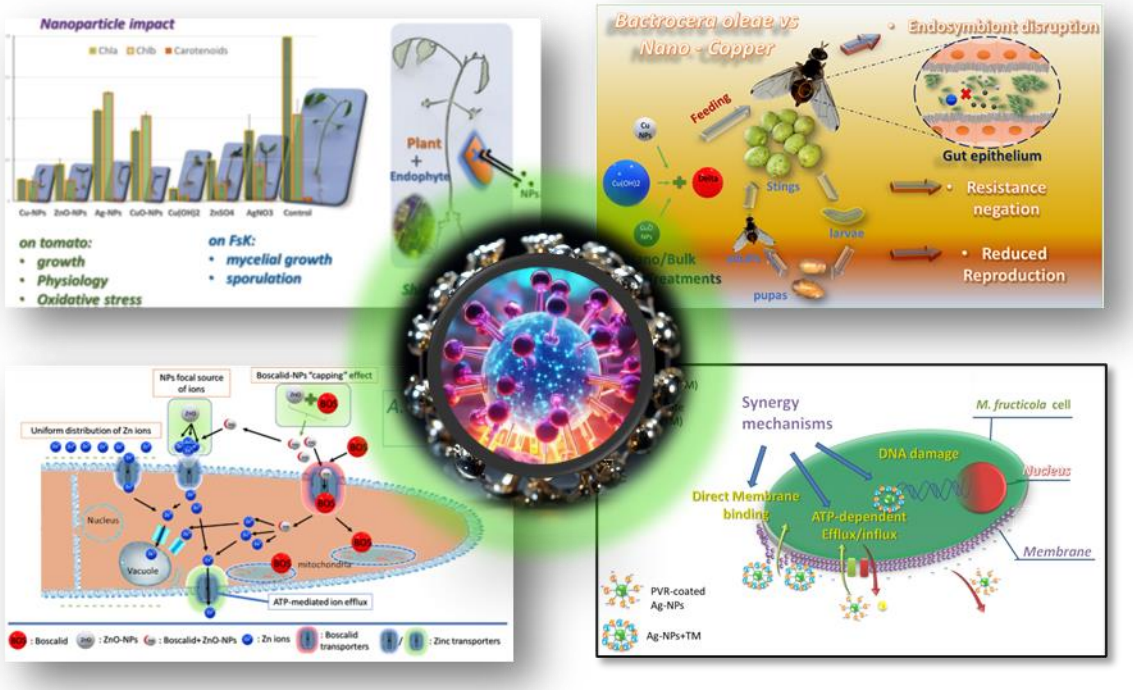
TECHNICAL UNIVERSITY OF CRETE

SCHOOL OF CHEMICAL AND ENVIRONMENTAL ENGINEERING

NANOPARTICLES VS PESTICIDES: APPLICATIONS AND IMPACT ON AGRO-ECOSYSTEMS



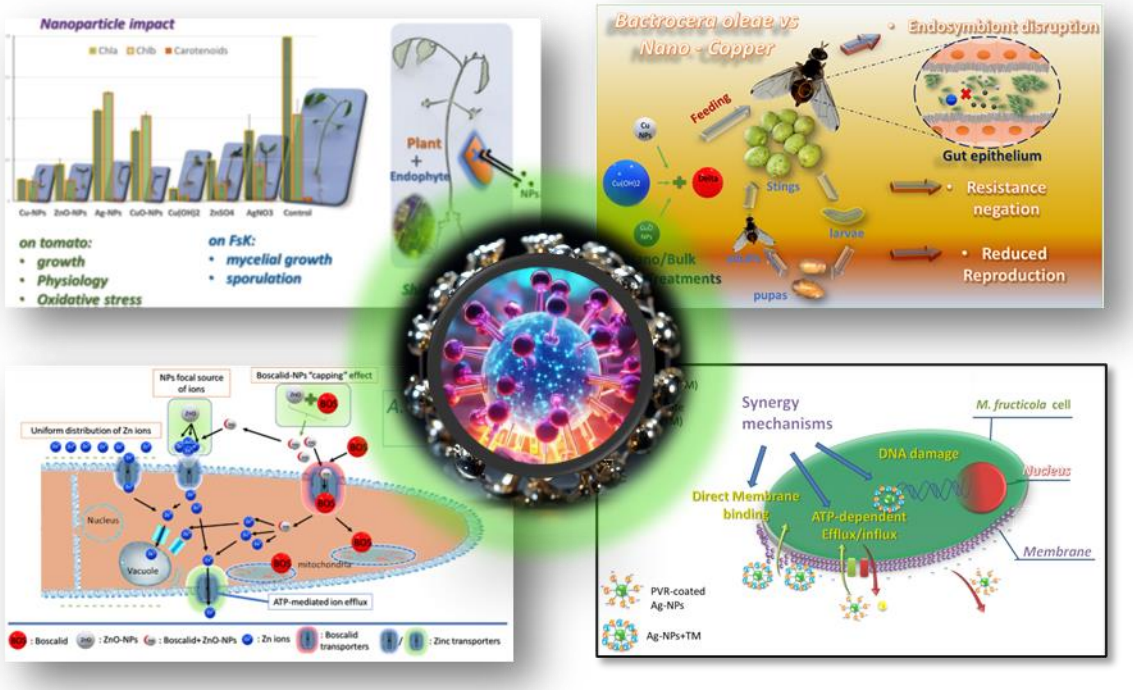
Anastasios A. Malandrakis

Chania, October 2024

ΠΟΛΥΤΕΧΝΕΙΟ ΚΡΗΤΗΣ

ΣΧΟΛΗ ΧΗΜΙΚΩΝ ΜΗΧΑΝΙΚΩΝ ΚΑΙ ΜΗΧΑΝΙΚΩΝ ΠΕΡΙΒΑΛΛΟΝΤΟΣ

ΝΑΝΟΣΩΜΑΤΙΔΙΑ-ΓΕΩΡΓΙΚΑ ΦΑΡΜΑΚΑ: ΕΦΑΡΜΟΓΕΣ ΚΑΙ ΕΠΙΔΡΑΣΗ ΣΤΟ ΑΓΡΟΟΙΚΟΣΥΣΤΗΜΑ



Αναστάσιος Α. Μαλανδράκης

Χανιά, Οκτώβριος 2024

Thesis Committee

Professor Dr Constantinos Chrysikopoulos – Supervisor

School of Chemical and Environmental Engineering, Technical University of Crete, Chania

Professor Dr Danae Venieri – Advisory Committee

School of Chemical and Environmental Engineering, Technical University of Crete, Chania

Research Director Dr Nektarios Kavroulakis – Advisory Committee

Institute of Olive Tree, Subtropical Plants and Viticulture, Hellenic Agricultural Organization (ELGO) “DIMITRA”, Chania

Professor Dr Petros Gikas - Examination Committee

School of Chemical and Environmental Engineering, Technical University of Crete, Chania

Assistant Professor Dr Aristeidis Koutroulis - Examination Committee

School of Chemical and Environmental Engineering, Technical University of Crete, Chania

Professor Dr John Vontas - Examination Committee

School of Plant Sciences, Department of Crop Science, Agricultural University of Athens

Professor Dr Constantinos Ehaliotis - Examination Committee

Department of Natural Resources Development and Agricultural Engineering, Agricultural University of Athens

Acknowledgments

This work was carried out in the Technical University of Crete, Environmental Engineering Laboratory (TUCeeL) Laboratory and the Institute of Olive Tree, Subtropical Plants and Viticulture under the supervision of Professor Constantinos Chrysikopoulos.

I am deeply grateful to Professor Constantinos Chrysikopoulos, my supervisor, for entrusting me with this PhD opportunity, inspiring me, and supporting my efforts in countless ways. His guidance and mentorship have been instrumental in shaping my research and professional development. I would like to express my sincere appreciation to Dr. Nektarios Kavroulakis, a valued member of my advisory committee and a dear friend, for providing laboratory facilities, engaging in fruitful brainstorming sessions, and offering invaluable assistance throughout my work. I am also thankful to Professor Danae Venieri for her trust, advice, and valuable suggestions regarding my research. I extend my gratitude to the members of the examination committee - Professor Petros Gikas, Associate Professor Aristeidis Koutroulis, Professor John Vontas, and Professor Constantinos Ehaliotis - for their willingness to evaluate this thesis and provide their expertise. A special note of thanks goes to my colleague and close friend, Ms. Roika Sarika, the TUCeeL general manager, for her unwavering support at both the technical and psychological levels. Her presence has been a source of strength and encouragement throughout my journey. I am grateful for the scientific discussions, encouragement, and help provided by my dear friend Dr. Sosa Fountouli during our time as PhD candidates. Our shared experiences and camaraderie have been a constant source of motivation. Finally, I would like to express my heartfelt gratitude to my family for their patience, love, and support throughout my PhD journey. Their unwavering belief in me has been a driving force in my pursuit of academic excellence.

ΠΕΡΙΛΗΨΗ

Η σύγχρονη γεωργία αντιμετωπίζει σημαντικές προκλήσεις για την επίτευξη βιώσιμης παραγωγής τροφίμων, ελαχιστοποιώντας παράλληλα την εξάντληση των φυσικών πόρων και τη ρύπανση του περιβάλλοντος. Οι πρόσφατες εξελίξεις στην τεχνολογία, ιδιαίτερα στη νανοτεχνολογία, παρουσιάζουν πολλά υποσχόμενες λύσεις σε αυτά τα ζητήματα. Η έρευνα της τελευταίας δεκαετίας υπογραμμίζει τις δυνατότητες της νανοτεχνολογίας σε διάφορες γεωργικές εφαρμογές, όπως τα νανο-λιπάσματα, που ενισχύουν την παροχή θρεπτικών ουσιών μέσω στοχευμένης απελευθέρωσης, μειώνοντας έτσι τα απόβλητα και τις περιβαλλοντικές επιπτώσεις. Επιπλέον, τα φυτοπροστατευτικά προϊόντα με τη βοήθεια νανοτεχνολογίας μπορούν να βελτιώσουν τις αποδόσεις των καλλιεργειών, ενώ μειώνουν το οικολογικό αποτύπωμα των συμβατικών αγροχημικών. Η ενσωμάτωση των νανοαισθητήρων στη γεωργία ακριβείας επιτρέπει την παρακολούθηση σε πραγματικό χρόνο της υγείας των καλλιεργειών, των συνθηκών του εδάφους και των περιβαλλοντικών παραγόντων, οδηγώντας σε πιο αποτελεσματικές γεωργικές πρακτικές. Επιπλέον, η νανοτεχνολογία μπορεί να βοηθήσει στην ανάπτυξη καλλιεργειών που είναι πιο ανθεκτικές στις ακραίες καιρικές συνθήκες που προκαλούνται από την κλιματική αλλαγή. Συνολικά, η εφαρμογή της νανοτεχνολογίας όχι μόνο ενισχύει την παραγωγικότητα, αλλά προάγει επίσης τη βιωσιμότητα βελτιστοποιώντας τη χρήση των παραγωγικών πόρων και μειώνοντας τις περιβαλλοντικές επιπτώσεις, τοποθετώντας την ως κρίσιμο στοιχείο για το μέλλον της βιώσιμης γεωργίας. Παρόλα τα δυνητικά πλεονεκτήματα και τις εφαρμογές των νανοσωματιδίων, η δυνατότητα χρήσης τους για τη διαχείριση της ανθεκτικότητας των φυτοπαθογόνων και εχθρών των καλλιεργειών καθώς και οι επιπτώσεις τους στα φυτά και σε οργανισμούς μη-στόχους έχει ελάχιστα διερευνηθεί.

Υπό αυτό το πρίσμα, οι κύριοι στόχοι της παρούσας διατριβής ήταν α) να μελετηθεί η αποτελεσματικότητα των μεταλλικών νανοσωματιδίων (MNPs) έναντι των φυτοπαθογόνων μυκήτων τόσο *in vitro* όσο και *in vivo,* β) να διερευνηθεί ο μηχανισμός της μυκητοτοξικής δράσης των MNPs σε βιοχημικό επίπεδο, γ) να εξεταστεί η δυνατότητα χρήσης των MNPs είτε μεμονωμένα είτε σε συνδυασμό με συμβατικά φυτοπροστατευτικά σκευάσματα για την καταπολέμηση ανθεκτικών στελεχών φυτοπαθογόνων, καθώς και για τη μείωση των συνιστώμενων δόσεων και του περιβαλλοντικού αποτυπώματος των φυτοφαρμάκων, δ) να εξεταστεί η πιθανή χρήση των MNPs κατά ανθεκτικών σε εντομοκτόνα πληθυσμών του Δάκου της ελιάς, και η επίδρασή τους στους ενδο-συμβιωτικούς μικροοργανισμούς του πεπτικού συστήματος του Δάκου, και ε) να διερευνηθούν οι τοξικολογικές επιδράσεις των MNPs στην ανάπτυξη και τις φυσιολογικές παραμέτρους των φυτών ντομάτας, καθώς και στους συμβιωτικούς οργανισμούς του ριζικού τους συστήματος.

Μεταλλικά νανοσωματίδια ως εναλλακτικά μυκητοκτόνα

Σε μια προσπάθεια αξιολόγησης της δυνατότητάς τους να χρησιμοποιηθούν ως εναλλακτικοί των φυτοφαρμάκων παράγοντες, νανοσωματίδια που περιείχαν χαλκό (Cu-NPs, CuO-NPs), άργυρο (Ag-NPs) και ψευδάργυρο (ZnO-NPs) αξιολογήθηκαν *in vitro* κατά επτά οικονομικά σημαντικών φυτοπαθογόνων μυκήτων, οι οποίοι προσβάλλουν το εναέριο και το υπόγειο μέρος των φυτών. Μεταξύ των παραπάνω, τα Cu-NPs αποδείχθηκαν ως οι πλέον αποτελεσματικοί μυκητοτοξικοί παράγοντες, ακολουθούμενα από τα ZnO-NPs και Ag-NPs, ενώ τα CuO-NPs παρουσίασαν περιορισμένη αποτελεσματικότητα. Οι Cu-NPs έδειξαν μεγαλύτερη μυκητοτοξικότητα στα περισσότερα υπό εξέταση φυτοπαθογόνα σε σχέση με το μυκητοκτόνο αναφοράς που περιείχε $Cu(OH)_2$. Τα περισσότερα NPs που δοκιμάστηκαν ήταν πιο τοξικά έναντι στα χονδροειδή τους ανάλογα, υποδεικνύοντας ότι υπάρχουν διαφορές στον τρόπο δράσης μεταξύ των νανοσωματιδίων και των ιοντικών τους αναλόγων. *In vitro*

βιοδοκιμές έδειξαν ότι τα NPs ήταν πολύ πιο τοξικά κατά την βλάστηση των σπορίων σε σχέση με τη μυκηλιακή αύξηση των μυκήτων, ενώ ήταν γενικά πιο αποτελεσματικά από το $Cu(OH)_2$. Επιπλέον, τα NPs μείωσαν σημαντικά τα συμπτώματα της τεφράς σήψης σε φρούτα δαμάσκηνου, ιδιαιτέρως τα Ag-NPs, τα οποία παρεμπόδισαν πλήρως την ανάπτυξη της ασθένειας. Αυτά τα ευρήματα υπογραμμίζουν το πολλά υποσχόμενο δυναμικό των νανοσωματιδίων να χρησιμοποιηθούν ως προστατευτικοί παράγοντες για την αντιμετώπιση των ασθενειών των φυτών. Στη συνέχεια, επιλεγμένα MNPs δοκιμάστηκαν έναντι ανθεκτικών στα μυκητοκτόνα στελεχών.

Μεταλλικά νανοσωματίδια έναντι ανθεκτικών σε μυκητοκτόνα στελεχών φυτοπαθογόνων μυκήτων

Η αντιμετώπιση και διαχείριση της ανθεκτικότητας των φυτοπαθογόνων στα μυκητοκτόνα είναι ένα απαιτητικό εγχείρημα, ειδικά λόγω της έλλειψης διαθέσιμων εναλλακτικών φαρμάκων με διαφορετικούς/πολλαπλούς μηχανισμούς δράσης. Στο δεύτερο μέρος αυτής της διατριβής, εξετάστηκε η δυνατότητα των MNPs να χρησιμοποιηθούν έναντι ανθεκτικών στα μυκητοκτόνα στελεχών φυτοπαθογόνων, η πιθανή συνεργιστική τους δράση με συμβατικά μυκητοκτόνα, καθώς και οι υποκείμενοι μηχανισμοί σε τέσσερις περιπτώσεις παθογόνων-νανοσωματιδίων. Στην πρώτη περίπτωση, νανοσωματίδια χαλκού (Cu-NPs) ήταν αποτελεσματικά τόσο έναντι σε ευαίσθητα όσο και ανθεκτικά στελέχη του *Botrytis cinerea* στα μυκητοκτόνα thiophanate methyl (TM) και pyraclostrobin που έφεραν τις E198A και G143A μεταλλαγές ανθεκτικότητας στα γονίδια της *β*-τουμπουλίνης και *cyt*b, αντίστοιχα. Παρατηρήθηκε συνεργιστική δράση μεταξύ Cu-NPs, TM και fluazinam τόσο *in vitro* όσο και *in vivo* όταν δοκιμάστηκαν σε μήλα. Η μυκητοτοξική δράση των Cu-NPs οφειλόταν εν μέρη στην απελευθέρωση ιόντων χαλκού καθώς και σε άλλους μηχανισμούς σχετιζόμενους με τις ιδιότητες των νανοσωματιδίων όπως ο ATP-εξαρτώμενος μεταβολισμός. Παρόμοια αποτελέσματα παρατηρήθηκαν στην περίπτωση του *M. fructicola* όπου τα Cu-NPs παρεμπόδιζαν αποτελεσματικά την μυκηλιακή αύξηση τόσο σε ευαίσθητα (BEN-S) όσο και σε ανθεκτικά (BEN-R) στελέχη που έφεραν τη E198A μεταλλαγή ανθεκτικότητας στα βενζιμιδαζόλικά μυκητοκτόνα, ενώ ήταν πιο αποτελεσματικά από το $Cu(OH)_2$. Και σε αυτήν την περίπτωση η απελευθέρωση ιόντων χαλκού και ο ATP-εξαρτώμενος μεταβολισμός βρέθηκαν επίσης να συμβάλλουν στη μυκητοτοξική δράση των Cu-NPs. Σημαντική συνεργιστική δράση παρατηρήθηκε μεταξύ των Cu-NPs και TM ενάντια στα ευαίσθητα στο TM στελέχη του μύκητα τόσο *in vitro* όσο και *in vivo*, ενώ οι πειραματικές ενδείξεις υποδεικνύουν την αυξημένη βιοδιαθεσιμότητα του TM ή την υποβοηθούμενη από τα NPs μετατροπή του TM σε carbendazim -το οποίο είναι πιο τοξικό από το TM- ως πιθανούς μηχανισμούς υπεύθυνους για το συνεργισμό. Στην τρίτη περίπτωση, η ευαισθησία των παραπάνω στελεχών του *M. fructicola* στα Ag-NPs διερευνήθηκε τόσο *in vitro* όσο και *in vivo*. Τα Ag-NPs παρεμπόδισαν αποτελεσματικά την μυκηλιακή ανάπτυξη τόσο στα ευαίσθητα όσο και στα ανθεκτικά στελέχη, ενώ παρατηρήθηκε επίσης συνεργισμός με το TM ανεξαρτήτως φαινοτύπου ανθεκτικότητας (BEN-R/S) τόσο *in vitro* όσο και όταν εφαρμόστηκαν σε φρούτα μήλου. Αντίθετα με το προφίλ που παρατηρήθηκε στην περίπτωση των Cu-NPs, η συνεργιστική δράση αποδόθηκε σε αυξημένη δραστικότητα των Ag-NPs και όχι στην αυξημένη διαθεσιμότητα του TM. Επιπλέον, ο ρόλος της απελευθέρωσης ιόντων αργύρου στη μυκητοτοξική δράση των Ag-NPs κατά του *M. fructicola* ήταν περιορισμένος, ενώ ο ATP-εξαρτώμενος μεταβολισμός εξακολουθούσε να συμβάλλει στη μυκητοτοξική τους δράση. Στην τελευταία περίπτωση, μελετήθηκε η μυκητοτοξική δράση νανοσωματιδίων ZnO έναντι σε ευαίσθητα και ανθεκτικά στελέχη του παθογόνου *Alternaria alternata* στον αναστολέα της αφυδρογονάσης του ηλεκτρικού οξέος (SDHI) boscalid. Όπως και στις παραπάνω περιπτώσεις, τα ZnO-NPs ανέστειλαν αποτελεσματικά την μυκηλιακή αύξηση τόσο στα ευαίσθητα όσο και στα ανθεκτικά στελέχη. Η απελευθέρωση ιόντων ψευδαργύρου ήταν εν μέρει υπεύθυνη για την μυκητοτοξική δράση των ZnO-NPs μαζί με πρόσθετους μηχανισμούς που σχετίζονται με την κυτταρική ιονική ομοιόσταση, όπως η

ATP-εξαρτώμενη απέκκριση ιόντων και η παραγωγή ROS. Τα ZnO-NPs έδειξαν συνεργιστική δράση με το μυκητοκτόνο boscalid σε όλες τις φαινοτυπικές περιπτώσεις (BOSC-S/R) τόσο *in vitro* όσο και *in vivo*. Αυτή τη συνεργιστική δράση πιθανώς οφείλονταν στην "τυποποιητική" δραση του boscalid το οποίο περιβάλλει τα ZnO-NPs κατά την εφαρμογή του, προκαλώντας σημαντική μείωση του μεγέθους των νανοσωματίδιων, αυξάνοντας επομένως την τοξική τους δράση.

Τα παραπάνω αποτελέσματα δείχνουν ότι τα μεταλλικά NPs μπορούν να χρησιμοποιηθούν για τη διαχείριση του φαινομένου ανθεκτικότητας στα μυκητοκτόνα, ειδικά όταν χρησιμοποιούνται σε συνδυασμό με το ίδιο μυκητοκτόνο στο οποίο το παθογόνο έχει μειωμένη ευαισθησία. Αυτή η σημαντική δράση κατά της ανθεκτικότητας μπορεί να αποδοθεί σε αρκετούς μηχανισμούς, όπως η αυξημένη βιοδιαθεσιμότητα του μυκητοκτόνου που προκαλείται από τα NPs που δρουν ως φορείς, η χημική ευαισθητοποίηση του κυττάρου των μυκήτων που προκαλείται από τα NPs ή μια έμμεση αυξημένη τοξικότητα των NPs λόγω της "τυποποιητικής" δράσης του μυκητοκτόνου που έχει ως αποτέλεσμα μικρότερα και επομένως πιο τοξικά NPs. Σε κάθε περίπτωση, εκτός από τη διαχείριση της ανθεκτικότητας στα μυκητοκτόνα, ο συνδυασμός των MNPs με τα μυκητοκτόνα αποδείχθηκε ότι μειώνει δραστικά τις συνιστώμενες δόσεις μυκητοκτόνων, γεγονός που μπορεί να συμβάλει στην μετρίαση του προβλήματος της περιβαλλοντικής ρύπανσης που προκαλείται από τη υπερβολική χρήση αγροχημικών.

