., 2020; Nisar et al., 2019). There is an ongoing debate between scientists whether the observed antimicrobial action is caused by the metal ions escaping nanoparticle surfaces (Sun et al., 2018; Hoseinzadeh et al., 2017; Król et al., 2017). In an attempt to elucidate this claim, a number of studies have utilized NaCl or KCl in combination with NPs in order to capture metal cations released by NPs using chlorine anions (Jo et al., 2009). In the present study, the use of NaCl in combination with Ag-NPs did not result any decrease in the fungitoxic action of Ag-NPs against *M. fructicola* but, on the contrary, resulted a significant synergistic effect. This result indicates that the role of $[Ag^+]$ ions in the fungitoxic action of the silver nanoparticles in *M. fructicola* could be minor. Furthermore, the lack of any correlation observed between $AgNO_3$ and Ag-NPs also indicates differences between the mode of fungitoxic action between silver NPs and their bulk counterpart, which agrees with the above hypothesis. Opposite results were obtained in our previous study where NaCl practically inactivated Cu-NPs against *B. cinerea* suggesting potential differences in the mode of

action of various metal NPs (Malandrakis et al., 2020). In a number of studies, Ag-NPs have been shown to interfere with ergosterol biosynthesis, which is essential for the structural integrity of the fungal membrane (Prasher et al., 2018). Sun et al. (2016) observed a synergistic effect between Ag-NPs with fluconazole – an ergosterol biosynthesis inhibitor (EBI)- against drug-resistant *C. albicans* strains proposing dysregulation of the ergosterol biosynthesis pathway and efflux pumps inhibition as possible mechanisms responsible for the synergy. In this study, combination of Ag-NPs with the EBI tebuconazole resulted in an additive effect in most isolate cases indicating a possible role of Ag-NPs in ergosterol biosynthesis although no correlation was found between Ag-NPs and tebuconazole sensitivity.

In conclusion, Ag-NPs were effective against *M. fructicola* sensitive and resistant to benzimidazoles phenotypes and exhibited an enhanced antifungal activity when applied was in mixtures with thiophanate methyl or fluazinam both *in vitro* and in apple fruit. A potential increase in Ag-NPs bioavailability is a probable cause for the observed synergy with TM while ATP-dependent metabolism is more involved in the fungitoxic action of Ag-NPs than [Ag+] ions. Effectiveness against sensitive and resistant isolates and synergy observed in this study point out the promising potential of Ag-NPs to be used as antifungal alternatives, providing the means for both effective anti-resistance strategies and reducing environmental impact of synthetic fungicides.

## Ethical approval

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

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## CRediT authorship contribution statement

**Anastasios A. Malandrakis:** Conceptualization, Investigation, Methodology, Writing - original draft. **Nektarios Kavroulakis:** Investigation, Visualization, Writing - review & editing. **Constantinos V. Chrysikopoulos:** Supervision, Visualization, Writing - review & editing.

## 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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