MNPs έναντι πληθυσμών του Δάκου της ελιάς ανθεκτικών στα εντομοκτόνα

Εκτός από τις αντιμικροβιακές τους ιδιότητες, τα MNPs έχουν δείξει μεγάλη αποτελεσματικότητα και μπορούν να χρησιμοποιηθούν ως εναλλακτικά εντομοκτόνα κατά των αρθροπόδων οικονομικής σημασίας, όπως οι μύγες, τα κουνούπια, οι ψείρες και τα τσιμπούρια. Σε αυτή τη μελέτη, νανοσωματίδια που περιείχαν χαλκό (Cu-/CuO-NPs) δοκιμάστηκαν ως εντομοτοξικοί παράγοντες κατά πληθυσμών του δάκου της ελιάς (*B. oleae*) με μειωμένη ευαισθησία στο εντομοκτόνο deltamethrin. Επίσης μελετήθηκε η επίδρασή τους στις αναπαραγωγικές παραμέτρους του εντόμου καθώς και σε ενδο-συμβιωτικούς μικροοργανισμούς του πεπτικού του συστήματος. Τόσο τα νανοσωματίδια όσο και ο συμβατικός χαλκός που εφαρμόστηκαν μέσω σίτισης προκάλεσαν σημαντικά επίπεδα θνησιμότητας στα ενηλίκα άτομα του δάκου, τα οποία ήταν συγκρίσιμα ή ανώτερα από αυτά που επιτεύχθηκαν με το εντομοκτόνο deltamethrin στις συνιστώμενες δόσεις. Όταν συνδυάστηκαν με το deltamethrin, τα Cu-NPs αύξησαν σημαντικά την αποτελεσματικότητα του εντομοκτόνου κατά των ενηλίκων του *B. oleae*, μείωσαν τον μέσο συνολικό αριθμό απογόνων σε σύγκριση με το μάρτυρα καθώς και τον αριθμό των τσιμπημάτων, των προνυμφών, των θηλυκών και του συνολικού αριθμού απογόνων σε σύγκριση με την επέμβαση που περιείχε μόνο το εντομοκτόνο. Τόσο ο συμβατικός χαλκός όσο και τα νανοσωματίδια χαλκού μείωσαν σημαντικά την αφθονία του ενδοσυμβιωτικού βακτηρίου *Candidatus Erwinia dacicola*, το οποίο είναι απαραίτητο για την επιβίωση των προνυμφών του *B. oleae*. Αυτά τα ευρήματα υποδεικνύουν ότι οι Cu-NPs μπορούν να συμβάλουν στη διαχείριση του *B. oleae* τόσο μειώνοντας την επιβίωση των προνυμφών του όσο και ενισχύοντας την αποτελεσματικότητα του deltamethrin όσον αφορά την τοξικότητα και τη μειωμένη γονιμότητα και αποτελούν ένα αποτελεσματικό εργαλείο κατά της ανθεκτικότητας ενώ μπορούν να ελαχιστοποιήσουν το περιβαλλοντικό αποτύπωμα των συνθετικών εντομοκτόνων μειώνοντας τις απαιτούμενες δόσεις για τον έλεγχο του παρασίτου.

Τοξικολογικές επιδράσεις των MNPs σε φυτά και σε συμβιωτικούς οργανισμούς του ριζικού συστήματος της τομάτας

Ως αναπόσπαστο μέρος όλων των οικοσυστημάτων, τα φυτά αναμένεται να είναι δυνητικοί αποδέκτες άμεσων ή έμμεσων αλληλεπιδράσεων με νανοσωματίδια, τα οποία θα μπορούσαν ενδεχομένως να

οδηγήσουν σε τοξικότητα, συσσώρευση μέσω της πρόσληψης ή διατάραξη των αλληλεπιδράσεων των φυτών με ωφέλιμους/συμβιωτικούς μικροοργανισμούς. Πολλές μελέτες αξιολόγησης της φυτοτοξικότητας των μεταλλικών NPs αναφέρουν αρνητικές επιδράσεις στην ανάπτυξη και φυσιολογία των φυτών σε πολλά είδη. Αν και οι αναφορές συχνά είναι αντικρουόμενες, οι περισσότερες συνηγορούν στη άποψη ότι το επίπεδο φυτοξικότητας των NPs εξαρτάται από το είδος και ότι κάθε περίπτωση θα πρέπει να αξιολογείται ξεχωριστά. Η τομάτα είναι μια δημοφιλής καλλιέργεια με μεγάλη οικονομική σημασία παγκοσμίως, και αν και υπάρχουν μελέτες που αξιολογούν τη φυτοτοξικότητα της ντομάτας που προκαλείται από NPs που περιέχουν άργυρο, οξείδιο του ψευδαργύρου, διοξείδιο του τιτανίου, χαλκό και σίδηρο, η τοξικότητά τους σε σύγκριση με τις χονδροειδείς/ιοντικές μορφές τους, καθώς και η αλληλεπίδρασή τους με ενδοφυτικούς συμβιωτικούς μικροοργανισμούς της ντομάτας δεν είναι επαρκώς μελετημένη. Στα πλαίσια της παρούσας μελέτης, διερευνήθηκε η επίδραση νανοσωματιδίων χαλκού (Cu-NPs, CuO-NPs), αργύρου (Ag-NPs) και οξειδίου του ψευδαργύρου (ZnO-NPs) στην ανάπτυξη, τις φυσιολογικές ιδιότητες και τις συμβιωτικές αλληλεπιδράσεις φυτών ντομάτας με το στέλεχος του ενδοφυτικού μύκητα *Fusarium solani* FsK. Τα αποτελέσματα έδειξαν ότι το FsK ήταν πιο ευαίσθητο στα Cu-NPs και ZnO-NPs σε σύγκριση με τα CuO-NPs και Ag-NPs, με όλα τα νανοσωματίδια να παρουσιάζουν μεγαλύτερη τοξικότητα σε σχέση με τις χονδροειδείς/ιοντικές μορφές τους, εκτός από το $AgNO_3$, το οποίο ήταν σημαντικά πιο τοξικό από τα AgNPs. Ενώ τα υπό εξέταση NPs δεν επηρέασαν τη βλάστηση των σπόρων, μείωσαν το μήκος και το ξηρό βάρος των ριζών, ιδίως στις περιπτώσεις των Cu-NPs και ZnO-NPs. Το μήκος των ριζών και των βλαστών των αναπτυγμένων φυτών ντομάτας επηρεάστηκε επίσης από τις επεμβάσεις, ενώ οι διαφορές μεταξύ NPs και των ιοντικών τους μορφών ήταν ποικίλες. Τα NPs και τα ιοντικά τους ανάλογα προκάλεσαν μείωση των επιπέδων χλωροφύλλης-a και καροτενοειδών και προκάλεσαν έντονο οξειδωτικό στρες, όπως υποδεικνύεται από την αύξηση των επιπέδων MDA και H_2O_2 στα φυτά που εφαρμόστηκαν. Τα NPs και οι χονδροειδείς τους μορφές δεν επηρέασαν τον αποικισμό του FsK στις ρίζες, υποδηλώνοντας πιθανή προστατευτική επίδραση των φυτών ντομάτας μόλις ο ενδοφυτικός μικροοργανισμός εγκατασταθεί μέσα στις ρίζες. Ενδιαφέρον παρουσιάζει το γεγονός ότι η παρουσία της FsK στις ρίζες φαίνεται να μετριάζει την τοξικότητα των νανοσωματιδίων, το οποίο υποδηλώνει την ύπαρξη σύνθετων αλληλεπιδράσεων φυτού-συμβιωτικού μικροοργανισμού οι οποίες θα μπορούσαν να προσδώσουν ανθεκτικότητα στα φυτά τομάτας έναντι σε αυτούς τους τοξικούς παράγοντες.

Συνοψίζοντας, η δυνατότητα των μεταλλικών νανοσωματιδίων (MNPs) να χρησιμοποιηθούν ως εναλλακτικά, οικολογικά συμβατά φυτοφάρμακα διερευνήθηκε σε διάφορα συστήματα παθογόνων/παρασίτων. Επιπλέον, τα αποτελέσματα ανέδειξαν τη δυνατότητά τους να αντιμετωπίσουν το ζωτικής σημασίας για τη γεωργία ζήτημα της ανθεκτικότητας στα φυτοφάρμακα. Συγκεκριμένα, ο συνδυασμός των MNPs με φυτοφάρμακα οδήγησε σε αύξηση της αποτελεσματικότητας των τελευταίων κατά ευαίσθητων και ανθεκτικών στελεχών, επιτρέποντας την αποτελεσματική διαχείριση του φαινομένου ανθεκτικότητας, τη μείωση των δόσεων και, κατά συνέπεια, την ελαχιστοποίηση του περιβαλλοντικού αποτυπώματος αυτών των φαρμάκων. Ωστόσο, παρά την πολλά υποσχόμενη προστασία των καλλιεργειών, η χρήση των MNPs μπορεί να εγκυμονεί γνωστούς και άγνωστους κινδύνους για την υγεία και το περιβάλλον. Δοκιμές φυτοτοξικότητας σε τομάτες αποκάλυψαν αρνητικές επιδράσεις των μεταλλικών νανοσωματιδίων στην ανάπτυξη και τη φυσιολογία των φυτών τομάτας, καθώς και στις συμβιωτικές σχέσεις με το FsK στελέχος του μύκητα *F. solani* το οποίο προστατεύει τις τομάτες από ασθένειες του εδάφους και συνθήκες φυσιολογικού στρες. Επομένως, δεδομένου ότι η επίδρασή τους στα βιολογικά συστήματα δεν έχει ακόμη πλήρως κατανοηθεί, τα MNPs θα πρέπει να μελετηθούν περαιτέρω για ανεπιθύμητες επιδράσεις σε οργανισμούς μη στόχους, όπως η φυτοτοξικότητα, η τοξικότητα για τους ανθρώπους και η οικοτοξικότητα στο περιβάλλον, πριν από την εμπορική τους αξιοποίηση ως νανο-φυτοφάρμακα σε μεγάλη κλίμακα.

ABSTRACT

Modern agriculture faces significant challenges in achieving sustainable food production while minimizing the depletion of natural resources and environmental pollution. Recent advancements in nanotechnology hold great promise in addressing these challenges. This has been highlighted by several studies focusing on various agricultural applications of nanotechnology, such as the development of nano-fertilizers, which enhance nutrient delivery through targeted release, thereby reducing waste and environmental impact. A major challenge chemical control faces is the loss of available conventional active ingredients due to strict environmental safety regulations which, combined with the loss of fungicide efficacy due to resistance development, constitute major issues of contemporary crop protection. Metal containing nanoparticles appear to have all the credentials to be the next-generation, eco-compatible fungicide alternatives and a valuable anti-resistance management tool. Could the introduction of metal NPs as nano-fungicides be the answer to both reducing environmental footprint of xenobiotics and dealing with fungicide resistance? Furthermore, and despite their "golden" potential to be used as pesticide alternatives and anti-resistant agents, safety issues concerning undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity, should be considered before their commercial introduction as nano-pesticides.

Under this light the main objectives of the present thesis were a) to study the effectiveness of metallic nanoparticles (MNPs) against phytopathogenic fungi both *in vitro* and *in vivo*, b) to investigate the mechanism of fungitoxic action of MNPs at the biochemical level, c) to explore the potential use of MNPs alone or in combination with conventional plant protection products to combat resistant phytopathogenic strains, as well as to reduce recommended doses and the environmental footprint of pesticides, d) to examine the potential use of MNPs against populations of the olive fruit fly, resistant to insecticides, and their impact on the endosymbiotic microorganisms of the fly's gut flora, and e) to investigate the toxicological effects of MNPs on the growth and physiological parameters of tomato plants, as well as on the symbiotic organisms of their root system.

Metallic nanoparticles as fungicide alternatives

In an attempt to evaluate their potential to be used as fungicide alternatives, copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc (ZnO-NPs) containing nanoparticles (NPs) were assessed *in vitro* against seven economically important fungal species, known to cause foliar and soil-borne plant diseases. Cu-NPs emerged as the most effective, followed by ZnO-NPs and Ag-NPs, while CuO-NPs and showed limited effectiveness. Cu-NPs demonstrated greater fungitoxicity than the reference fungicide $Cu(OH)_2$ across most species. Most NPs tested outperformed their bulk counterparts in terms of toxicity, indicating distinct mechanisms of action between nanoparticles and their ionic counterparts. All NPs were significantly more toxic to fungal spores than to hyphae and overall more effective than $Cu(OH)_2$, as confirmed by *in vitro* bioassays. Additionally, NPs notably reduced grey mold symptoms on plum fruit, particularly Ag-NPs, which completely inhibited disease development. These findings highlight the promising potential of these nanoparticles as protective antifungal agents.Following this initial screening, selected MNPs were tested against fungicide-resistant fungal pathogens.

MNPs combating fungicide resistance

Combating drug-resistance is a daunting task, especially due to the shortage of available drug alternatives with multisite modes of action. In the second part of this thesis, the potential of MNPs to be used against fungicide-resistant fungal pathogens, their potential synergy with conventional fungicides as well as the underlying mechanisms were investigated in four pathogen-NP cases. In the first case, copper nanoparticles (Cu-NPs) were found to be effective against both sensitive and resistant to thiophanate-methyl (TM) and pyraclostrobin *Botrytis cinerea* isolates bearing the E198A and G143A resistance mutations in the *β*-tubulin and *cytb* genes, respectively. A synergistic effect was observed between Cu-NPs, TM and fluazinam both *in vitro* and when tested on apple fruit. Copper ion release contributed to the fungitoxic activity of Cu-NPs, but nano-specific mechanisms including ATP-dependent metabolism were also involved. Similar results were observed in the case of Cu-NPs, which could suppress mycelial growth in both sensitive (BEN-S) and resistant (BEN-R) to TM *M. fructicola* isolates harbouring the E198A benzimidazole resistance mutation, more effectively than $Cu(OH)_2$. Copper ion release and ATP-dependent metabolism were also found to contribute to the fungitoxic action of Cu-NPs against *M. fructicola.* A significant synergy of Cu-NPs with TM was observed against TM-sensitive isolates both *in vitro* and when applied on plum fruit suggesting an enhanced availability or nanoparticle-induced transformation of TM to carbendazim. In the third case, to test whether the effectiveness of MNPs could be affected by the metallic basis of NPs, Ag-NPs were tested against the above *M. fructicola* isolates in terms of effectiveness and fungicide resistance management both *in vitro* and *in vivo.* Ag-NPs effectively suppressed mycelial growth in both sensitive (BEN-S) and resistant isolates. Ag-NPs also exhibited synergy with TM regardless resistant phenotype (BEN-R/S) both *in vitro* and when applied on apple fruit. Contrary to the profile observed in the case of Cu-NPs, the synergistic action was attributed to an enhanced Ag-NPs activity rather than increased TM availability. Furthermore, the role of released silver ions on the fungitoxic action of Ag-NPs against *M. fructicola* was found to be limited, while ATP-dependent metabolism probably contribute to their fungitoxic action. In the last NP-pathogen case, the antifungal potential of ZnO-NPs against *Alternaria alternata* isolates with reduced sensitivity to the succinate dehydrogenase inhibitor (SDHI) boscalid, resulting from target site modifications, was assessed. Similarly to the above cases, ZnO-NPs could effectively inhibit mycelial growth in both sensitive (BOSC-S) and resistant (BOSC-R) isolates. Zinc ion release was partly responsible for the fungitoxic effect of ZnO-NPs as well as additional mechanisms involving cellular ion homeostasis such as ATP-dependent ion efflux and ROS production. ZnO-NPs showed a synergistic effect with boscalid in all phenotypic cases (BOSC-S/R) both *in vitro* and *in vivo*. A "capping" effect of boscalid when applied with ZnO-NPs probably accounts for this synergistic effect by causing a significant reduction of the nanoparticle size.

All the above cases indicate that metal NPs can combat fungicide resistance especially when used in combination with the same fungicide that the pathogen has developed resistance to. This extraordinary resistance-negating effect can be attributed to several mechanisms such as increased bioavailability of the fungicide caused by a carrier effect of the NP, chemo sensitization of the fungal cell caused by the NPs or an indirect increased toxicity of the NPs due to a "capping" effect of the fungicide which results in smaller, and thus more toxic, NPs. In any case, besides managing fungicide-resistance, the combination of MNPs with fungicides was shown to drastically reduce the recommended fungicide doses which can contribute to the mitigation of the problem of environmental contamination caused by the excessive use of agrochemicals.

MNPs against insecticide-resistant *Bactrocera oleae* populations

Besides their antimicrobial properties, MNPs such as those containing silver, zinc oxide, titanium oxide and copper, have exhibited a great potential as pesticide alternatives against arthropod pests of economic importance, including flies, moths, mosquitos, lice and ticks. In this study, copper nanoparticles (NPs)

were tested as an alternative control agent against olive fruit flies (*B. oleae*) with reduced sensitivity to the insecticide deltamethrin and their impact on the insect's reproductive and endosymbiotic parameters was investigated. Both nanosized and bulk copper applied by feeding resulted in significant levels of adult mortality, comparable to or surpassing those achieved with the insecticide deltamethrin at recommended doses. When combined with deltamethrin, Cu-NPs significantly enhanced the insecticide's efficacy against *B. oleae* adults, reduced the mean total number of offspring compared with the control, and the number of stings, pupae, female and total number of offspring compared with the insecticide alone. Both bulk and nanosized copper significantly reduced the abundance of the endosymbiotic bacterium *Candidatus Erwinia dacicola* which is essential for the survival of *B. oleae* larvae. These findings indicate that Cu-NPs can contribute to the management of *B. oleae* both by reducing larval survival and by enhancing deltamethrin performance in terms of toxicity and reduced fecundity, providing an effective anti-resistance tool and minimizing the environmental footprint of synthetic insecticides by reducing the required doses for the control of the pest.

Toxicological effects of MNPs on tomato and its root-symbiotic organisms

Plants, being an essential part of all ecosystems, are expected to interact directly or indirectly with nanoparticles, that could potentially inflict toxicity, accumulate via uptake or disturb interactions of plants with beneficial/symbiotic organisms. Various studies evaluating phytotoxicity of metal NPs have been conducted reporting adverse effects on various aspects of plant growth and physiology in numerous plant species although reports are often conflicting, highlighting that toxicity threshold of NPs towards plants is species dependent and each case should be evaluated separately. Tomato is a vegetable crop with great popularity and economic importance worldwide, and although studies evaluating phytotoxicity of tomato caused by silver, zinc oxide, titanium oxide, copper, and ferric NPs are available, their toxicity compared with their bulk counterparts, as well as their interaction with tomato's endophytic symbiotic microorganisms is limited. Therefore, this study investigated the effect of copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc oxide (ZnO-NPs) nanoparticles (NPs) on plant growth, physiological properties of tomato plants and their symbiotic relationships with the endophytic *Fusarium solani* FsK strain. Results indicated that the FsK strain was more sensitive to Cu-NPs and ZnO-NPs compared to CuO-NPs and Ag-NPs, with most nanoparticles exhibiting greater toxicity than their bulk forms. While NPs did not affect seed germination, they reduced root length and dry weight in a dose-dependent manner, with notable effects from $AgNO_3$, Cu-NPs and ZnO-NPs. Root and shoot length of grown tomato plants was also affected by treatments while differences between NPs and bulk counterparts varied. NPs and bulk counterparts resulted in reduced chlorophyl-a and carotenoid levels and a marked oxidative stress response as indicated by increased MDA and H_2O_2 levels of treated plants. NPs and counterparts did not affect FsK colonization of roots indicating a possible shielding effect of tomato plants once the endophyte was established inside the roots. Interestingly, the presence of FsK in roots appeared to mitigate some of the nanoparticle toxicity, suggesting a complex interaction that could provide resistance against these toxic agents in tomato plants.

Summarizing, the potential of MNPs to be used as alternative, eco-compatible pesticides was demonstrated in several disease/pest pathosystems. Results also highlighted their potential for addressing the crucial agricultural issue of pesticide resistance, especially when combined with pesticides which leads to synergy, enabling effective management of the resistance phenomenon while simultaneously allowing for reduced dosages and consequently minimizing the environmental footprint of these drugs. However promising for crop protection, MNPs may pose both known and unknown health and environmental risks. Phytotoxicity tests on tomato plants revealed adverse effects of metal NPs on growth and physiological properties as well as on an endosymbiotic fungal strain which shields tomato plants against soil diseases and stress conditions. Therefore, since their effect on biological

systems is not yet completely understood, MNPs should be further studied for undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity before their commercial introduction as nano-pesticides at a large scale.

Table of Contents

Table of Figures

List of Tables

Αφιερώνεται στους γονείς και στους γιούς μου

Published Papers

This phD thesis is based on the following published papers:

1. Malandrakis, A.A., Varikou, K., Kavroulakis, N., Nikolakakis, A., Dervisi, I., Reppa, C., Papadakis, S., Holeva, M.C., Chrysikopoulos, C.V. Copper nanoparticles interfere with insecticide sensitivity, fecundity and endosymbiont abundance in olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) (2024) Pest Management Science, DOI: 10.1002/ps.8068

2. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Metal nanoparticles against fungicide resistance: alternatives or partners? (2022) Pest Management Science, 78 (10), pp. 3953-3956. DOI: 10.1002/ps.7014.

3. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant *Alternaria alternata* isolates (2022) Science of the Total Environment, 829, art. no. 154638, DOI: 10.1016/j.scitotenv.2022.154638.

4. Malandrakis, A.A., Kavroulakis, N., Avramidou, M., Papadopoulou, K.K., Tsaniklidis, G., Chrysikopoulos, C.V. Metal nanoparticles: Phytotoxicity on tomato and effect on symbiosis with the *Fusarium solani* FsK strain (2021) Science of the Total Environment, 787, art. no. 147606, DOI: 10.1016/j.scitotenv.2021.147606.

5. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates (2021) Pesticide Biochemistry and Physiology, 173, art. no. 104796, DOI: 10.1016/j.pestbp.2021.104796.

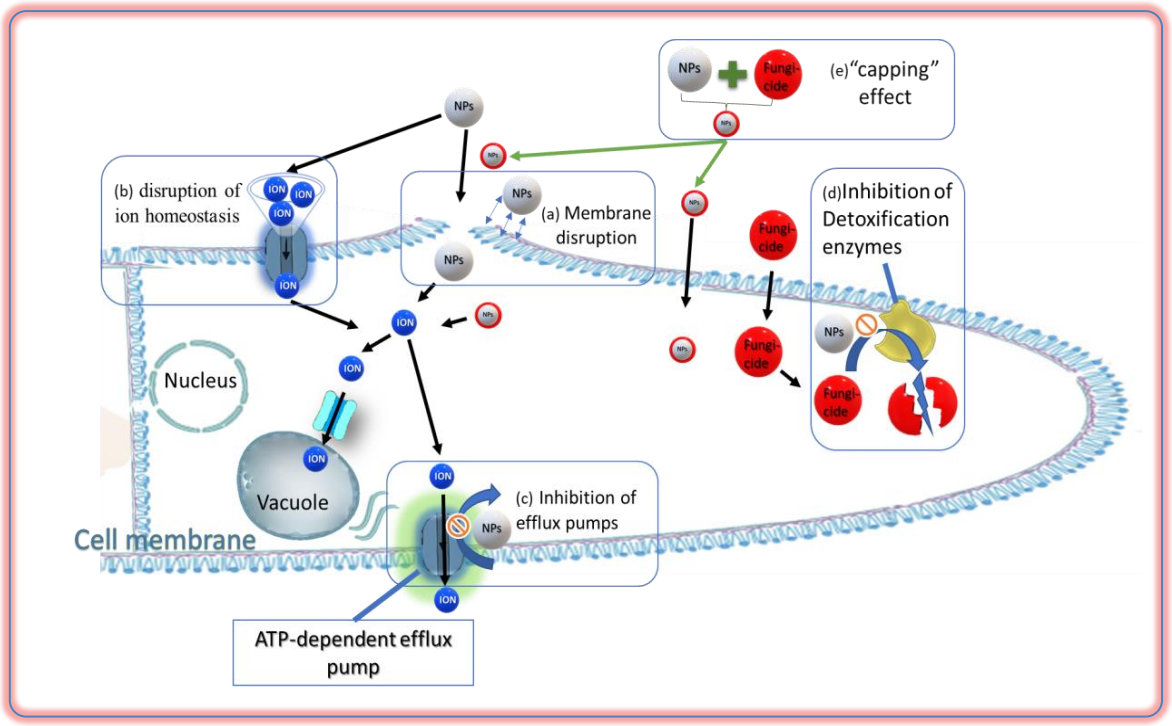
6. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola* (2020) Science of the Total Environment, 747, art. no. 141287, DOI: 10.1016/j.scitotenv.2020.141287.

7. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Synergy between Cu-NPs and fungicides against *Botrytis cinerea* (2020) Science of the Total Environment, 703, art. no. 135557, DOI: 10.1016/j.scitotenv.2019.135557.

8. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019) Science of the Total Environment, 670, pp. 292-299. DOI: 10.1016/j.scitotenv.2022.154638.

1 Nanoparticles and Pesticides in Agriculture:

Applications and challenges



1. Nanoparticles and pesticides in Agriculture: Applications and challenges

1.1 Nanoparticles - definition and classification

According to the European Union's definition, nanoparticles (NPs) are characterized on the basis of their particle size, which typically ranges between 1 to 100 nm and overlaps with that of colloid particles (size ranging from 1 to 1,000 nm). As a result, it is common to find literature that uses the terms "nanoparticles" and "colloidal particles" interchangeably, particularly for particles smaller than 100 nm.
EU's definition of NPs was revised in 2022 to provide a more coherent and efficient regulatory framework for nanomaterials across all sectors in the EU. Specifically, the European Commission (EC) Recommendation states:

« "Nanomaterial" means a natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50% or more of these particles in the number-based size distribution fulfil at least one of the following conditions:

1. one or more external dimensions of the particle are in the size range 1 nm to 100 nm;

2. the particle has an elongated shape, such as a rod, fiber or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm;

3. the particle has a plate-like shape, where one external dimension is smaller than 1 nm and the other dimensions are larger than 100 nm.

4. In the determination of the particle number-based size distribution, particles with at least two orthogonal external dimensions larger than 100 μm need not be considered.

However, a material with a specific surface area by volume of $2/cm^3$ shall not be considered a nanomaterial.»

NPs have a size comparable to viruses, DNA, and proteins, while microparticles have a size comparable to cells, organelles, and larger physiological structures. The unique features of NPs primarily arise from the reduction of particle size to less than 100 nm, leading to properties that include higher thermal stability, strength, high conductivity and low permeability (Rauscher et al., 2019). The decrease in particle size also increases the surface area-to-volume ratio, making NPs more reactive than their bulk counterparts. As a result, it is challenging to predict the physicochemical characteristics and toxicities of NPs, even if the bulk materials they are derived from are well understood.

A relevant categorization of NPs can stem from their a) main material, a) dimensions, and c) source:

Material-based classification

This category includes:

i. **Carbon-based NPs** contain carbon and include a variety of NPs such as nanodiamonds, graphene (Gr), carbon nanotubes (CNTs) and nanofibers (CNFs), and fullerenes (C60) (Kumar and Kumbhat, 2016).

ii. **Inorganic-based NPs** containing **inorganic** molecules such as **metals** (Cu, Zn, and Ag), **metal oxides** (CuO, TiO_2 and ZnO), and **semiconductors** (ceramics).

iii. **Organic-based NPs**, consisting of organic materials (polymers, dendrimers, liposomes, and micelles.

iv. **Composite-NPs**, are nanostructured materials that consist of at least two different types of materials, one of which is a nanoscale material. These composites can be made up of carbon-based, metal-based, or organic-based nanomaterials combined with any form of metal, ceramic, or polymer bulk materials.

Dimension-based classification

According to Poh, et al. (2018), NPs can be classified based on their dimension as:

i) **Zero-dimensional** (**0D**) in which all three dimensions are in a nanometric range such as quantum dots in light-emitting diodes (LEDs), solar cells, and lasers.

ii) **One dimensional** (**1D**) in which two dimensions are in a nanometric range such as nanowires, nanorods, and nanotubes,

iii) **Two dimensional (2D**) in which one dimension is in a nanometric range such as nano-thin films, nanosheets, and nanoplates,

iv) **Three dimensional (3D)** in which all three dimensions are outside of a nanometric range, as shown in Figure 1.1 These NPs may consist of nanofiber, nanotube groups or of different NP-containing configurations.

Classification of NPs based on their source

According to their origin, NPs can be classified into natural, incidental and engineered (Khan, 2020):

i) **Natural NPs** are naturally occurring nanoscale materials produced by living organisms or through anthropogenic activities. A number of natural processes are responsible for their production including photochemical reactions, volcanic eruptions, erosion, skin shedding or animal hair.

ii) **Incidental NPs** consisting of anthropogenic side- or by-products of processes and activities (transportation vehicles), or some incidental natural processes such as forest fires.

iii) **Engineered nanoparticles (ENPs)** are specifically manufactured NPs with tuned properties. They are manufactured via physical, chemical or hybrid methods, such as milling (from their macro-scale counterparts) or self-assembly (atoms or molecules). Applications of ENMs include food contact materials, cosmetics, textiles, improved diagnosis, and disease treatment, energy storage and computer products.

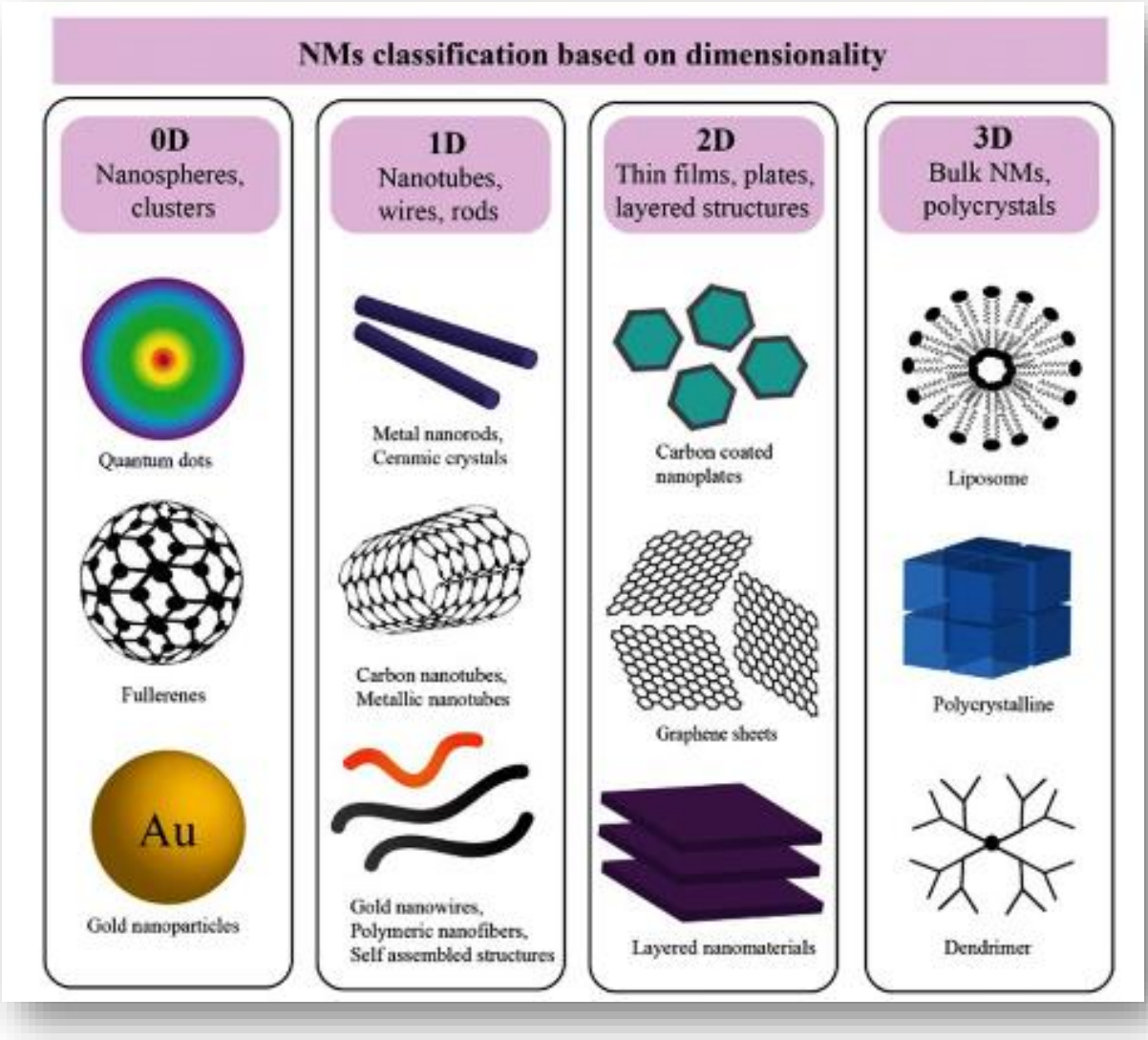


Figure 1.1 Classification of Nanoparticles (NPs) based on their dimensions (adopted from Poh et al., 2018).

1.2 Nanotechnology applications in agriculture

Modern Agriculture faces multiple challenges in its effort to provide sustainable production of food and at the same time avoid exhausting natural resources or polluting the environment. Recently, to address these challenges, significant technological advancements and innovations have been made. Among the proposed technologies, nanotechnology has the greatest potential to provide effective solutions to challenging agriculture-related problems. A significant amount of research, spanning at least a decade, has been undertaken on

nanotechnology applications in agriculture. Sustainable agricultural production demands certain inputs such as fertilizers and agrochemicals. Fertilizers play a pivotal role in increasing yield, although their excessive use or misuse alters the chemical ecology of soil and undermines soil fertility while reducing the available area for crop production. Nano-fertilizers increase the efficiency of agricultural inputs by facilitating site-targeted controlled delivery of nutrients, thereby ensuring optimal use of agri-inputs. Furthermore, the use of nanotechnology-assisted plant protection products has a great potential for increasing crop yield and reducing the environmental footprint of agrochemicals. Another major concern of sustainable agricultural production is the ability of plants to adapt to the accelerated climate change which causes extreme temperatures, water deficiency, salinity, alkalinity and environmental pollution, and `at the same time to preserve existing sensitive ecosystems. Additionally, the development of precision farming utilizing nanosensors, provides the means for monitoring crop growth, soil conditions, diseases, fate of agrochemicals and environmental pollution with obvious benefits for sustainable agriculture and the environment. Nanomaterial engineering is the cutting-edge track of research that supports the development of high-tech agricultural fields by offering a wider specific surface area crucial for the sustainable development of agriculture systems.Therefore, nanotechnology not only reduces the uncertainty, but can also provide alternative to conventional solutions to optimize agricultural production (Shang et al., 2019). A summary of nanotechnology applications in agriculture is shown in Figure 1.2.

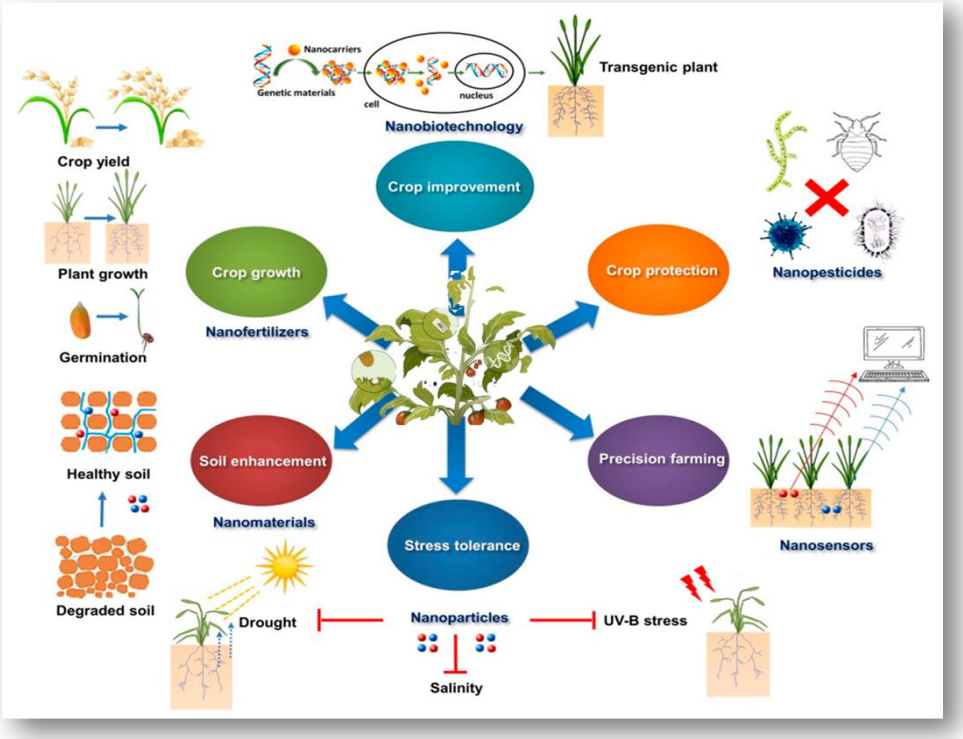


Figure 1.2 Applications of nanotechnology in agriculture (adopted from Shang et al., 2019).

1.2.1 Nanoparticles in Plant Protection

Plant pests and pathogens cause substantial decreases in crop production, with global losses estimated at 20%–40% per year. The current reliance on pesticides (including insecticides, fungicides, and herbicides) for pest management, has led to harmful side effects on non-target organisms, pest population resurgence, and resistance development, with an estimated 90% of pesticides being lost during application. As a result, there is a growing need to develop cost-efficient, high-performing and, at the same time, less harmful to the environment pesticides (Worrall et al.,2018). Nanotechnology has the potential to provide solutions for the above-mentioned pesticide-related challenges through the development of novel, highly effective nano-pesticides. Although nanotechnology has been substantially exploited in medicine, this is not the case for agricultural applications. Recently, the main applications of this technology include seed germination, nano-sensors, improved water management, target gene transfer, nano-barcoding, and delivery/controlled release of pesticides and hormones (Hayles et al., 2017).

Engineered nanoparticles with specific properties such as shape, pore size, and surface characteristics, can serve as protectants or targeted delivery agents. These nanoparticles can be used to encapsulate or conjugate active ingredients, such as pesticides, for precise and targeted delivery. The advancement of agricultural nanotechnology is expected to provide a new generation of pesticides and other active ingredients for the of plant diseases. Nanoparticles can be used to protect plants through two mechanisms: (a) directly, acting themselves as toxic agents, or (b) indirectly, acting as carriers for active ingredients such as conventional/green pesticides or novel biotechnological agents such as double-stranded RNA (dsRNA). These nanocarriers have several advantages, including enhanced shelf-life, improved solubility of water-insoluble pesticides, reduced toxicity to non-target organisms and farms, and increased targeted uptake by pests (Worrall et al.,2018). Additionally, nanocarriers can increase in the efficacy and resilience of the pesticides under adverse environmental conditions such as intense UV-irradiation and rain, reducing the number of applications and toxicity while reducing costs (Figure 1.3).

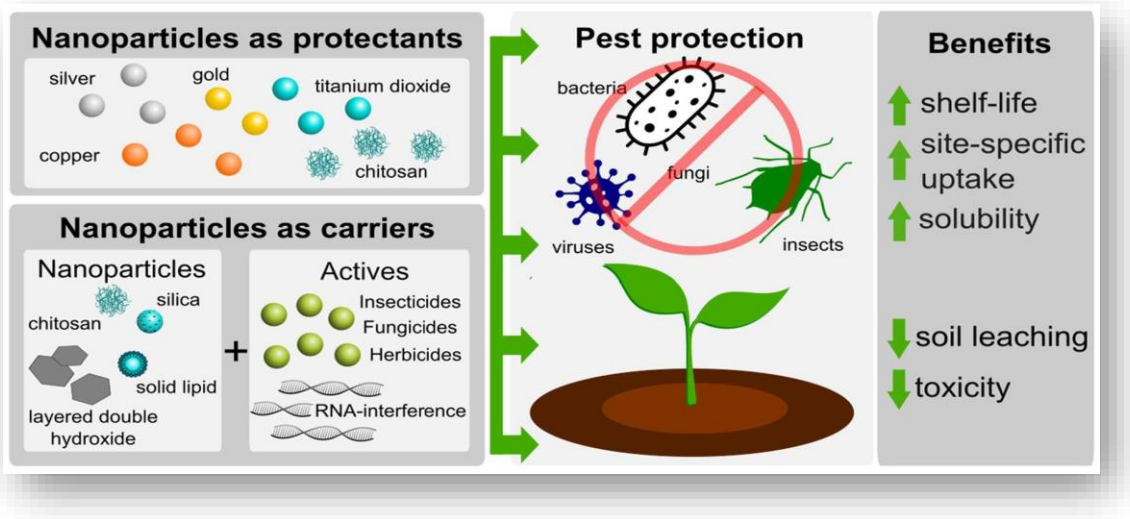


Figure 1.3 Nanomaterials in crop protection. Different nanomaterials used in plant protection as either A) protectants or B) carriers for active ingredients. C) Potential benefits of nanomaterial applications (adopted from Worrall et al.,2018).

1.2.2 Nanoparticles as Pesticide Alternatives

1.2.2.1 Nanoparticles used as protectant agents

Nanoparticles have demonstrated a high potential to be directly applied to plant seeds, foliage, or roots and to provide protection against pests and pathogens, such as insects, bacteria, fungi, and viruses. Metal nanoparticles such as silver, copper, zinc oxide, and titanium dioxide have been intensively researched for their antibacterial and antifungal properties and are also shown to have antiviral properties (Worrall et al., 2018).

Recently, silver nanoparticles have increased in popularity, due to "green synthesis" production in plants, bacteria, fungi, or yeast. Silver nanoparticles have shown antifungal properties against *Alternaria alternata, Sclerotinia sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, Botrytis cinerea, and Curvularia lunata* tested by well diffusion assays (Worrall et al., 2018). When silver nanoparticles were sprayed onto bean leaves, complete suppression of the sun-hemp rosette virus was observed. In another study, faba bean plants challenged with bean yellow mosaic virus produced remarkably better results when sprayed with silver nanoparticles 24 h post-infection, compared to spray applications before or simultaneously at the time of inoculation (Elbeshehy et al., 2015). Silver nanoparticles have shown immense potential for plant disease management against fungal and bacterial pathogens, but there are significant hurdles associated to them, such as production, toxicity, and soil interaction.

Similar reports exist on the effectiveness of other metal nanoparticles including copper, titanium dioxide, and gold against plant pests.

Cu and TiO_2 NPs are mainly utilized as fertilizers, whereas relevant research on plant disease management is limited. A number of studies have been conducted on the antimicrobial or insecticidal action of TiO_2, Ag, Al, and Cu nanoparticles. In some cases, the application of nanoparticles directly or indirectly via fertilizers has induced plant resistance against bacteria and viruses. For example, poly-dispersed gold NPs introduced through a mechanical abrasive was shown to confer plant resistance by deactivating the viral particles of Barley yellow mosaic virus (Worrall et al., 2018).

Another nanoparticle with a promising potential as a protective agent is Chitosan. It is characterized by numerous advantageous biological properties, including biocompatibility, antimicrobial activity, biodegradability, non-allergenicity, and low toxicity to non-target organisms including humans. Chitosan NPs have been shown to possess anti-viral properties against infections caused by plant pathogens including the alfalfa mosaic virus infecting, peanut, snuff, cucumber and potato plant tissues, Bean mild mosaic virus infecting beans, Tobacco mosaic virus, and Tobacco necrosis virus infecting tobacco plants (Worrall et al., 2018). They have also exhibited substantial antimicrobial properties and have been shown to be effective against tomato crown and root rots caused by *Fusarium* spp, gray mold caused by *B. cinerea*, and rice blast caused by *Phyricularia grisea* (Kashyap t al., 2015), and to a much lesser extent against bacterial plant pathogens. A number of potential mechanisms involved in the antimicrobial activity of chitosan have been proposed including, cell membrane disruption, inhibition of toxin production and H^+-ATPase activity, agglutination, inhibition messenger RNA and protein synthesis, and inhibition of microbial growth through blocking nutrient flow.

Besides their effectiveness against plant pathogens, chitosan NPs have been shown to be effective against plant pests such as the aphid *Aphis nerii*, pear psylla *Cacopsylla pyricola*, the leafworm *Spodoptera littoralis* and the root-knot nematode *Meloidogyne javanica* (Worrall et al., 2018). Overall, chitosan nanoparticles have immense potential as plant protectant agents, rivaling their use as nanocarriers for other active ingredients.

1.2.2.2 Nanoparticles as Pesticide Carriers

Another extremely significant application of nanoparticles is their use as carrier-molecules for entrapping, absorbing, encapsulating, or attaching other active molecules to produce agricultural formulations with enhanced properties. The most popular NPs used as nano-carriers for fungicides, herbicides, insecticides and RNAi-s, include silica, chitosan, solid lipid nanoparticles (SLN), and layered double hydroxides (LDH) nanoparticles.

Silica NPs are among the highly advantageous delivery vehicles because they can be easily synthesized with a controllable size, shape, or structure. The most widely studied silica nano-carriers are porous hollow silica nanoparticles (PHSNs) and mesoporous silica nanoparticles (MSNs). Typically, pesticides are loaded into the inner core of these NPs, providing protection against UV light degradation and a sustained release of the active ingredients. According to existing literature, silicon has been utilized as a means for enhancing plant tolerance against various abiotic and biotic stresses, making silica nanoparticles a promising candidate for use as a pesticide-carrier (Worrall et al., 2018).

Another promising drug-delivery molecule is chitosan. Typically, chitosan NPs are hydrophobic which leads to limited water-solubility, requiring the use of various organic or inorganic copolymers to address this issue. The reactive amine and hydroxyl groups it contains, provide great flexibility in terms of property improvement including ionic interactions, desired modifications, and graft reactions. A critical property of chitosan is its ability to adhere well to leaf and stem, enhancing the contact time and promoting bioactive molecule uptake.

SLNs consist of solid at room temperature lipids with emulsion-like properties. This enables them to form a matrix for entrapping lipophilic active ingredients without using organic solvents [28]. Furthermore, the solid matrix of SLNs limits the mobility of active ingredients enabling the controlled release of encapsulated lipophilic molecules. Stabilization of SLN during their dispersal in water is typically achieved using surfactants. Certain drawbacks of SLNs stem from their limited loading capacity and the instability of the structure leading to leakage of the a.i. during storage (Tamjidi et al., 2013).

LDHs are clays formed into hexagonal layered sheets trapping active molecules between their interlayer space. Under acidic conditions, e.g. under the presence of moisture and atmospheric carbon dioxide, LDH NPs break down releasing their biologically active content (Mitteret al., 2017). Specific LDH NPs, such as positively charged delaminated LDH lactate nanoparticles, can facilitate the uptake of biologically active substances across the plant cell wall.

1.2.2.2.1 Nanoparticles as Insecticide Carriers

Most insecticides have low water-solubility and require organic solvents to be solubilized, increasing costs and the toxicity of the formulation. Alternatively, nanoparticles can be used to address these solubility-toxicity issues. Currently, insecticides with limited water-solubility have been successfully conjugated with porous silica and modified chitosan nanoparticles (Worrall et al., 2018), although environmental safety issues regarding these NPs have not been investigated. Modified chitosan NPs loaded with the hydrophobic insecticide azadirachtin exhibited sustained drug release and a significant inhibition of cell proliferation in *S. litura* ovarian cell lines. An increased uptake of dendrimers loaded with thiamethoxam and a higher mortality to *H. armigera* larvae have been also reported (Worrall et al., 2018). This is of great significance considering that *H. armigera* is normally insensitive to thiamethoxam. A similar synergistic effect was observed when anacardic acid was incorporated into LDH NPs and directly sprayed onto *S. litura* or mustard leaves, leading to an increase in mortality compared to treatments with anacardic acid alone (Nguyen et al., 2015). The above studies showcase the potential advantages of NPs in improving the solubility of active molecules.

The loss of insecticide active ingredients after application due to volatilization or evaporation is another challenge for effective control of insects. Various substances such as essential oils, although effective against insects, are unstable and rapidly evaporate at high temperatures and increased air, light, and moisture conditions (Liu et al., 2001). *Artemisia arborescens* L. essential oil encapsulated in SLN NPs evaporated 35% less when sprayed to glass vials compared to the essential oil alone, in a time period of 48 h. In another case, polyethylene glycol (PEG) was used to encapsulate garlic essential oil and its effectiveness against the red flour beetle *Tribolium castaneum* was tested in rice post harvest. The resulting nanoparticle formulation caused an 80% mortality in contrast to 11% observed in the treatment consisting of the essential oil alone. In another study, pinene and linalool-encapsulated silica NPs resulted in a significant reduction in the feeding activity of *S. litura* fed on treated leaves ultimately causing insect mortality due to starvation (Yang et al., 2009).

Other desired properties of nano-insecticides include increased stability and controlled-sustained release, which can reduce insecticide doses and improve their safety. Field trials, using silica-encapsulated chlorfenapyr to treat *Brassica chinese* revealed that a similar or more effective control could be achieved against *Plutella xylostella* compared to the insecticide used alone (Song et al., 2012).

A drawback of the NP-induced slow release of pesticides is a potential reduction in the toxic action of the insecticide against its targets. So far, few studies have tested this hypothesis. For example, Kumar et al. (2016) reported a significant loss of cytotoxicity imidacloprid when loaded onto sodium alginate NPs, although this was not validated via statistical analysis. Toxicity tests using mouse fibroblast cell lines treated with botanical repellents encapsulated in zein-NPs, revealed a reduction in toxicity compared with the sole use of botanical repellents. Similarly, chitosan NPs loaded with carvacrol and linalool, exhibited a decreased toxicity to fibroblast cell lines tested. Furthermore, this decreased toxicity may be beneficial, especially in the case of non-target organisms. This was demonstrated in the case of cyhalothrin

encapsulated in hollow polymeric shell-NPs, which was less toxic to zebrafish compared to the unformulated cyhalothrin (Worrall et al., 2018). In any case, additional studies are required to confirm the above hypothesis of reduced insecticide toxicity caused by NP encapsulation.

1.2.2.2.2 Nanoparticles as Fungicide Carriers

Studies on the use of nano-fungicides emerged as early as 1997, mostly concerning ways to control wood diseases (Worrall et al., 2018). Since then, the number of studies on nanoparticles with antifungal action has increased exponentially (Ray et al., 2023), with the most studied nanoparticles used as fungicide carriers being silica, chitosan and polymeric mixtures. However, although many studies have evaluated the effectiveness of nano-fungicides against a wide range of fungal pathogens *in vitro*, efficacy of these formulations *in planta* and toxicity against non-target organisms have been poorly studied.

Similarly to insecticides, nanoparticle properties have been exploited for improving effectiveness, water-solubility, high volatilization, stability, and controlled release of fungicides. Several studies exist on the use of nanoparticles used for alleviating the limited water-solubility of fungicides (Worrall et al., 2018). Hatfaludi et al. (2004) used empty cell envelopes of *Pectobacterium cypripedii* as a nanosized bacterial vessel to improve leaf adherence and low water-solubility of tebuconazole. The authors tested six plants (rice, soya, cabbage, cotton, barley, and corn), sprayed with ghost-loaded tebuconazole or with two reference tebuconazole commercial fungicides (WP 25 and EW 250), against several plant pathogens. Ghost-loaded tebuconazole was equally or more effective than WP 25 but not to EW 250-treated. In another study, chitosan–lactide copolymer NPs loaded with pyraclostrobin, a fungicide with low water solubility, was applied at different concentrations. Although no significant differences were found at 3 to 5 days post-application, a significant increase in inhibition against the fungal target was recorded 7 days post-treatment, compared to pyraclostrobin alone (Xu et al., 2014). *In vitro* experiments of lecithin/chitosan loaded with kaempferol, demonstrated a 67% inhibition efficacy against *Fusarium oxysporum* incubated for 60 d (Worrall et al., 2018).

Nanoparticles loaded with fungicides can provide sustained delivery of the active substance and reduce the required doses and maintain the same effectiveness against plant pathogens. For example, tebuconazole loaded to NPs could provide the same level of control against decay requiring 1/10 of the recommended dose. Encapsulating chlorothalonil and tebuconazole using a surfactant-free method, resulted in stable NPs with small diameters capable of penetrating wood, and exhibited an excellent performance against wood decay of southern pine infected by *G. trabeum* and birch infected by *T. versicolor* (Worrall et al., 2018).

A major drawback of essential oils with antimicrobial action is their rapid evaporation which makes them unsuitable for use in the field. In a recent study, 5 essential oils were successfully encapsulated into MSN and exhibited superior antifungal activity against *Aspergillus niger* 14 d post-infection, compared to the original essential oil formulations. This was also demonstrated by the increased effectiveness against six fungi exhibited by SLN-stabilized *Zataria multiflora* essential oil (Worrall et al., 2018).

One of the major pesticide environmental challenges is leaching, which causes the translocation of pesticides through the soil to water bodies and eventually polluting aquifers. MSN-loaded metalaxyl observed 76% leaching in soil of a conventional formulation of metalaxyl compared to 11.5% exhibited by encapsulated metalaxyl over a 30-d period. This release rate of the encapsulated metalaxyl increased when tested in water but was still lower than that of conventional formulation (Wanyika, 2013). Similarly, carbendazim and tebuconazole were loaded into solid lipid and polymeric NPs, and their leaching rates were tested in comparison with conventional fungicides containing these active ingredients. Results revealed that the encapsulation of these active ingredients with nanoparticles decreased their release rate in soil compared to the commercial formulations (Campos et al., 2015).

Slow release of the active ingredients of fungicides is a desirable property when protection is needed for longer periods such as in the case of soil pathogens or pathogens with multiple generations over a cultivation period. Such a prolonged release was achieved using validamycin loaded to nanosized calcium carbonate. Validamycin-loaded NPs showed less effectiveness against *Rhizoctonia solani* in the short-term, but two weeks later, the surpassed the effectiveness of validamycin applied alone.

The effectiveness of certain fungicides has been shown to increase when used in combination or loaded to NPs. For example, carbendazim-loaded polymeric nanoparticles achieved an increased inhibition rate against *Fusarium oxysporum* and *Aspergillus parasiticus* compared to a commercial fungicide containing only carbendazim (Worrall et al., 2018).

Several studies have investigated the use of nano-carriers to enhance the safety of fungicides in terms of phytotoxicity. Nano-formulated carbendazim was safer in terms of root growth and seed germination in *Zea mays*, *Lycopersicum esculentum* and *Cucumis sativa* compared to the fungicide used alone. The uptake of pyrimethanil-loaded MSNs in cucumber plants was studied over 48 days using HPLC-TM, revealing that the risk of pyrimethanil-loaded MSN accumulation in edible parts of the plant is minimal (Worrall et al., 2018).

1.2.2.3 Nanoparticles as RNAi Carriers

The RNA interference (RNAi) pathway was recently discovered and is expected to revolutionize pests and pathogen management in plants. RNAi is a conserved mechanism in eukaryotes that plays a crucial role in growth, development, and host defense against viruses and transposons. This mechanism can be exploited to target insects, fungi, viruses, and weeds. (Worrall et al., 2018). In plants, this pathway is triggered by double-stranded RNA (dsRNA), which is subsequently sliced and converted by dicer-like (DCL) enzymes to small-interfering RNA (siRNA). siRNAs are then introduced into an RNA-induced silencing complex, which uses base pairing to direct the degradation of the target pathogen's RNA, preventing it from being used as a translation template. The exploitation of the RNAi mechanism has been used as a powerful method for combating plant pests and pathogens through genetic modification (Worrall et al., 2018). Because most countries are skeptical of or against the use of genetically modified organisms, research has focused on new methods such as the topical application of dsRNA. Although appealing, this method faces similar challenges to those of conventional pesticides including environmental degradation and limited uptake by the plant and the target

organism. This fact makes nanoparticles promising vessels to be used for shielding dsRNAs from degradation and enhancing their efficiency to be delivered in their target sites.

Nanoparticles have been used as RNAi-inducing molecules against viruses, aphids and mosquitoes. LDH nanoparticles loaded with dsRNA (BioClay) provided resistance to CMV and PMMoV viruses when sprayed on cucumber and pepper plants (Mitter et al., 2017). A single spray of BioClay was sufficient to protect plants for 20 days after application both on sprayed and newly emerged-unsprayed leaves, while naked dsRNA failed to protect plants against viral infection.

The first step in achieving effective protection against plant infecting insects was the development of a perfluorocarbon-siRNA nanotechnology used against the Acyrthosiphon *pisum, Aphis glycines, and Schizaphis graminum* aphid species. Results revealed that NPs significantly enhanced the gene knockdown effect of siRNA compared to free siRNA. Several studies have focused on using this technology against human health-threatening mosquitoes. For example, dsRNA-loaded chitosan NPs were used against the malaria and dengue and yellow fever vectors *Anopheles gambiae* and *Aedes aegypti*, respectively (Worrall et al., 2018). In another study, carbon quantum dots, silica, and chitosan nanoparticles were used as carriers of dsRNA against the larvae of *A. aegypti*. This study revealed that polyethyleneimine-coated carbon quantum dots were the most efficient for dsRNA delivery and mosquito control. Although promising for crop protection, exogenous RNAi-inducing molecule application, further studies are needed to resolve the challenges topical application of RNAi faces before being used in a wider scale (Worrall et al., 2018).

1.2.3 Environmental fate of Nanopesticides

Despite their advantages and great potential as pesticide alternatives, nanopesticides can pose a threat to the environment and non-target organisms. Compared with conventional pesticides containing the same active ingredients, nanopesticides may differ significantly in terms of toxicity and environmental fate, a fact that merits additional attention and caution (Ding et al., 2023).

Nanoparticle encapsulation of active ingredients often results in enhanced activity and bio-efficacy partly due to their small size and thus greater potential to enter non-target organisms. This can lead to enhanced ecotoxic effects to non-target organisms. It has been demonstrated is several cases that nanopesticides can be up to an order of magnitude more toxic than conventional formulations and that they can be harmful or kill aquatic and other non-target organisms. Furthermore, they can undergo several transformations during their "aging process" in soil making them more toxic to non-target organisms. For example, Ag-NPs treated with environmental soil resulted in a greater toxicity to earthworms was greater in soil samples containing compared untreated Ag-NPs, indicating an additive role of organic matter affecting their toxicity (Ding et al.,2023). Organic matter has been shown to modify NP properties by forming surface coatings or being adsorbed onto their surface, significantly impacting their toxicity and bioavailability. Overall, it can be stated that mechanisms and processes determining the environmental fate and impact of nanopesticides on non-target organisms may differ from those of conventional pesticides, necessitating improved or novel risk assessment

methods to address these differences. This would require the introduction of regulatory risk assessment systems which considers all components of the nano-pesticide instead of not solely the individual pesticide active ingredients.

1.2.3.1 Environmental fate and toxicity in soil

An integral part of nanopesticide risk assessment concerns the fate and behavior of nanoparticles in the soil, which is the major recipient of pesticides (Figure 1.4). Parameters such as bioavailability, mobility, transformation and degradation of nanoparticles are crucial and determine their fate in the soil (Taverna et al., 2018). Specifically, the behavior of nanopesticides in the soil is governed by a series of complex processes, such as runoff and leaching, adsorption, desorption, absorption by plants, volatilization, chemical and biological degradation. The interaction between nanomaterials and various environmental media can change particle properties, such as surface binding with organic matter such as humic acids. It has been shown that the exposure of nanoparticles to organic matter can alter their bioavailability and toxicity (Coutris et al., 2012). Mobility is another nanopesticide property affected by this type of formulation. For example, bifenthrin, remains relatively immobile in soil and is considered relatively safe in terms of groundwater contamination. However, bifenthrin nano-formulations have been shown to possess enhanced soil mobility facilitated by the colloidal nature of the nano-carrier molecules used for its formulation. Additionally, studies have demonstrated that soil sorption capacity of nanopesticides as well as their toxicity to non-target organisms can be altered. Nanopesticides may interact with soil organic matter and affect microbial communities. This has been demonstrated in the case of CuO NPs, which accelerated organic matter mineralization and the reduction of iron when applied in concentrations of 1000 mg/kg. Although organic matter could partially mitigate the toxicity of the above NPs, soil microbial biomass was severely reduced by CuO NPs. Several other studies have reported harmful effects of carbon or metallic NPs on soil micro- and macro-organisms such as soil fauna and beneficial microorganisms, as well as and plants (Ding et al., 2023). For example, carbon NPs have been shown to significantly affect bacterial community composition in various soil types. Furthermore, once in soil, transformation, degradation or nanoparticle-mediated slow release of active ingredients could significantly affect their bioavailability, bioaccumulation, and potential toxicity to non-target organisms. In any case, the expected increased use of nanopesticides in agriculture could lead to an unprecedented increase in nanoparticles entering the soil, making nanopesticide risk assessment a necessity.

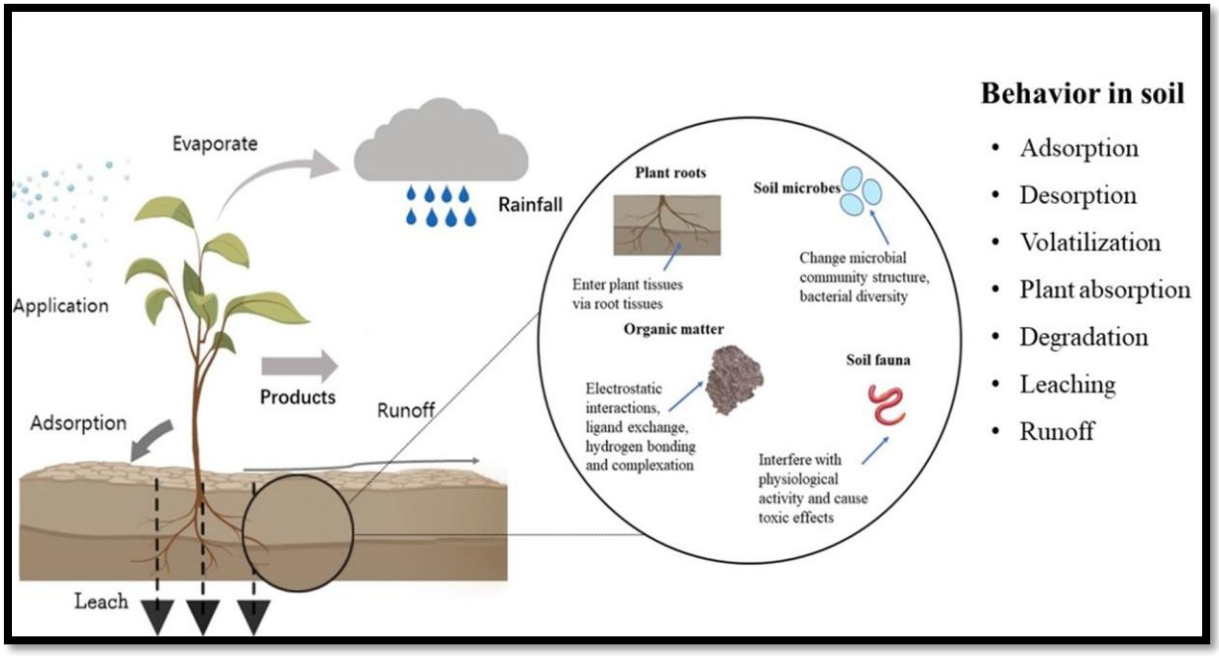


Figure 1.4 Environmental behavior of nanopesticides (adopted from Ding et al., 2023).

1.2.3.2 Environmental fate and toxicity in air and water

The use of nanopesticides in the soil environment may reduce the risk of pesticide residues compared to traditional pesticides. However, nanopesticides are more likely to evaporate and diffuse into the atmosphere, where they can then be carried by rainfall into the broader biosphere. As a result, the potential harm of nanopesticides to air quality and biological health should not be underestimated. In addition to volatilization, the direct release of nanopesticide particles into the air may also be an important exposure pathway. Furthermore, undegradable nanopesticides that flow into water bodies can pose a significant burden on aquatic environments, potentially harming not just targeted pests but also beneficial and non-target aquatic organisms, thereby impacting the overall health of aquatic ecosystems.

1.2.3.3 Environmental fate and toxicity in organisms

Nanoparticles pose significant toxicological risks to all animals, especially mammals, due to their unique biological properties and high capacity for distribution and bioaccumulation in soil, water, and the food chain. Nanomaterial formulations tend to persist for longer in plants, soil, and water compared to their conventional counterparts, and can inadvertently migrate into various food and agricultural products (Ding et al., 2023). This increases the likelihood that hazardous substances from these nanomaterials will enter the body through the food chain. Furthermore, nanopesticides can more easily penetrate cell walls and membranes compared to conventional pesticides, allowing them greater access to both targeted and non-targeted organisms due to their small size.

1.2.3.4 Effects of NPs on plants

1.2.3.4.1 Effect of NPs on plant physiological indices, hormones and crop quality

The toxic effects of nanoparticles (NPs) on plants can be assessed through various physiological indices, including germination percentage, root elongation, biomass, and leaf number. NPs can have substantial negative impacts on plant growth and development, such as reduced seed germination, suppressed plant elongation, and even plant death. Several previous studies have examined the inhibitory effects of different types of NPs, including multi-walled carbon nanotubes, single-wall carbon nanotubes, zinc oxide NPs, silver NPs, and iron NPs, on various plant species like soybean, maize, wheat, ryegrass, and barley (Yang et al. 2017). These studies have found that NP exposure can affect multiple aspects of plant growth, including seed germination, shoot length, biomass, and gene expression. Growth inhibition has also been observed in *Bacillus thuringiensis* (Bt)-transgenic cotton exposed to silica NPs, as well as in wheat plants grown in a sand matrix with copper oxide NPs, which led to changes in root structure. Additionally, studies have shown that copper oxide NPs can significantly reduce the fresh weights, root lengths, germination rates, and biomass of Arabidopsis seedlings and rice seeds.

Several researchers discovered that rare earth oxide nanoparticles (CeO_2, La_2O_3, Gd_2O_3 and Yb_2O_3) significantly inhibited plant growth in radish, tomato, rape, lettuce, wheat, cabbage, cucumber, and corn when applied to roots at high concentrations. Conversely, TiO_2 nanoparticles enhanced the content of total chlorophyll and catalase (CAT) while decreasing ascorbate peroxidase (APX) levels in leaves, as reported by Servin et al. (2013).

Plant hormones are biologically active organic compounds produced through plant metabolism that regulate physiological responses during plant growth and mediate responses to various challenges. The content and activity of these plant hormones are considered an important indicator of toxicity in plants. Research has shown that exposure to certain nanoparticles can significantly influence the production of plant hormones. For example, studies have found that cerium oxide nanoparticles had no significant effect on indole-3-acetic acid (IAA), abscisic acid (ABA), and gibberellic acid (GA) in the leaves of Bt-transgenic and conventional cotton but did decrease the content of trans-zeatin-riboside (t-ZR) in conventional cotton. Similarly, γFe_2O_3were found to increase IAA and ABA content in the roots of both transgenic and non-transgenic rice, while carbon nanotubes decreased phytohormone concentrations in rice seedlings. Additionally, silver ions were observed to inhibit the production of the plant hormone ethylene, which would substantially weaken the interaction between IAA and ethylene. These findings highlight the importance of understanding how various nanoparticles can impact the production and regulation of critical plant hormones (Yang et al., 2017).

Previous research on hydroponically grown plants has shown that the accumulation of NPs in the environment can significantly impact the quality and yield of soil-based food crops. For example, studies have found that the protein content of plants was less sensitive to stimulation by AgNPs compared to carbohydrates, with protein only increasing at high AgNP concentrations. Other work has demonstrated that zinc oxide ZnONPs can increase the starch

and protein content of cucumbers while decreasing their micronutrient (copper and molybdenum) levels. Similarly, cerium oxide (CeO_2) NPs were found to reduce the iron, sulfur, prolamin, glutelin, lauric acid, valeric acid, and starch content of rice, as well as weaken its antioxidant properties. Additionally, CeO_2NPs were shown to alter the amino acid, fatty acid, non-reducing sugar, and phenolic content of plants. Researchers have concluded that the main factors influencing the effects of NPs on plants include the dose and inherent nanomaterial characteristics (size, shape, and stability), the plant seed (size and species), the growth medium, the plant growth stage, and the NP capping material (Yang et al. 2017).

1.2.3.4.2 NPs-induced phytotoxicity mechanisms

Nanoparticles can cause a variety of adverse effects on plants at the cellular level including damage on the cell membrane, chromosomal aberration and disruption of chlorophyll and carotenoid synthesis, and at the physiological level leading to biomass reduction and inhibition of root length among others (Figure 1.2.3.1).

Reactive oxygen species (ROS) production is a major phytotoxicity-causing mechanism in plants. ROS production includes both free radicals like hydroxyl and superoxide radicals, as well as non-radical molecules such as singlet oxygen and hydrogen peroxide (Gill and Tuteja, 2010). In plants, ROS is a product of aerobic metabolism and function as signaling molecules. However, excessive ROS can lead to oxidative stress, which occurs when the ROS level exceeds the plant's defense mechanisms. This can result in DNA damage, protein oxidation, electrolyte leakage, lipid peroxidation, and membrane damage, ultimately causing cell death (Yang et al., 2017). Studies on metal and metal-based nanoparticle (NP) phytotoxicity suggest that NPs can induce oxidative stress in various plant species. For instance, ZnO-NPs have been shown to cause particle-dependent ROS formation and lipid peroxidation on cellular membranes, while Ag NPs have been linked to ROS generation, which correlates with a decrease in viable cells.

Recent studies have used the ROS-sensitive dye DAB to detect H_2O_2 accumulation in plant roots treated with CeO_2 and La_2O_3 NPs. The results clearly showed the formation of a visible deep brown product, indicating H_2O_2 presence under NP treatment. Exposure to 800 mg/kg CeO_2 NPs increased H_2O_2 levels 10-fold (35 μM) compared to controls, despite no lipid peroxidation or ion leakage, suggesting maintained membrane integrity (Zhao et al. 2012). However, H_2O_2 can be converted to highly reactive •OH, which causes cellular damage and cannot be enzymatically detoxified (Ma, White, et al. 2015). •OH is the most reactive ROS due to its unpaired electron, allowing it to interact with and damage various biomolecules, ultimately leading to cell death (Freinbichler et al. 2011).

Oxidative stress and protein damage are key mechanisms by which nanoparticles can exert phytotoxic effects on plants. Reactive oxygen species levels can directly impair normal cellular functioning and also exacerbate oxidative stress through the production of lipid-derived radicals. This lipid peroxidation, indicated by increased malondialdehyde (MDA) levels, can damage cell membranes. Oxidative stress can also induce covalent modifications to proteins, such as fragmentation, aggregation, and increased susceptibility to proteolysis. Proteins containing sulfur and thiol groups are particularly vulnerable to ROS-induced damage. Studies have shown that exposure to metal-based nanoparticles, such as silver, can lead to altered abundance of proteins involved in various physiological and biochemical processes, including oxidative stress response, cell division, and apoptosis.

Reactive oxygen species (ROS) can lead to oxidative damage in the nuclear, chloroplastic, and mitochondrial DNA of cells. DNA is the genetic material of cells, and damage to DNA can cause changes in the encoded proteins, leading to malfunctions or inactivation. Metal and carbon NPs have been shown to induce DNA degradation in plants. For example, high concentrations of cerium oxide NPs have been shown to have negative effects on the DNA of soybean plants, while bismuth oxide NPs caused chromosomal aberrations and increased mitotic index in onion roots. Genotoxic effects of titanium dioxide NPs such as micronuclei formation, chromosome damage, and DNA shearing have been reported in various plant species. In some cases, however, NP-driven increased ROS resulted in a plant growth promoting effect. For example, AgNPs exposure of castor bean seeds, enhanced antioxidant defense mechanisms, including increased peroxidase and superoxide dismutase activity, as well as increased phenolic acid synthesis, which promoted root elongation in a dose-dependent manner (Yasur and Rani, 2013). These findings suggest that the effects of NPs on plants can be complex, with both negative and potentially beneficial outcomes depending on the specific nanoparticle and plant species.

Nanoparticles (NPs) can induce the generation of reactive oxygen species (ROS) either directly or indirectly, which plays a crucial role in the phytotoxicity mechanism. The production of ROS is influenced by the physicochemical properties of NPs as well as the species in study. Various factors, such as size, shape, solubility, particle dissolution, ion release, biotransformation of NPs, and light exposure, can contribute to ROS generation and phytotoxicity. A study by Zhang et al. (2015) compared the toxicity of three types of CeO_2 NPs to different species of the Lactuca genus. Compared to the control, 7 nm CeO_2 caused a significant increase in the malondialdehyde (MDA) level, indicating membrane damage in root cells. However, there was no obvious difference in the MDA levels among the control, 25 nm CeO_2, and its bulk counterpart treatments. Other reports have also studied the effects of size and shape of NPs on various plant species demonstrating that plant responses to NP exposure may vary with particle sizes and plant growth stages.

The phytotoxicity of MNPs has been investigated in several studies. While metal ion release plays a role, direct NP interactions with plant tissues appear to be a key mechanism of phytotoxicity for these NPs. For example, Ag-NPs were more toxic to Arabidopsis in terms of root elongation compared to silver ions, probably due to a disruption of the oxidation-antioxidant balance and small molecule homeostasis (Qian et al. 2013), while phytotoxic activity of ZnONPs was attributed to its reactive oxygen-promoting photocatalytic activity (Ma et al. 2013). Various factors may affect the phytotoxic activity of NPs including their size, morphology, composition, concentration, and surface. Therefore, advanced molecular approaches such as proteomics and genomics must be utilized to enhance our understanding on the toxicity mechanisms of NPs.

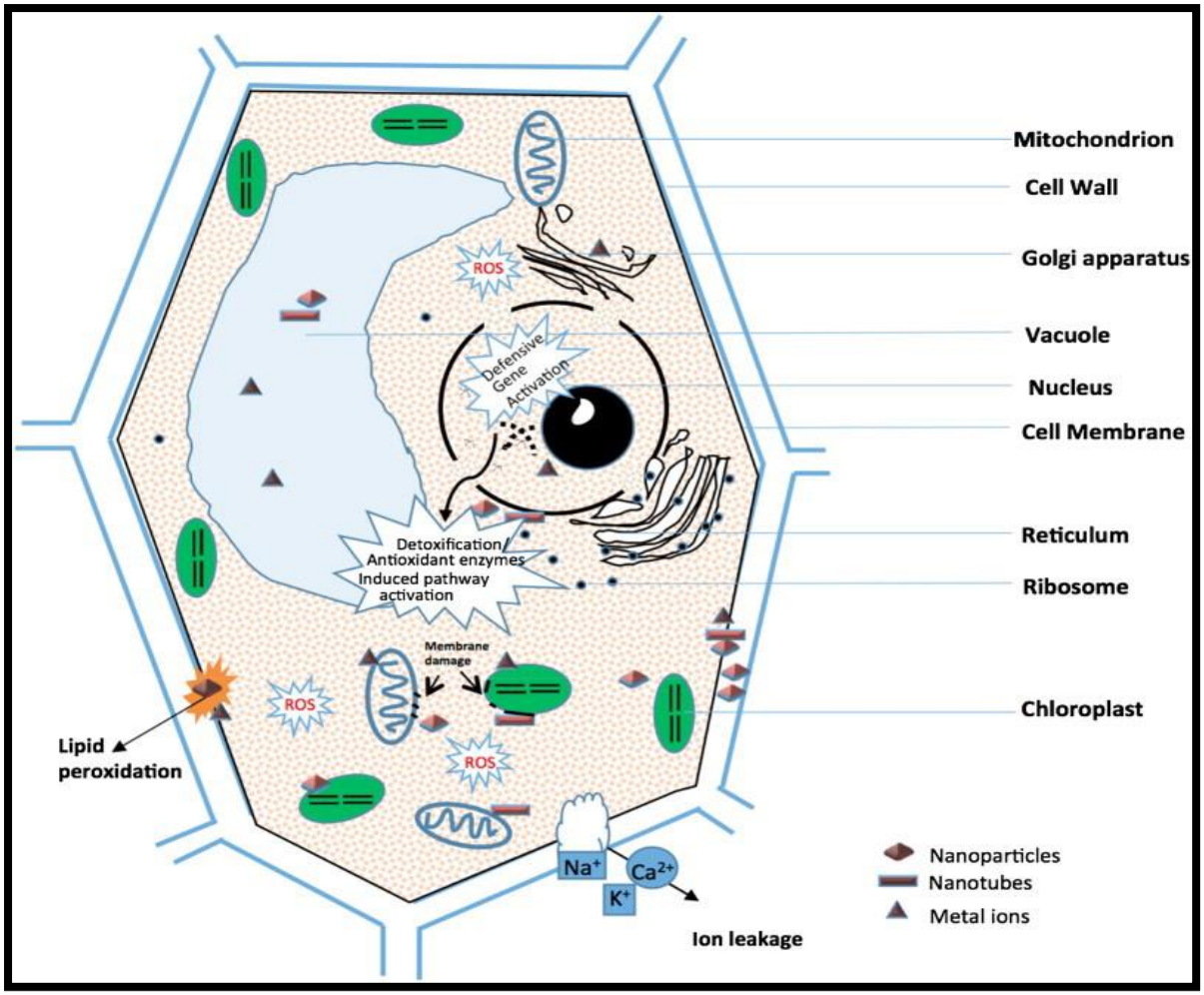


Figure 1.5 Potential adverse effects of NPs at the cellular level, and consequent detoxification pathways triggered as a response (adopted from Yang et al.,2017).

1.2.4 References

Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner MR. 2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nat Nanotechnol. 4:634–641.

Batley GE, Kirby JK, McLaughlin MJ. 2013. Fate and risks of nanomaterials in aquatic and terrestrial environments. Acc Chem Res. 46:854–862. Bleecker AB, Kende H. 2000. Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol. 16:1–18.

Campos, E.V.R.; De Oliveira, J.L.; Da Silva, C.M.G.; Pascoli, M.; Pasquoto, T.; Lima, R.; Abhilash, P.;Fraceto, L.F. Polymeric and solid lipid nanoparticles for sustained release of carbendazim and tebuconazole in agricultural applications. Sci. Rep. **2015**, 5, 13809.

Chen G, Ma C, Mukherjee A, Musante C, Zhang J, White JC, Dhankher OP, Xing B. 2016. Tannic acid alleviates bulk and nanoparticle Nd 2 O3toxicity in pumpkin: a physiological and molecular response.Nanotoxicology,10:1243–1253.

Cheng Y, Song C. 2006. Hydrogen peroxide homeostasis and signaling in plant cells. Sci China. Ser C Life Sci/Chinese Acad Sci. 49:1–11.

Coutris C, Hertel-Aas T, Lapied E, Joner EJ, Oughton DH (2012) Bioavailability of cobalt and silver nanoparticles to the earthworm *Eisenia fetida*. Nanotoxicology 2:186–195. https://doi.org/10.3109/17435390.2011.569094.

Cui D, Zhang P, Ma Y, He X, Li Y, Zhang J, Zhao Y, Zhang Z. 2014. Effect of cerium oxide nanoparticles on asparagus lettuce cultured in an agar medium. Environ Sci Nano.1:459–465.

Demir E, Kaya N, Kaya B. 2014. Genotoxic effects of zinc oxide and titanium dioxide nanoparticles on root meristem cells of *Allium cepa* by comet assay. Turk J Biol. 38:31–39.

Dimkpa CO, McLean JE, Latta DE, Manangón E, Britt DW, JohnsonWP, Boyanov MI, Anderson AJ. 2012. Cuo and ZnO nanoparticles: phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. J Nanopart Res. 14:1–15.

Dimkpa CO, McLean JE, Martineau N, Britt DW, Haverkamp R,Anderson AJ. 2013. Silver nanoparticles disrupt wheat (Triticum aestivum L.) growth in a sand matrix. Environ Sci Technol. 47:1082–1090.

Ding, Y., Wang, Q., Zhu, G. *et al.* Application and perspectives of nanopesticides in agriculture. *J Nanopart Res* **25**, 159 (2023). https://doi.org/10.1007/s11051-023-05811-5

Diplock AT, Machlin L, Packer L, Pryor W. 1989. Vitamin E: biochemistry and health implications. New York: The New York Academy of Sciences.

El-Temsah YS, Joner EJ. 2012. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. Environ Toxicol. 27:42–49.

Elbeshehy, E.K.F.; Elazzazy, A.M.; Aggelis, G. Silver nanoparticles synthesis mediated by new isolates of Bacillus spp., nanoparticle characterization and their activity against Bean Yellow Mosaic Virus and human pathogens. *Front. Microbiol.* **2015**, *6*, 453.

Faisal M, Saquib Q, Alatar AA, Al-Khedhairy AA, Hegazy AK, Musarrat J. 2013. Phytotoxic hazards of NiO-nanoparticles in tomato: a study on mechanism of cell death. J Hazard Mater. 250–251:318–332.

Feizi H, Kamali M, Jafari L, Moghaddam PR. 2013. Phytotoxicity and stimulatory impacts of nanosized and bulk titanium dioxide on fennel (*Foeniculum vulgare* Mill). Chemosphere. 91:506–511.

Foyer CH, Halliwell B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta. 133:21–25.

Foyer CH, Lopez-Delgado H, Dat JF, Scott IM. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stresstolerance and signaling. Physiol Plant. 100:241–254.

Foyer CH, Noctor G. 2003. Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant. 119:355–364.

Freinbichler W, Colivicchi MA, Stefanini C, Bianchi L, Ballini C, MisiniB, Weinberger P, Linert W, Varešlija D, Tipton KF, Corte LD. 2011.Highly reactive oxygen species: detection, formation, and possible functions. Cell Mol Life Sci. 68:2067–2079.

Fridovich I. 1989. Superoxide dismutases. An adaptation to a paramagnetic gas. J Biol Chem. 264:7761–7764.

Garg N, Manchanda G. 2009. ROS generation in plants: boon or bane? Plant Biosyst. 143: 81–96.Ghisla S, Massey V. 1989. Mechanisms of flavoprotein-catalyzed reactions. Eur J Biochem. 181:1–17.

Ghosh M, Bandyopadhyay M, Mukherjee A. 2010. Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere. 81:1253–1262.

Ghosh M, Bhadra S, Adegoke A, Bandyopadhyay M, Mukherjee A. 2015. MWCNT uptake in Allium cepa root cells induces cytotoxic and genotoxic responses and results in DNA hyper-methylation. Mutat Res/ Fundam Mol Mech Mutagen. 774:49–58.

Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 48:909–930.

Gottschalk F, Lassen C, Kjoelholt J, Christensen F, Nowack B. 2015.Modeling flows and concentrations of nine engineered nanomaterialsin the Danish environment. Int J Environ Res Public Health. 12:5581–5602.

Hatfaludi, T.; Liska, M.; Zellinger, D.; Ousman, J.P.; Szostak, M.; Jalava, K.; Lubitz, W. Bacterial ghost technology for pesticide delivery. J. Agric. Food Chem. **2004**, 52, 5627–5634.

Hayles, J.; Johnson, L.; Worthley, C.; Losic, D. Nanopesticides: A review of current research and perspectives. *New Pestic. Soil Sens.* **2017**, 193–225.

Kashyap, P.L.; Xiang, X.; Heiden, P. Chitosan nanoparticle based delivery systems for sustainable agriculture. *Int. J. Boil. Macromol.* **2015**, *77*, 36–51.

Khan FA. Nanomaterials: types, classifications, and sources. In: Khan FA, Khan F, editors. Applications of nanomaterials in human health. Singapore: Springer; 2020. p. 1–13.

Kumar N, Kumbhat S. Carbon-based nanomaterials. Chapter 5 in Essentials in Nanoscience and Nanotechnology". 2016:189–236.

Kumar, D.R.; Kumar, P.S.; Gandhi, M.R.; Al-Dhabi, N.A.; Paulraj, M.G.; Ignacimuthu, S. Delivery of chitosan/dsRNA nanoparticles for silencing of wing development vestigial (vg) gene in Aedes aegypti mosquitoes. Int. J. Boil. Macromol. **2016**, 86, 89–95.

Liu, Y.; Yan, L.; Heiden, P.; Laks, P. Use of nanoparticles for controlled release of biocides in solid wood.J. Appl. Polym. Sci. 2001, 79, 458–465.

Ma H, Williams PL, Diamond SA. 2013. Ecotoxicity of manufactured ZnO nanoparticles – a review. Environ Pollut. 172:76–85.

Mitter, N.; Worrall, E.A.; Robinson, K.E.; Li, P.; Jain, R.G.; Taochy, C.; Fletcher, S.J.; Carroll, B.J.; Lu, G.; Xu, Z.P. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nat. Plants 2017, 3, 16207.

Nguyen, T.N.Q.; Le, V.A.; Hua, Q.C.; Nguyen, T.T. Enhancing Insecticide Activity of Anacardic Acid by Intercalating it into MgAl Layered Double Hydroxides Nanoparticles; Institut für Abfallwirtschaft und Altlasten, Fakultät Umweltwissenschaften, Technische Universität Dresden: Dresden, Germany, 2015.

Poh TY, et al. Inhaled nanomaterials and the respiratory microbiome: clinical, immunological and toxicological perspectives. Part Fibre Toxicol. 2018;15:1–16.

Qian H, Peng X, Han X, Ren J, Sun L, Fu Z. 2013. Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. J Environ Sci. 25:1947–1956.

Rauscher H, et al. Identification of nanomaterials through measurements. Publications Office of the European Union. 2019.

Ray M.K., Mishra A.K., Mohanta Y.K., Mahanta S., Chakrabartty I., Kungwani N.A., Avula S.K., Panda J., Pudake R.N. Nanotechnology as a Promising Tool against Phytopathogens: A Futuristic Approach to Agriculture. (2023) Agriculture (Switzerland), 13 (9), art. no. 1856. DOI: 10.3390/agriculture13091856

Servin AD, Morales MI, Castillo-Michel H, Hernandez-Viezcas JA, Munoz B, Zhao L, Nunez JE, Peralta-Videa JR, Gardea-Torresdey JL. 2013. Synchrotron verification of TiO2 accumulation in cucumber fruit: a possible pathway of TiO2 nanoparticle transfer from soil into the food chain. Environ Sci Technol. 47:11592–11598.

Shang, Y.; Hasan, M.K.; Ahammed, G.J.; Li, M.; Yin, H.; Zhou, J. Applications of Nanotechnology in Plant Growth and Crop Protection: A Review. *Molecules* **2019**, *24*, 2558. https://doi.org/10.3390/molecules24142558

Song, M.-R.; Cui, S.-M.; Gao, F.; Liu, Y.-R.; Fan, C.-L.; Lei, T.-Q.; Liu, D.-C. Dispersible silica nanoparticles as carrier for enhanced bioactivity of chlorfenapyr. J. Pestic. Sci. **2012**, 37, 258–260.

Tamjidi, F.; Shahedi, M.; Varshosaz, J.; Nasirpour, A. Nanostructured lipid carriers (NLC): A potential delivery system for bioactive food molecules. *Innov. Food Sci. Emerg. Technol.* **2013**, *19*, 29–43.

Taverna ME, Busatto CA, Lescano MR, Nicolau VV, Zalazar CS, Meira GR, Estenoz DA (2018) Microparticles based on ionic and organosolv lignins for the controlled release of atrazine. J Hazard Mater 359:139–147. https://doi.org/10.1016/j.jhazmat.2018.07.010.

Wanyika, H. Sustained release of fungicide metalaxyl by mesoporous silica nanospheres. J. Nanopart. Res. **2013**, 15, 1831.

Worrall, E.A.; Hamid, A.; Mody, K.T.; Mitter, N.; Pappu, H.R. Nanotechnology for Plant Disease Management. *Agronomy* **2018**, *8*, 285. https://doi.org/10.3390/agronomy8120285)

Xu, L.; Cao, L.-D.; Li, F.-M.;Wang, X.-J.; Huang, Q.-L. Utilization of chitosan-lactide copolymer nanoparticles as controlled release pesticide carrier for pyraclostrobin against Colletotrichum gossypii Southw. J. Dispers. Sci. Technol. **2014**, 35, 544–550.

Yang J, Cao W & Rui Y (2017) Interactions between nanoparticles and plants: phytotoxicity and defense mechanisms, Journal of Plant Interactions, 12:1, 158-169, DOI: 10.1080/17429145.2017.1310944

Yang, F.-L.; Li, X.-G.; Zhu, F.; Lei, C.-L. Structural characterization of nanoparticles loaded with garlic essential oil and their insecticidal activity against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). J. Agric. Food Chem. 2009, 57, 10156–10162.

Yasur J, Rani PU. 2013. Environmental effects of nanosilver: impact on castor seed germination, seedling growth, and plant physiology. Environ Sci Pollut Res. 20:8636–8648. doi: 10.1007/s11356-013-1798-3.

Zhao L, Peng B, Hernandez-Viezcas JA, Rico C, Sun Y, Peralta-Videa JR, Tang X, Niu G, Jin L, Varela-Ramirez A, et al. 2012. Stress response and tolerance of *Zea mays* to CeO_2 nanoparticles: cross talk among H_2O_2, heat shock protein, and lipid peroxidation. ACS Nano. 6:9615–9622. doi: 10.1021/nn302975u.

Zhang P, Ma Y, Zhang Z, He X, Zhang J, Guo Z, Tai R, Zhao Y, Chai Z. 2012. Biotransformation of ceria nanoparticles in cucumber plants. ACS Nano. 6:9943–9950. doi: 10.1021/nn303543n.

Zhang P, Ma Y, Zhang Z, He X, Li Y, Zhang J, Zheng L, Zhao Y. 2015. Species-specific toxicity of ceria nanoparticles to Lactuca plants. Nanotoxicology. 9:1–8. doi: 10.3109/17435390.2013.855829.

1.3 Metal Nanoparticles against fungicide resistance: alternatives or partners?

Abstract

Chemical control suffers from the loss of available conventional active ingredients due to strict environmental safety regulations which, combined with the loss of fungicide efficacy due to resistance development, constitute major problems of contemporary crop protection. Metal containing nanoparticles appear to have all the credentials to be the next-generation, eco-compatible fungicide alternatives and a valuable anti-resistance management tool. Could the introduction of metal NPs as nano-fungicides be the answer to both reducing environmental footprint of xenobiotics and dealing with fungicide resistance? The potential of metal nanoparticles to be utilized as nano-fungicides both as alternative to conventional fungicides or/and as partners in combating fungicide resistance is discussed in terms of effectiveness, potential antimicrobial mechanisms as well as synergy profiles with conventional fungicides. However, their "golden" potential to be used both as alternatives and partners of conventional fungicides to combat resistance and reduce environmental pollution, is challenged by undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity, constituting risks that should be considered before their commercial introduction as nano-pesticides at a large scale.

1.3.1 Introduction

All available plant disease control methods considered, chemical control via the use of synthetic fungicides remains the most cost-efficient, food sustainability and safety-ensuring disease management strategy so far. [1,2] This great asset of the plant protection arsenal is seriously challenged by a number of inherent shortcomings of fungicides: high environmental footprints, side effects to non-target organisms, increasing costs on RnD and registration of new active ingredients and the loss of their effectiveness due to resistance development. Environmental pollution issues -especially water contamination– have resulted in the withdrawal of an unprecedently large number of older fungicide active ingredients enforced by the implementation of EU regulations and a halt in the development of new ones.[3] The limited number of available fungicides has rendered resistance even more difficult to manage highlighting the necessity for alternative disease management agents capable of controlling both sensitive and fungicide-resistant pathogens and, at the same time, minimizing environmental risks.

Nanotechnology has made a dramatic entrance in modern agriculture providing novel means for improving crop production and protection introducing nanoparticles (NPs) acting as nano-fertilizers, nano-pesticides or as carriers for relevant active ingredients. Metal nanoparticles (MNPs), taking advantage of their unique physico-chemical properties, have exhibited a significant effectiveness in controlling numerous plant pathogens both sensitive and drug-resistant, requiring lower doses compared to their bulk/ionic protective counterparts.[3] Lower doses required for anti-microbial action and their potential to be synthesized by green

methods of synthesis utilizing plants or microorganisms (or their metabolites) make them eco-compatible alternatives to synthetic fungicides.[4] The question that arises is: Should those potent plant protection agents be used instead of or as fungicide partners?

1.3.2 Metal-Nanofungicides

Nanomaterials with a potential to play an important role in plant disease management include organic or inorganic nanoparticles (typically 10 to 100 nm in size) acting directly as nano-pesticides or as formulating agents. Their unique physico-chemical properties enable them to maximize pathogen control in lower doses, achieve optimized drug delivery or to increase residual action by controlled-release in slower rates, making NPs ideal, environmentally compatible fungicide alternatives.[1,5-8] Nanopesticides containing metals such as zinc, silver, or copper have demonstrated significant antibacterial, antifungal or even antiviral activity and are being increasingly scrutinized by scientists for their application potentials against several plant pathogens.[6] Metal NPs (MNPs) effectiveness in controlling fungal plant pathogens including *Monilinia fructicola, Fusarium* sp., *Sclerotinia homoeocarpa*, *Botrytis cinerea*, *Aspergillus niger*, *Penicillium expansum* and *Rhizopus stolonifer* has been evaluated in a number of studies both *in vitro* and *in vivo*.[2,9,10-13] Positively charged MNPs adhere to the microbial cell membrane due to electrostatic attraction with the negatively charged cell membrane of microbes causing damage in the membrane integrity eventually leading to cell death. Other mechanisms of anti-microbial action of metallic NPs against plant pathogens include: protein/enzyme deactivation, production of reactive oxygen species (ROS) and antioxidant depletion, ion-homeostasis disruption and DNA damage.[14] These mechanisms result from ion-release or are nanoparticle-specific and often interlinked, resulting in a multitargeted action ideal for effectively controlling plant pathogens.[4,15] Although metal NPs share this multi-site mode of action with their bulk/ionic metal counterparts, in the majority of cases exceed their efficacy against pathogens.[3] Differences in the level of toxicity between MNPs and their ionic counterparts could be due to either the way they are distributed around the fungal cell or an additional nanoparticle-properties dependent mechanism. While metal ions are uniformly distributed around fungal cells without specific localization, larger MNPs are unevenly distributed creating focal sources of continuously unleased ions. This large NP-generated ion concentration induces ion penetration and impairs ion homeostatic mechanisms that cannot cope with the ion release rates produced, thus causing a more profound toxic effect than individual metal ions.[11,16,17] Besides ion release, nanoparticles can cause additional cell mechanical damage directly (due to their large surface to volume ratio or physical defects in their nanostructure) or by producing H_2O_2 able to penetrate the membrane and cause internal damage.[17] This could explain the superior fungitoxic effect of MNPs against fungal pathogens compared to their ionic counterparts, an advantage that makes them preferable to ionic forms both in terms of effectiveness and eco-compatibility since smaller xenobiotic quantities produce a lower environmental footprint.

1.3.3 Anti-resistance agents

The multi-site anti-microbial properties make MNPs ideal for anti-resistance management tools to be used in rotation with or in mixtures with conventional drugs, a fact initially demonstrated in studies involving human bacterial pathogens resistant to one or more antibiotic drugs. This is especially important in the case of life-threatening multi drug resistant (MDR) pathogens with the list of success stories of Ag or other metal-containing NPs in the control of hard to kill, highly adaptive, drug-resistant bacterial or fungal human pathogens is continually being populated.[20,22] In the case of fungicide resistant plant pathogens, reports on the potential of metal NPs to counter resistance are scarce but quite promising. Copper and silver containing NPs were shown to provide effective control against both sensitive and benzimidazole-resistant *B. cinerea* and *Monilia fructicola* isolates.[3,9] Ag-NPs have been able to effectively inhibit mycelial growth of *Fusarium graminearum* isolates resistant to carbendazim, tebuconazole, prochloraz, fudioxonil, phenamaril and pyriflumetofen *in vitro*.[23] This was also the case for ZnO-NPs that exhibited excellent activity against both sensitive and boscalid-resistant *A. alternata* isolates *in vitro* and *in vivo*. [10]

Fungal pathogens achieve fungicide resistance utilizing a number of biochemical mechanisms that help them escape inhibition. The most important mechanism involves target site modification/overexpression which reduces affinity of the fungicide with/or modifies abundance of its target molecule. The rest of the biochemical resistance mechanisms either prevent the active form of the active ingredient (a.i) to reach its target (reduced influx, increased efflux, detoxification) usually resulting in non-specific/multi drug resistance (MDR) or utilize alternative biochemical pathways (e.g Alternative Oxidase in the case of QoIs) to circumvent inhibition by the a.i.[24] Multiple targeting of both metal ions and MNPs make them low resistance risk antimicrobial agents at least as far as the target site modification mechanism is concerned. Resistance to metal ions may occur via mutations that result in the disruption of the cellular ion-homeostasis mechanisms that regulate ion influx/efflux and sequestration of excess ion loads that are toxic to the cell. Since the mode of action of MNPs extends beyond mere ion release, their risk for resistance development should be even lower than that of respective metal containing protective fungicides. Their ability to control bacterial pathogens is also very important since antibiotics are not allowed in agriculture and alternatives are rare (mostly consisting of copper containing inorganic/organic compounds). Differences between NPs and their bulk counterparts could also mean that they could be used for controlling copper resistant bacteria – which have already been reported.[25] The above make MNPs ideal tools for any effective resistance management strategy against plant pathogens.

1.3.4 Synergy with conventional drugs

Suitability of MNPs for use as alternatives or partners of conventional fungicides is evident both theoretically due to their multisite mode of action and practically as shown by their effectiveness against fungicide-resistant plant pathogens. What is not self-evident, is the "hidden" property of metal NPs to exhibit a synergistic effect when combined with

conventional drugs. In a large number of human disease cases, NPs containing Ag, Fe or Zn increased antimicrobial activity of antibiotic drugs against both sensitive and drug-resistant bacteria such as *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumonia, Eschericia coli, Staphylococcus aureus,* and *Enterococcus faecalis*.[22,26,27,28] Recently, a number of studies have confirmed a similar pattern concerning the combination of MNPs with conventional fungicides. Ag-NPs and ZnO-NPs combined with fungicides carbendazim, mancozeb and thiram exhibited enhanced toxicity against *B. cinerea, A. alternata, Fusarium oxysporum, Aspergilus niger* and, *Penicillium expansum*.[29] A similar synergistic effect was reported for ZnO-NPs when applied with the fungicide thiram in *Phytophthora capsici*.[21] To make things even more interesting, Ag-NPs, Cu-NPs and ZnO-NPs have been shown to "neutralize" fungicide resistance when applied against benzimidazole or boscalid-resistant isolates of *B. cinerea, M. fructicola* or *A. alternata* respectively: a profound synergistic effect was found when combining MNPs with fungicides that where otherwise ineffective due to target site resistance. [3,9,10]

The exact mechanisms by which metal NPs can achieve such a synergy with fungicides and bypass of fungicide resistance are largely unknown, even though certain suggestions exist based on the mode of action of MNPs and their interaction with the fungicides. MNPs can act as chemo-sensitizing agents, complementing fungicide action, or synergists that neutralize fungicide resistance mechanisms. Synergy between MNPs and fungicides may result from a) enhanced membrane perturbation, b) disruption of ion homeostasis, c) inhibition of efflux pumps, d) inhibition of detoxification enzymes, and e) a "capping" effect resulting the formation of a NPs-fungicide conjugate (Fig. 1.3.1).[10,18-20] Most of the above mechanisms are associated with the bioavailability of the fungicide inside the fungal cell (the amount of a.i. that finally reaches its target) facilitated by the action of MNPs. Membrane openings caused by Ag_3PO_4-NPs promoted Sodium o-phenyl phenolate entrance and enhanced its toxic action against *Phytophthora capsici* and *B. cinerea*. [21] A combination of membrane damage and disruption of a major efflux pump was reported to contribute to the synergy observed between fluconazole and Ag-NPs against drug-resistant *Candita albicans* while a depletion of the detoxifying enzyme GSH caused by thiram resulted in a synergistic toxic effect when combined with ZnO-NPs against *P. capsici*. [20,21] Boscalid acted as a capping agent of ZnO-NPs, reducing nanoparticle aggregation and resulting in a reduction of NP size which probably enhanced the toxic effect of ZnO-NPs against *A. alternata*. [10]

Most of the above-mentioned mechanisms are especially important when we are dealing with multidrug isolates of plant pathogens where decreased influx/increased efflux or detoxification are the main causes of resistance. Nevertheless, MNPs can also suppress target site resistance when the MNPs/fungicide combination result in a higher nanoparticle bioavailability by e.g. reducing nanoparticle size.[10] This fact highlights that the benefits of the combination of MNPs with conventional fungicides extend beyond the typical use of alternative and/or mixing partners with different modes of action for combating resistance. In order to exploit MNPs' full potential as control agents against sensitive and fungicide-resistant pathogens and broaden the application range of this phenomenon, their synergy profile with fungicides should be further studied and their synergy mechanisms elucidated.

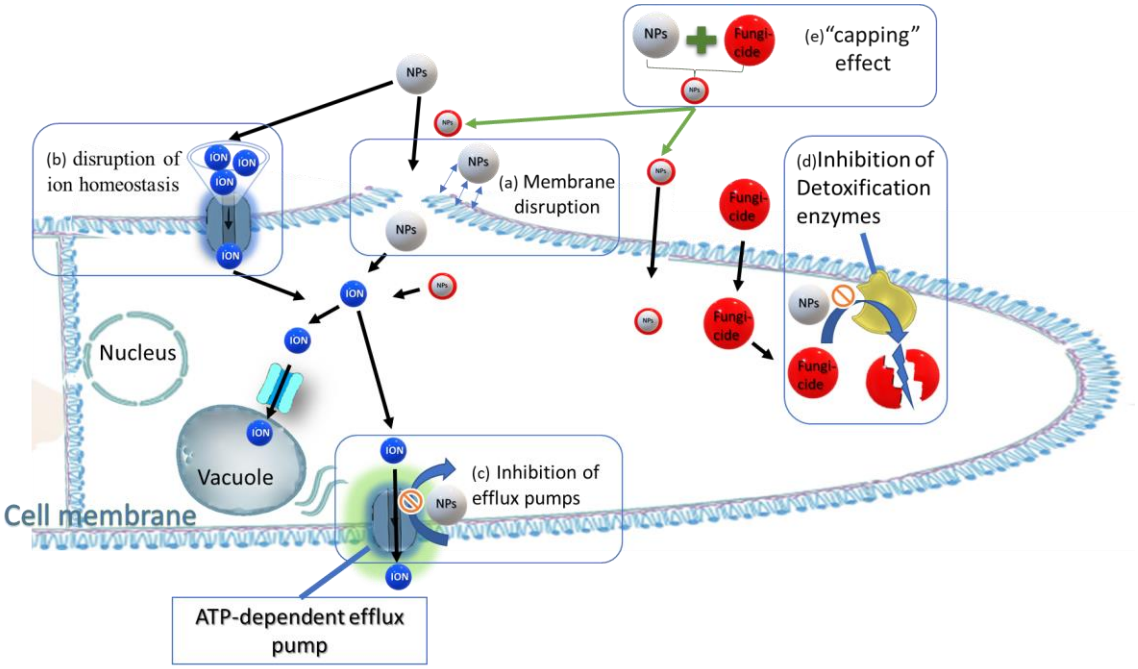


Figure 1.6 Schematic representation of proposed synergy mechanisms between NPs and fungicides: a) Enhanced membrane perturbation, b) Disruption of ion homeostasis, c) Inhibition of efflux pumps, d) Inhibition of detoxification enzymes, and e) "Capping" effect resulting the formation of a NPs-fungicide conjugate that deters agglomeration and reduces NP size.

1.3.5 Safety issues

MNPs can reduce the environmental footprint of plant protection by reducing pesticide doses and, especially green-synthesized ones, could be considered eco-compatible. However promising as they are for crop protection, MNPs may pose both known and unknown health and environmental risks. Their very same unique properties that enable them to have increased fungitoxic action compared to their bulk counterparts could have undesirable/toxic effects towards non-target organisms. Their ability to penetrate fungal cell membranes -especially in the case of smaller NPs- could extend to plant or even human cells and cause toxic responses. [30] Ecotoxicity tests typically used for their bulk counterparts may prove insufficient to evaluate NP toxicity since their effect on biological system is not yet completely understood. Phytotoxicity, human and environmental safety issues should be systematically investigated before their commercial release for use as nano-pesticides. The same applies for their combinations with fungicides which may result in a special, combined toxicity to non-target organisms.

1.3.5 Conclusion

In an era of continuously increasing limitations in the availability of active ingredients against plant pathogens, metal nanoparticles exhibit a great potential to be used both as alternative to conventional fungicides or/and as their partners in combating fungicide resistance. Their demonstrated effectiveness against several sensitive or fungicide-resistant plant pathogenic fungi, alone or when combined with conventional fungicides at lower than recommended doses highlight their "golden" potential to be used both as alternatives and partners of conventional fungicides to combat resistance and reduce environmental impact of xenobiotics. Targets of NPs fungitoxic action in a sub-cellular level could be key for inactivating resistance mechanisms of pathogens while their interaction with organic fungicides could facilitate an increase in the bioavailability of both antifungal agents leading to enhanced toxicity. However, since their effect on biological systems is not yet completely understood, MNPs should be further studied for undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity before their commercial introduction as nano-pesticides at a large scale.

1.3.6 References

1. Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks. Proceedings of the National Academy of Sciences India Section B - Biological Sciences **88**: 1287–1308 (2018).

2. Malandrakis, A.A., Vattis, K.N., Markoglou, A.N., Karaoglanidis, G.S. Characterization of boscalid-resistance conferring mutations in the SdhB subunit of respiratory complex II and impact on fitness and mycotoxin production in *Penicillium expansum* laboratory strains. Pesticide Biochemistry and Physiology **138**: pp. 97-103, (2017)

3. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates. Pesticide Biochemistry and Physiology **173**: art. no. 104796 (2021).

4. Alghuthaymi, M.A., Almoammar, H., Rai, M., Said-Galiev, E., Abd-Elsalam, K.A. Myconanoparticles: Synthesis and their role in phytopathogens management. Biotechnology and Biotechnological Equipment **29**: pp. 221-236 (2015).

5. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola.* Science of the Total Environment **747**: art. no. 141287 (2020).

6. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens. Science of the Total Environment **670**: 292-299 (2019).

7. Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D., A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. Nature Nanotechnology **13:** 677–684 (2018).

8. Sun, Q., Li, J., Le, T., Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives. Journal of Agricultural and Food Chemistry **66**: 11209–11220 (2018).

9. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Synergy between Cu-NPs and fungicides against *Botrytis cinerea.* Science of the Total Environment **703**: art. no. 135557 (2020).

10. Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant *Alternaria alternata* isolates Science of the Total Environment **829**: art. no. 154638 (2022).

11. Li, J., Sang, H., Guo, H., Popko, J.T., He, L., White, J.C., Parkash Dhankher, O., Jung, G., Xing, B. Antifungal mechanisms of ZnO and Ag nanoparticles to *Sclerotinia homoeocarpa*. Nanotechnology **28:** art. no. 155101 (2017).

12. Ashajyothi, C., Prabhurajeshwar, C., Handral, H.K., Kelmani, C.R. Investigation of antifungal and anti-mycelium activities using biogenic nanoparticles: An eco-friendly approach Environmental Nanotechnology. Monitoring and Management **5**: 81-87 (2016).

13. Sardella, D., Gatt, R., Valdramidis, V.P Physiological effects and mode of action of ZnO nanoparticles against postharvest fungal contaminants. Food Research International **101**: 274-279 (2017).

14. Avila-Quezada, G. D. , Espino-Solis, G. P. . Silver Nanoparticles Offer Effective Control of Pathogenic Bacteria in a Wide Range of Food Products. In: Kırmusaoğlu, S. , Bhardwaj, S. B. , editors. Pathogenic Bacteria [Internet]. London: IntechOpen; (2019). DOI: 10.5772/intechopen.89403

15. Abd-Elsalam, K.A., Prasad, R. Preface Nanotechnology in the Life Sciences, pp. v-vi. (2018).

16. Robinson, J.R., Isikhuemhen, O.S., Anike, F.N. Fungal–metal interactions: A review of toxicity and homeostasis. Journal of Fungi **7**: art. no. 225 (2021).

17. Slavin, Y.N., Asnis, J., Häfeli, U.O., Bach, H. Metal nanoparticles: Understanding the mechanisms behind antibacterial activity Journal of Nanobiotechnology **15**: art. no. 65 (2017).

18. Jampilek, J., How can we bolster the antifungal drug discovery pipeline? Future Medicinal Chemistry **8**: 1393-1397 (2016).

19. Xue, J., Zan, G., Wu, Q., Deng, B., Zhang, Y., Huang, H., Zhang, X. Integrated nanotechnology for synergism and degradation of fungicide SOPP using micro/nano-Ag_3PO_4. Inorganic Chemistry Frontiers **3**: 354-364 (2016).

20. Sun, L., Liao, K., Li, Y., Zhao, L., Liang, S., Guo, D., Hu, J., Wang, D., Synergy between polyvinylpyrrolidone-coated silver nanoparticles and azole antifungal against drug-resistant *Candida albicans*. Journal of Nanoscience and Nanotechnology **16**: 2325-2335 (2016).

21. Xue, J., Luo, Z., Li, P., Ding, Y., Cui, Y., Wu, Q., A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles. Scientific Reports **4**: art. no. 5408 (2014).

22. Punjabi, K., Mehta, S., Chavan, R., Chitalia, V., Deogharkar, D., Deshpande, S., Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens. Frontiers in Microbiology **9**: art. no. 2207 (2018).

23. Jian, Y., Chen, X., Ahmed, T., Shang, Q., Zhang, S., Ma, Z., Yin, Y. Toxicity and action mechanisms of silver nanoparticles against the mycotoxin-producing fungus *Fusarium graminearum.* Journal of Advanced Research, DOI: 10.1016/j.jare.2021.09.006 (2021)

24. FRAC (2007). Fungicide resistance in crop pathogens: How can it be managed? https://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf?sfvrsn=769d419a_8. Accessed January 2022.

25. Lee YA, Hendson M, Panopoulos NJ, Schroth MN. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small blue copper proteins and multicopper oxidase. J Bacteriol. **176**:173-88 (1994).

26. Gabrielyan, L., Hakobyan, L., Hovhannisyan, A., Trchounian, A., Effects of iron oxide (Fe_3O_4) nanoparticles on *Escherichia coli* antibiotic-resistant strains. Journal of Applied Microbiology **126**: 1108-1116 (2019).

27. Nejabatdoust, A., Salehzadeh, A., Zamani, H., Moradi-Shoeili, Z., Synthesis, Characterization and Functionalization of ZnO Nanoparticles by Glutamic Acid (Glu) and Conjugation of ZnO@Glu by Thiosemicarbazide and Its Synergistic Activity with Ciprofloxacin Against Multi-Drug Resistant *Staphylococcus aureus*. Journal of Cluster Science **30**: 329-336 (2019).

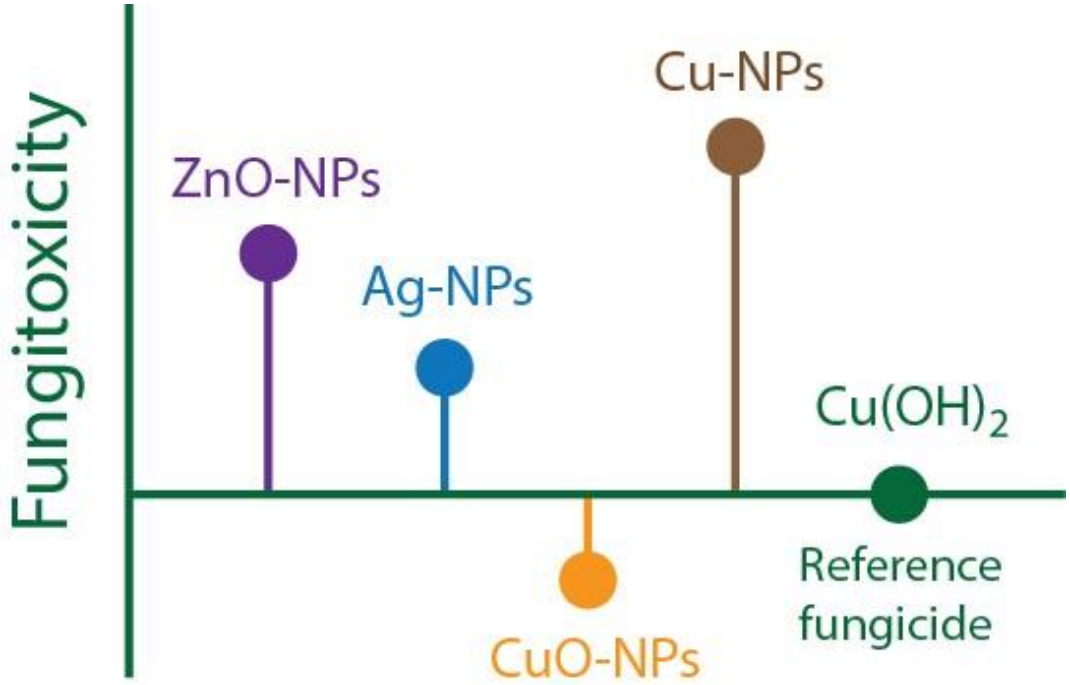
28. Paralikar, P., Ingle, A.P., Tiwari, V., Golinska, P., Dahm, H., Rai, M., Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria. Journal of Environmental Science

and Health - Part A Toxic/Hazardous Substances and Environmental Engineering **54**: 381-390 (2019).

29. Jamdagni, P., Rana, J.S., Khatri, P., Comparative study of antifungal effect of green and chemically synthesised silver nanoparticles in combination with carbendazim, mancozeb, and thiram. IET Nanobiotechnology **12**: 1102-1107 (2018).

30. Rudramurthy, G.R., Swamy, M.K., Sinniah, U.R., Ghasemzadeh, A. Nanoparticles: Alternatives against drug-resistant pathogenic microbes. Molecules **21**: art. no. 836, (2016).

2 Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens



Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019) Science of the Total Environment, 670, pp. 292-299. DOI: 10.1016/j.scitotenv.2022.154638

2. Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens

Abstract

Nano-fungicides are expected to play an important role in future plant disease management as eco-friendly alternatives of conventional synthetic fungicides. In the present study, the sensitivity of seven fungal species, known to cause foliar and soil-borne diseases, to nanoparticles (NPs) containing copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc (ZnO-NPs) was assessed *in vitro*. Mycelial growth assays revealed that Cu-NPs with mean inhibition rates, EC_{50}, ranging between 162 and 310 μg/mL were most effective among the NPs tested in inhibiting fungal growth, followed by ZnO-NPs with EC_{50} ranging between 235 and 848 μg/mL. All fungal species were practically insensitive to CuO-NPs and Ag-NPs except for *B. cinerea*, which was equally sensitive to Ag-NPs and Cu-NPs (EC_{50}=307 μg/mL). Cu-NPs were more fungitoxic in terms of mycelial growth, to almost all species tested, than a protective fungicide containing $Cu(OH)_2$, which was used as a reference. Fungitoxicity experiments with the NPs tested and bulk size reagents containing the respective metals revealed that ZnO-NPs were more toxic to all fungal species tested than $ZnSO_4$, whereas Cu-NPs were more fungitoxic than $CuSO_4$ in all cases, except for *B. cinerea, A. alternata* and *M. fructicola*. The existence of a positive correlation between Cu-NPs and CuO-NPs toxicity and, at the same time, the absence of any correlation between NPs tested and their respective bulk metal counterparts indicated potential differences in the mode of action between bulk and nanosized antifungal ingredients. Although there was considerable variation between fungal species, all NPs were generally 10 to 100 fold more fungitoxic to spores than hyphae and in the majority of cases more effective than $Cu(OH)_2$, as revealed by colony formation bioassays. NPs significantly suppressed grey mold symptoms on plum fruit, especially Ag-NPs, which completely inhibited disease development. Consequently, tested NPs have the potential to be used as protective antifungal agents.

2.1 Introduction

Crop losses due to plant parasites are a considerable challenge that the current agricultural production system faces worldwide with diseases accounting for at least 25% of the losses (Pantley et al., 2016). Conventional synthetic fungicides are largely considered as the most effective and cost-efficient means for disease management. However, the intensity of usage and the site-specific mode of action of most synthetic fungicides eventually lead to resistance problems and an increased environmental cost due to elevated drug residues to water reservoirs. Nanoparticles (NPs) are expected to play an important role in resolving this challenge in the future (Pandey et al., 2016; Kah et al., 2018; Sun et al., 2018). NPs provide a novel eco-friendly alternative to synthetic chemical fungicides, due to their promising properties that improve drug delivery, slow active ingredient release, and increase effectiveness in lower doses. NPs containing silver (Ag-NPs) have been proven to exert a wide range of antimicrobial activity against bacteria, fungi and viruses (Huang et al., 2018). Bulk copper

compounds have been exploited to protect agricultural crops from many pests, including those causing a wide range of bacterial and fungal infections, due to their low cost, protective activity, and reduced risk for resistance development controlled by their multi-site mode of action against pathogens (Keller et al., 2017). The observed lack of systemic movement and ease of residue removal from plant tissues has enabled copper fungicides to be a part of disease control management in organic farming (Bakeret al., 2002; Winter and Davis, 2006). Taking advantage of the antifungal and antimicrobial properties of [Cu^{+2}], applications of NPs containing copper (Cu-NPs) in agriculture and food preservation are readily emerging in an attempt to exploit their unique nano-scale properties (Keller et al., 2017; Park et al., 2015).

Metal oxide NPs, compared to their bulk counterparts, are more stable in extreme conditions, exhibit antimicrobial activity at low concentrations and low or no toxicity to humans (Król et al., 2017). NPs containing zinc (ZnO-NPs) are very effective antibacterial agents against a broad spectrum of bacterial species, due to their high surface to volume ratio and unique physicochemical properties (Sun et al., 2018). Several studies have demonstrated the fungistatic potential of ZnO-NPs against fungal pathogens including *Fusarium sp., Botrytis cinerea, P. expansum*, *Aspergillus niger* and *Rhizopus stolonifer* (He et al., 2011; Król et al., 2017; Sharma et al., 2010).

Air-borne fungal strains tested in the present study include pathogens that cause important pre- and post-harvest plant diseases such as Alternaria tomato brown leaf spot, Grey mould caused by *Botrytis cinerea,* Monilia stone fruit Brown rot, and strawberry Anthracnose caused by *Colletotrichum spp.* Most of the above pathogens require a large number of fungicide applications for their control, while fungicide resistance development is seriously impairing effective control (FRAC, 2013; Malandrakis et al., 2013, 2018; Ziogas et al., 2003). Soil-borne pathogens also included in this study are causal agents of wilting (*V. dahliae*) or root rots (FORL and *F. solani*) in a wide range of hosts and are difficult to control with conventional fungicides except for high-cost fumigants (Paplomatas et al., 2005). Thus, it is essential to consider alternative compounds suitable for disease suppression with suitable properties to alleviate control disadvantages against the above pathogens, and at the same time minimize the environmental impact of conventional pesticides. Although numerous studies, especially in the case of silver, have examined metal nanoparticle fungitoxic activity, there are only very few data available concerning the efficacy of NPs in inhibiting both mycelial growth and spore germination of plant pathogens *in vitro* and even fewer *in vivo* (Nemati et al., 2015; Pandey et al., 2016; Kah et al., 2018; Sun et al., 2018). Under this light, *in vitro* and *in vivo* evaluation bioassays were carried out with the main objective of: (a) determining the mean sensitivity (in terms of EC_{50} values) of *Alternaria alternata, Botrytis cinerea, Monilia fructicola, Verticillium dahliae, Colletotrichum gloeosporioides, Fusarium oxysporum fsp Radicis Lycopersici* (FORL), and *Fusarium solani* strains to Cu-NPs, CuO-NPs, Ag-NPs and ZnO-NPs *in vitro*, and (b) comparing nanoparticle effectiveness against the above plant pathogens to bulk-size containing reagents and a reference fungicide containing $Cu(OH)_2$.

2.2 Materials and Methods

2.2.1 Nanoparticles, reagents and fungicide

NPs and reagents used in this study were purchased from Sigma Aldrich, MO, USA: silver [Ag-NPs] (<100nm particle size), zinc oxide [ZnO-NPs] (particle size <50 nm), copper [Cu-NPs] (particle size 25 nm), copper oxide [CuO-NPs] (particle size <50 nm), copper sulphate [$CuSO_4$], zinc sulphate [$ZnSO_4$] and silver nitrate [$AgNO_3$]. A commercial fungicide product (Copperblau-N 50 WP) containing the active ingredient copper hydroxide was purchased from NITROFARM (Hellas). All stock solutions of the antifungal compounds were prepared using sterilized distilled water as a solvent for all commercial or analytical grade active ingredients and were added aseptically to sterilized growth medium prior to inoculation. Nanoparticle suspensions were subjected to sonication for 30 min using a Transonic 420 (Elma, Germany) sonicator prior to incorporation in growth media.

2.2.2 Fungal strains and culture conditions

Seven fungal strains from the fungal collection of the Pesticide Science Lab (Agricultural University of Athens) originating from various crop fields, located in regions of Southern and Central Greece (Table 2.1), were used to evaluate the fungitoxic activity of the NPs studied. All strains were grown on Potato Dextrose Agar (PDA) medium in order to obtain inoculum for the fungitoxicity assays and kept in growth chambers in the dark at 25 °C and 70% humidity. For each strain, for long-term storage purposes, four 5mm mycelial plugs from the margin of rapidly growing fungal colonies were placed in 1.5 mL tubes containing 50% v/v of sterilized glycerol: water and kept at -20 °C.

Table 2.1. Fungal strains belonging to different species used in this study.

Fungal strain	Disease	Host	Origin (Region in Greece)
B. cinerea	Grey mold	tomato	Crete
A. alternata	Black rot	tomato	Crete
M. fructicola	Brown rot	peach	Naousa
C. gloeosporioides	Anthracnose	strawberry	Crete
F. solani	Fusarium root rot	tomato	Kalamata
F. oxysporum fsp Radicis Lycopersici	Crown root rot	tomato	Larisa
V. dahliae	Verticillium wilt	eggplant	Crete

2.2.3 *In vitro* mycelium growth inhibition tests

Sensitivity of fungal strains to NPs and fungicides was evaluated by measuring radial growth of strains on PDA. Fungitoxicity was expressed based on EC_{50} values (effective concentration causing 50% inhibition of mycelial growth) for each compound. PDA amended with 0, 1, 10, 100, 500, 1000, 5000 μg/mL Ag-NPs, 0, 1, 10, 100, 500, 1000 μg/mL $CuSO_4$,

$ZnSO_4$, $AgNO_3$, Cu-NPs, ZnO-NPs or CuO-NPs and 0, 50, 100, 500, 1000 μg/mL copper hydroxide was used to obtain fungitoxicity-curves for all strains. There were three replicate plates for each antifungal ingredient concentration - strain combination. Inoculum consisting of a 5-mm mycelial plug cut from the edge of 4-day old colony of each fungal strain grown on PDA was transferred to nanoparticle or fungicide-amended and non-amended (control) media. Cultures were incubated at 25 °C in the dark for 4 days. The mean diameter of the colony on the fungicide-amended plates was expressed as the percentage of the mean diameter of the untreated control. Tests for each isolate were repeated twice for each concentration and fungicide.

2.2.4 Inhibition of colony formation

The effect of NPs and fungicides on spore germination was assessed *in vitro* on PDA containing petri dishes. Each fungal strain was cultivated in appropriate medium in order to induce conidiation. In the case of *A. alternata, B. cinerea, M. fructicola* and *C. gloeosporioides,* a 5-mm mycelial plug cut from the edge of a rapidly growing colony was transferred on a 9-cm petri dish and incubated for 7 days at 25 °C in a growth chamber with a 12 h per day light-period. Conidia from each strain were harvested by scraping the surface of the plate, suspended in 10 mL sterilized-distilled water, and filtered using cheesecloth. In the case of *V. dahliae, FORL and F. solani* liquid cultures in 250 mL glass flasks containing Potato Dextrose Broth (PDB) were used to obtain conidia. Flasks were inoculated with four 5-mm mycelial plugs, cut from the edge of rapidly growing colonies from each fungal strain and incubated for 4 days in the dark at 25ºC in growth chambers under continuous shaking at 200 rpm. Conidia were then harvested by filtration of the liquid cultures using cheese cloth. The concentration of spores, in all cases, was determined using a haemocytometer (2 counts per replicate) and adjusted to 100 conidia/100 μL by serial dilutions in sterilized-distilled water.

Inhibition of colony formation assays was carried out on PDA petri dishes containing various nanoparticle and fungicide concentrations. PDA amended with 0, 1, 5, 10, 25, 50, 100, 250, 500, 1000, 2000 μg/mL Ag-NPs, ZnO or CuO; 0, 1, 2.5, 5, 10, 20, 50, 100 μg/mL Cu-NPs; and 0, 0.1, 1, 5, 10, 50, 100, 500 μg/mL copper hydroxide was used to obtain fungitoxicity-curves for all strains. There were three replicate plates of each compound concentration - strain combination. A volume of 100 μL from flasks containing a concentration of 10^3 conidia/mL from each fungal strain was transferred and spread on the surface of petri dishes containing PDA amended or not with the above-mentioned nanoparticle/fungicide concentrations. All dishes were incubated for 2 days in the dark at 25 ºC, and then, the number of forming colonies was counted. The percent inhibition of colony formation was calculated by dividing the mean number of colonies from each antifungal compound treatment by the mean number of colonies formed in the untreated control and multiplying the result with 100. EC_{50} values based on relative percent inhibition were calculated for each compound/strain combination. The experiment was conducted twice.

2.2.5 Effectiveness of nanoparticles in vivo

The effectiveness of NPs in suppressing grey mold symptoms caused by *B. cinerea in vivo* was tested on plum fruit (*Prunus domestica*) of uniform shape, size, maturity and without any wound. Cu-NPs, Ag-NPs and ZnO-NPs were included in tests as well as the reference fungicide Copperblau-N containing copper hydroxide. Four plum fruits per treatment with antifungal agents were used while distilled water was used as the control treatment. Prior to treatment and inoculation, plum fruit were surface disinfected in a 1% sodium hypochlorite solution by dipping for 10 min. Following disinfection, fruit were rinsed three times with distilled-sterilized water and left to dry. Subsequently, fruit were sprayed until ran off using concentrations of 100 and 1000 μg/mL for each NP/fungicide treatment. Plum fruit where air-dried for 2 hr and then a sterile needle was used to remove fruit skin creating a 4×4×2 mm [length × width × depth] wound at the front face of each fruit. Wounds were inoculated using a pipette with a 100 μL-drop transferred from a suspension containing *B. cinerea* conidia at a concentration of 10^4 conidia/mL. Inoculated fruit were placed on top of wet sterilized paper inside plastic boxes 24 × 34× 10 cm [length × width × height] covered by a lid and incubated in a growth chamber at 25 °C for 4 days. Symptom severity on NP/fungicide treated fruit was recorded by measuring the lesion diameter around each wound and expressed as a percent of the water-treated control. The experiment was repeated twice.

2.2.6 Morphological examination of nanoparticle-treated conidia

Morphological examination of germinating conidia treated with NPs was conducted by spreading a 20-μL volume of a conidial suspension (10^4 conidia/mL) on a 20×20 mm coverslip subsequently covered with sterile cellophane. A PDA plug was then placed on top of the cellophane. Conidia were left for at least 6 h on PDA blocks in order to germinate. Subsequently, untreated PDA plugs were replaced by other PDA plugs treated with NPs in doses equal to the respective EC_{50} values determined in the spore germination experiments. Cover slips were withdrawn and adhered conidia were examined with an x40 objective lens, on an OLYMPUS BX40 microscope.

2.3. Statistical analysis

The EC_{50} values for each strain and antifungal compound were calculated by regressing the relative inhibition of mycelial growth/colony formation against the Log_{10} compound concentrations. Cross sensitivity between antifungal compounds was evaluated using Pearson correlation coefficients. Data on efficacy of NPs were subjected to analysis of variance and mean separation using Tukey's HSD test ($\alpha = 0.05$). All analyses were conducted using SPSS (SPSS Inc., Chicago, IL, USA).

2.4 Results

2.4.1 Effect of NPs on mycelial growth *in vitro*

A dose-dependent decrease in growth was observed in most fungal strains treated with NPs while the respective EC_{50} values are shown in Table 2. Although sensitivity to tested NPs varied between strains, all fungal species were relative insensitive to CuO-NPs in terms of

mycelial growth, exhibiting EC_{50} values greater than 1000 μg/mL (see Table 2.2). This was also the case for Ag-NPs, which could not inhibit most of the fungal strains even at concentrations exceeding 5000 μg/mL. The only exception was *B. cinerea*, where mean inhibition by Ag-NPs was achieved at a concentration of 307 μg/mL (see Table 2.2). Among the four NPs, Cu-NPs exhibited the greater overall toxic activity against the 7 fungal species with EC_{50} values ranging from 162 (*V. dahliae*) to 328 μg/mL for the FORL, followed by ZnO-NPs with respective EC_{50} values ranging from 235 (*A. alternata*) to 847 μg/mL for the FORL strain (see Table 2.2). Copperblau-N, a protective broad-spectrum fungicide containing $Cu(OH)_2$, was included in this study as a reference antifungal agent. In most cases, Cu-NPs were more effective than $Cu(OH)_2$, while the rest of the NPs tested were equally or significantly less effective than the commercial fungicide except for ZnO-NPs which were more effective than $Cu(OH)_2$ against *M. fructicola*, *F. solani* and *V. dahliae* (see Table 2.2).

Table 2.2. Effect of NPs on mycelial growth of seven plant pathogenic fungal strains.

Fungal strain	EC_{50}[a] (μg m/L) (mean ± SD[b])				
	Ag-NPs	Cu-NPs	CuO-NPs	ZnO-NPs	$Cu(OH)_2$
B. cinerea	306.95± 4.75	310.23 ± 2.34	>1000	670.29 ±16.32	493.47 ±21.65
A. alternata	>5000	296.56 ± 8.72	>1000	235.48 ±15.09	210.07 ± 3.15
M. fructicola	4221.53 ± 56.70	219.46±10.12	>1000	628.22±24.05	861.04±44.18
C. gloeosporioides	1099.90 ± 80.43	161.78±14.42	>1000	551.05±18.22	563.44±9.82
F. solani	>5000	261.16±12.54	>1000	554.41±30.05	765.61±10.62
FORL[c]	>5000	328.12±20.30	>1000	847.44±24.18	856.96±73.82
V. dahliae	>5000	191.17±11.33	>1000	386.48 ±23.14	786.32±55.08

[a]EC_{50} = Effective concentration causing 50% reduction in mycelial growth rate.
[b]Standard Deviation
[c]FORL: *F. oxysporum fsp Radicis Lycopersici*

In order to investigate the effect of nanoparticle size to the observed mycelial growth inhibition, $CuSO_4$, $ZnSO_4$ and $AgNO_3$ reagents containing the respective metals in bulk size were included in fungitoxicity experiments. For all fungal strain cases examined, ZnO-NPs were significantly more fungitoxic compared to $ZnSO_4$ (see Fig. 2.1). The most profound differences was observed in the cases of *M. fructicola* and *A. alternata*, where EC_{50} values were equal to 628 and > 2000 μg/mL, 235 and 751 μg/mL for ZnO-NPs and $ZnSO_4$, respectively. A smaller but statistically significant difference in mycelial growth inhibition was observed in *C. gloeosporioides* between ZnO-NPs (EC_{50}=551 μg/mL) and $ZnSO_4$ (EC_{50}=667 μg/mL). Cu-NPs were significantly more fungitoxic than $CuSO_4$ towards *V. dahliae*, *C. gloeosporioides*, *F. oxysporum* (FORL) and *F. solani*, but that was not the case for the rest of the fungal species cases. Because silver NPs used in this study were hardly effective against mycelial growth in most of the strains studied, comparison with $AgNO_3$ was conducted only for the *B. cinerea* and *C. gloeosporioides* cases. In both cases $AgNO_3$ was more toxic to fungal

growth than Ag-NPs (see Fig. 1a,d), suggesting the probability of an additional mode of toxic action for $AgNO_3$, which is different than ion metal toxicity.

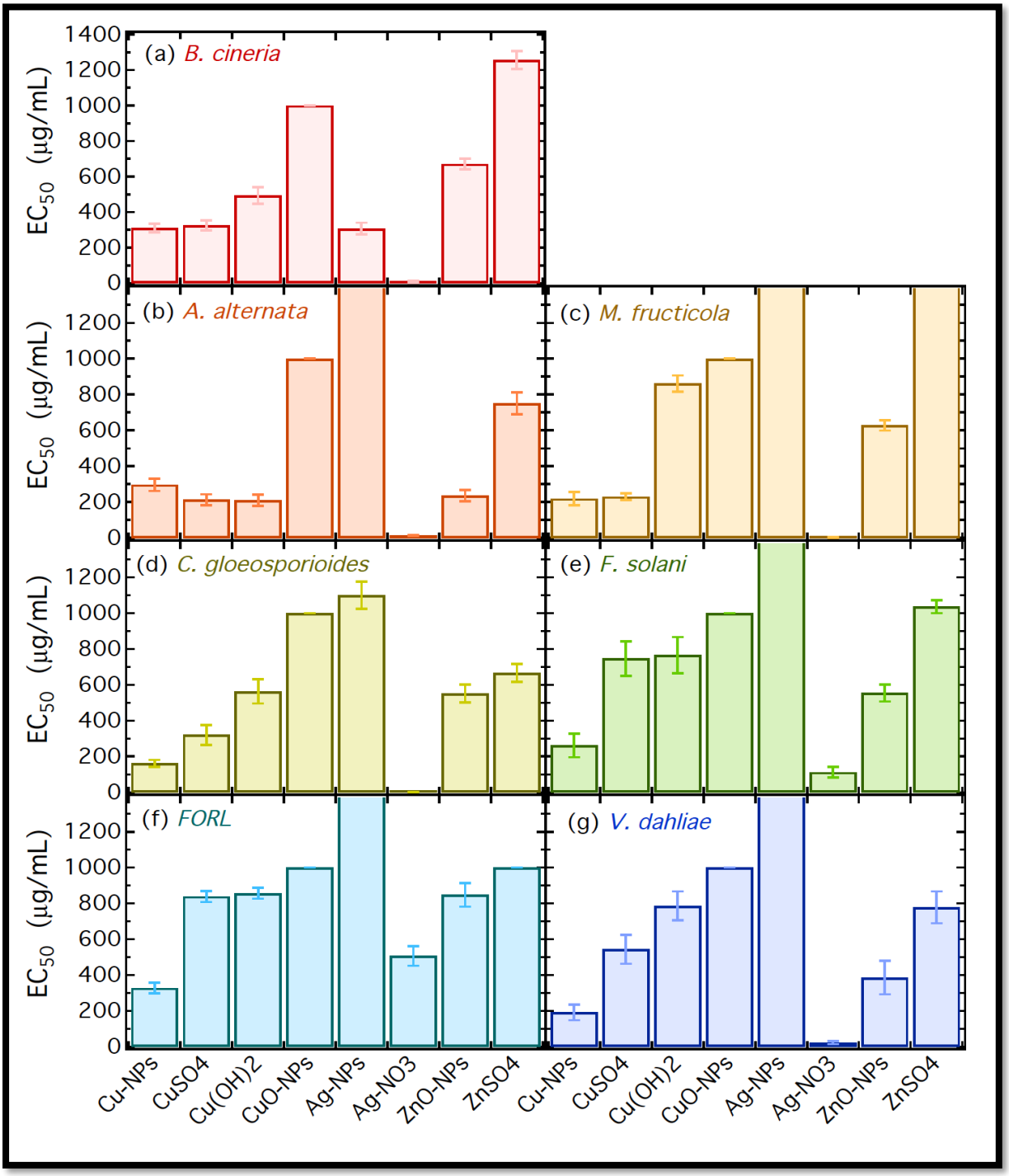


Figure 2.1. Sensitivity of fungal strains to NPs compared with bulk-sized metal containing reagents. Error lines represent the standard deviation of means (FORL: F. oxysporum fsp Radicis Lycopersici).

2.4.2 Cross sensitivity profiles of fungal strains to antifungal compounds

Growth inhibition profiles of the fungal strains to NPs, $AgNO_3$, $CuSO_4$, $ZnSO_4$ and $Cu(OH)_2$ were analyzed using Pearson correlation coefficients on EC_{50} or relative growth values. A statistically significant positive correlation between Cu-NPs and CuO-NPs was found (see Fig. 2.2a), while no significant correlation was found between Cu-NPs and other bulk-sized copper containing compounds (see Table 2.3). A positive correlation was observed between Ag-NPs and $CuSO_4$ or $Cu(OH)_2$ but not with $AgNO_3$ containing bulk sized silver (see Fig. 2.2b,c). No correlation was found between ZnO-NPs and any of the other antifungals used in this study (see Table 2.3).

Table 2.3. Correlation between sensitivity of fungal strains to NPs and their bulk counterparts.

	Cu-NPs	Ag-NPs	ZnO-Nps	CuO-NPs	$CuSO_4$	$Cu(OH)_2$	$ZnSO_4$	$AgNO_3$
Cu-NPs	1.0	-0.08 [a]	0.305	0.855*	0.287	-0.211	0.314	0.547
Ag-NPs	-	1.0	0.383	-0.016	0.808*	0.888**	0.136	0.524
ZnO-Nps	-	-	1.0	0.28	0.478	0.618	0.537	0.646
CuO-NPs	-	-	-	1.0	0.274	-0.115	0.149	0.716
$CuSO_4$	-	-	-	-	1.0	0.594	-0.08	0.485
$Cu(OH)_2$	-	-	-	-	-	1.0	0.403	0.433
$ZnSO_4$	-	-	-	-	-	-	1.0	0.018
$AgNO_3$	-	-	-	-	-	-	-	1.0

[a] Pearson correlation coefficient values.
* corresponds to a significance lever of P=0.05.
** corresponds to a significance lever of P=0.01.

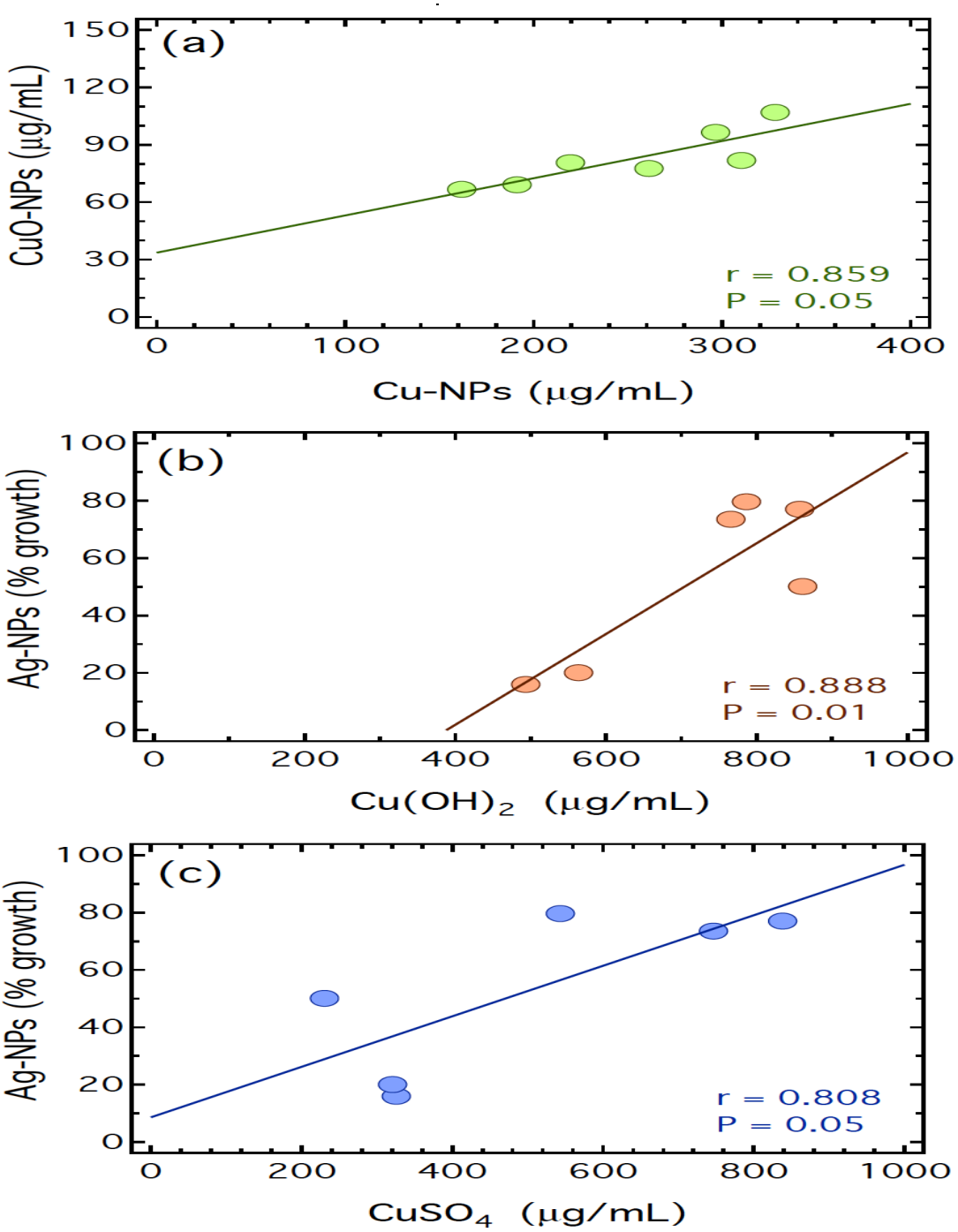


Figure 2.2. Correlation between sensitivities of fungal strains to (a) Cu-NPs (EC_{50} values) and CuO-NPs (relative growth at 1000 µg/mL), (b) Ag-NPs (relative growth at 5000 µg/mL) and $Cu(OH)_2$, and (c) Ag-NPs (relative growth at 5000 µg/mL) and $CuSO_4$ (EC_{50} values). Here, r is the Pearson correlation coefficient, and P the significance level.

2.4.3 Effect of NPs on colony formation

The ability of Cu, CuO, ZnO and Ag NPs to inhibit spore germination in the tested fungal species was assessed by colony formation inhibition experiments. Sensitivity of fungal strains to $Cu(OH)_2$ and NPs used in this study in terms of EC_{50} (concentration causing 50% colony inhibition) is presented in Table 2.4. Cu-NPs were more toxic to all species compared to the reference commercial fungicide, except in the case of FORL where toxicities did not difer significantly (see Table 2.4). Similarly, ZnO-NPs were more effective than $Cu(OH)_2$ in all cases but FORL (see Table 2.4). With the exception of the two Fusarium species tested, all other fungal strains were more sensitive to Ag-NPs than $Cu(OH)_2$ (see Table 2.4). Overall, NPs were significantly more effective against fungal strains at the spore germination level compared to mycelial growth. EC_{50} values of Cu-NPs ranged from 3 (*B. cinerea*) to 29 (FORL) μg/mL, indicating a 10 to 100-fold increased spore sensitivity to these NPs, compared to the respective hyphal sensitivity revealed by mycelia growth assays (compare Tables 2.2,2.4). A similar spore-inhibitory profile was observed for ZnO-NPs with EC_{50} ranging from 5 (*V. dahliae)* to 165 μg/mL (FORL). All fungal strains were highly sensitive to Ag-NPs in terms of colony formation except for *A. alternata* and *F. solani*, where EC_{50} values were 235 and >2000 μg/mL. Although significantly more toxic than in mycelial growth, CuO-NPs were less effective than both other NPs and the reference fungicide against fungal colony formation in all strain cases except for *M. fructicola* in which CuO-NPs were more effective than $Cu(OH)_2$ (see Table2. 4).

Table 2.4. Effect of NPs on spore germination in terms of colony formation of seven plant pathogenic fungal strains.

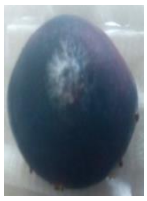
Fungal strain	EC_{50}[a] (μg m/L) (mean ± SD[b])				
	Ag-NPs	Cu-NPs	CuO-NPs	ZnO-NPs	$Cu(OH)_2$
B. cinerea	5.08 ± 0.30	3.05 ± 2.30	422.11 ± 15.39	16.29 ± 4.00	10.35 ± 0.10
A. alternata	235.55 ± 15.21	7.69 ± 1.00	678.20 ± 26.56	17.54 ± 3.61	255.82 ± 1.20
M. fructicola	50.60 ± 8.72	6.45 ± 0.27	215.02 ± 11.08	20.32 ± 2.10	355.51 ± 2.81
C. gloeospo-rioides	7.79 ± 1.14	17.44 ± 3.18	178.20 ± 23.12	10.36 ± 1.27	90.65 ± 12.34
F. solani	>2000	18.84± 2.44	>2000	6.81± 0.29	878.24± 30.17
FORL[c]	390.20± 35.34	29.04± 4.32	>2000	164.50± 24.31	25.23± 3.45
V. dahliae	2.36± 0.07	13.32± 0.81	252.12 ± 10.33	4.66± 0.14	15.25± 1.17

[a]EC_{50} = Effective concentration causing 50% reduction in number of colonies.
[b]Standard Deviation
[c]FORL: *F. oxysporum* fsp *Radicis Lycopersici*

2.4.4 Effect of NPs on grey mold symptom suppression

Grey mold symptom suppression properties of selected NPs in comparison with the reference fungicide containing $Cu(OH)_2$ are shown in Table 2.5. AgNPs were the most effective treatment exhibiting percent inhibition of *B. cinerea* disease symptoms equal with 85 and 100% at concentrations of 100 and 1000 μg/mL respectively. This was actually the only treatment that resulted in complete inhibition of symptoms. Cu-NPs treatment of plum fruit resulted in limited inhibition (16%) at the lower dose and a significantly higher inhibition (70%) compared to the reference fungicide at the higher dose (Table 2.5). ZnO-NPs showed inhibition rates which were not significantly different than the ones obtained by $Cu(OH)_2$ applied at any concentration. Disease suppression on plum fruit did not increase significantly by increasing doses 10-fold in both ZnO-NPs and $Cu(OH)_2$ cases (Table 2.5).

Table 2.5. Effect of NPs on symptom severity caused by *B. cinerea* spores inoculated on plum fruit.

Dose (μg/mL)	**% Inhibition**[a] Mean (± SD [b])				**Control** Lesion diameter in mm Mean (± SD [b])
	$Cu(OH)_2$	**ZnONPs**	**AgNPs**	**CuNPs**	
100	41.17 (± 5.32) b[c]	41.17 (± 7.56) b	85.29 (± 4.25) a	16.64 (± 3.22) c	17 (± 2.02)
					
1000	44.11 (± 4.11) c	47.05 (± 6.50) c	100 a	70.58 (± 8.10) b	
					

[a] Calculated as the percent inhibition in terms of observed lesion diameter compared to the untreated control.
[b] Standard Deviation of the Mean.
[c] Within rows, values followed by the same letter do not differ significantly according to Tukey's HSD test (α= 0.05).

2.4.5 Effect of NPs on hyphal and spore morphology

Microscopic observations under an optical microscope of NPs treated fungal conidia at non-lethal concentrations (equal to the respective EC_{50} values) have revealed certain

abnormalities in hyphae and germination tubes (see Fig. 2.3). Cu-NPs at a concentration of 3 μg/mL have resulted in deformation in hyphae, cytoplasm segmentation and exudates formation (see Fig. 2.3). Spores treated with NPs in EC_{50} value concentrations had abnormal tube elongation. Typical spore tube elongation involves hyphal branching in all directions in a concentric manner (see Fig. 2.3a). *B. cinerea* spores treated with copper NPs had germinating tubes advancing in a constantly changing direction without any branching as if searching for a less toxic path (see Fig. 2.2b). This apparently indicates the involvement of a chemiotactic mechanism for avoiding xenobiotics in this fungal strain.

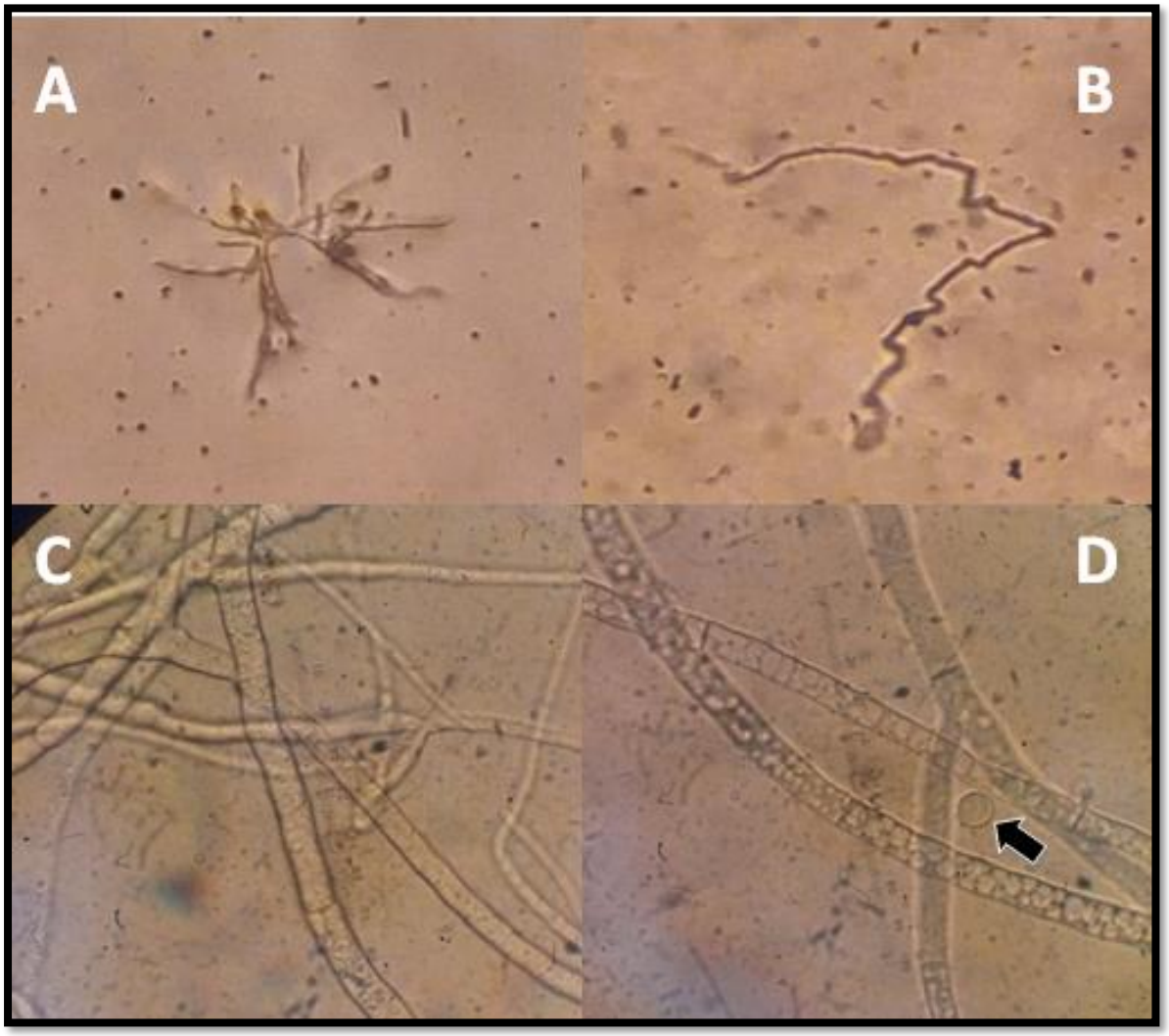


Figure 2.3. Microscopic observation of Botrytis cinerea conidia (top) and hyphae (bottom) treated (b, d) or untreated with Cu-NPs (a, c). Black arrow indicates abnormal hyphal swelling with cytoplasm exudates.

2.5 Discussion

In an attempt to evaluate the effectiveness of NPs against a number of important plant pathogenic fungi, the mean inhibitory concentrations of Cu, CuO, Ag and ZnO-NPs were determined in *in vitro* poison agar assays. Mycelial growth inhibition assays *in vitro* revealed

significant variations in sensitivity between fungal strains belonging to different species and between the NPs tested. Overall, the toxicity of NPs to fungal strains was in the order of: Cu-NPs>ZnO-NPs>Ag-NPs>CuO-NPs, considering the total number of sensitive strains and the range of sensitivity of tested species. Ashajyothi et al. (2016) reported that Ag-NPs were the most toxic to strains of *Aspergillus niger* and *Fusarium oxysporum* amongst Ag, Cu, Au and ZnO-NPs in zone inhibition mycelial growth assays. In another study, Ag-NPs exerted stronger overall inhibition than Cu-NPs against *Rhizoctonia solani, F. oxysporum and F. redolens* (Aleksandrowicz-Trzcinska et al., 2018), indicating that species-specific sensitivity probably accounts -at least partially- for variations between nanoparticle effectiveness.

In the present study, Cu-NPs was the most effective nano-sized compound against mycelial growth exhibiting significant inhibition at relatively low concentrations for all fungal strains tested. Mean sensitivity of all tested fungal strains to Cu-NPs was significantly higher than that of a commercial product containing $Cu(OH)_2$, except in the case of *A. alternata* that exhibited almost equal sensitivity to both compounds. Significant antifungal activity of Cu-NPs has been demonstrated in a number of fungal species including: *Fusarium sp., A. niger, R. solani, Alternaria solani, A.alternata, Phoma destructiva* (Pandey et al., 2016; Nemati et al., 2015; Aleksandrowicz-Trzcinska et al., 2018). Interestingly, copper-containing CuO-NPs were significantly less effective in inhibiting mycelial growth in all pathogens tested in this study. This is in alignment with previous studies on comparative toxicity of copper nanomaterials, which report a higher toxic effect of Cu-NPs compared with CuO-NPs (Keller et al., 2017). CuO-NPs effectiveness against *B. cinerea, A. alternata, A. fumigatus, F. solani* and *A. flavous* mycelial growth *in vitro* has been reported to be limited, while *B. cinerea* has been proposed to detoxify CuO-NPs by biotransformation to Cu-oxalate and subsequent extracellular secretion (Hao et al., 2017; Kovacec et al., 2017; Maqbool et al., 2017). ZnO-NPs were the second most effective NPs towards fungal strains used in this study. They exhibited satisfactory mycelial inhibition, which was greater or equal than that of $Cu(OH)_2$ in most of the fungal species tested.

Various size zinc oxide NPs acquired with different preparation methods were successfully tested against many bacteria and pathogenic fungi and their efficiency seems to depend on particle size and concentration (Sun et al., 2018). Silver NPs used in this study were practically effective against mycelial growth only in the case of *B. cinerea.* In fact, Ag-NPs and Cu-NPs were the most effective NPs against this fungus and performed better than the commercial fungicide containing copper hydroxide. Besides *B. cinerea*, several Ag-NPs studies have reported strong inhibition against a number of plant pathogenic fungi such as *C. gloeosporioides, Bipolaris sorokiniana, Rhizoctonia solani, Sclerotinia sclerotiorum, A. alternata and F. oxysporum* (Pandey et al., 2016). The larger particle sizes (<100 nm) of Ag-NPs used in the present study in comparison with the ones (4-21 nm) used in the above-mentioned studies could account for the lack of satisfactory mycelial inhibition of most of the fungal species tested.

Several mechanisms of antibacterial/antifungal action of NPs have been proposed including generation of Reactive Oxygen Species (ROS), disruption of microbial membranes by physical contact and the liberation of antimicrobial ions (Sun et al., 2018; Hoseinzadeh et

al., 2017; Król et al., 2017). Whether NPs act at a similar way as their bulk counterparts has been the subject of debate between researchers. In an attempt to answer this question, cross sensitivity of NPs used in the present study with reagents containing the respective bulk-sized metals was evaluated. Despite the potential differences in sensitivity between different fungal species tested, a positive correlation was found between both Cu containing NPs, but not with any of the bulk Cu containing antifungal agents [$CuSO_4$, $Cu(OH)_2$], indicating that Cu-NPs toxicity to fungal strains is not proportionally similar with bulk copper containing fungicides. Absence of statistical correlation between silver and zinc oxide NPs and their respective bulk counterparts also indicate differences in toxicity and potentially an alternate or additional mode of action, besides ion-release. Further studies at the biochemical level are needed to validate such a claim.

Regardless of their efficacy in inhibiting mycelial growth, toxicity of all NPs to fungal spores increased dramatically. Although there were differences in sensitivity between fungal species, in the majority of fungal cases, Cu-NPs were the most effective NPs followed by Zn-NPs, while both antifungal agents were able to inhibit colony formation at lower doses than the commercial fungicide $Cu(OH)_2$. Ag-NPs successfully inhibited spore germination/colony formation more effectively than $Cu(OH)_2$, except for soil-borne pathogen *F. solani*, which proved to be relatively tolerant to these NPs. Even though their activity against spore germination was drastically increased compared with that in mycelial growth, CuO-NPs were the less effective of the four NPs tested. It has been reported in the literature that the activity of silver, zinc oxide and gold NPs against *A. niger* and *F. oxysporum* was higher in spore germination than mycelial growth (Ashajyothi et al., 2016). Nemati et al. (2015) has demonstrated a stronger fungicidal effect of copper Cu-NPs against *F. expansum, A.solani* and *A. alternata* compared to a broad-spectrum fungicide containing copper (Bordeaux mixture) in terms of colony formation. Successful inhibition of colony formation, utilizing Ag-NPs against plant pathogenic fungi *B. sorokiniana* and *M. grisea*, was demonstrated by Jo et al. (2009). This increased toxic effect of NPs against fungal spores, compared to hyphal growth, could be attributed to structural differences between spore and vegetative fungal walls. Chitin content of many fungal species is significantly higher in hyphal walls, compared to spore walls, rendering the later more susceptible to heavy metals (Bartnicki-Garcia, 1968). Furthermore, during the process of spore germination, enzymes such as disulfide reductases and glucanases result in the softening of cell walls, in order to facilitate germ tube elongation, and thus create sensitive sites for toxic substances in contact with the fungal cell (Bartnicki-Garcia, 1968).

Potential suitability of NPs as alternative antifungal agents heavily depends on their effectiveness not only *in vitro* but primarily on their ability to exert their disease suppressing activity *in vivo* (Hao et al,2017). Assessing nanoparticle disease suppressive properties in field-scale experiments is hindered by practical difficulties mostly due to the lack of formulations of such substances. Detached fruit experiments provide an intermediate means of evaluating NPs effectiveness *in vivo* while very limited data is available in literature. In the present study, NPs spray applications on plum fruit resulted in significant disease suppression against *B. cinerea*. Ag-NPs and Cu-NPs were more effective than the reference fungicide at higher doses while Ag-NPs completely inhibited symptom development at the higher concentration. Hao et al

(2017) have reported successful *B. cinerea* symptom suppression on detached rose petals by carbon and copper nanoparticles while Ag-NPs were found to suppress disease symptoms of ryegrass pathogen *B. sorokiniana (*Jo et al, 2009). Although fungitoxic activity exerted by Cu-NPs *in vitro* against *B. cinerea* was similar to that of Ag-NPs, the later nanoparticles were dramatically more effective when applied *in vivo.* This observation demonstrates the importance of *in vivo* experiments for practical effectiveness evaluation of active substances. A possible explanation for such toxicity increase of Ag-NPs on plum fruit could be attributed to the differences between growth medium composition and the actual plant-tissue surface in contact with the fungal inoculum. PDA is a relatively rich growth medium and is expected to contain a higher protein content, rich in sulfide amino acids known to bind with [Ag^+], hindering dissolution of AgNPs or directly react with AgNPs to form precipitates without undergoing dissolution and thus could result in a reduction in toxicity compared with the plum fruit surface, where such substrate is not readily available (Levard et al., 2012; Zhang et al., 2016). In any case, interactions between AgNPs and organic matter is complex and a number of factors including nanoparticle coating and aggregation are involved, underlying mechanisms that differentiate toxicity of AgNPs between growth media and plant tissue have yet to be elucidated. Different inhibition levels of AgNPs against *B. cinerea* depending on the growth medium used in fungitoxicity tests *in vitro* have been reported by Kim et al. (2012).

All the above cases demonstrate a promising potential of NPs to be used as protective fungicides at early stages of disease initiation, by inhibiting spore germination of plant pathogenic fungi.

In summary, tested NPs were able to inhibit *in vitro* mycelial growth of fungal strains in a dose-response manner with the most effective being Cu-NPs and Zn-NPs. Although species dependent, the toxicity of NPs was not significantly correlated with the respective toxicity of their bulk counterparts, indicating potential differences in their mode of action. All NPs tested in this study were more toxic at the spore germination level than at mycelial growth and, in most cases, more effective than the commercial fungicide containing $Cu(OH)_2$. Furthermore, their suppressive antifungal properties extend to actual plant level, as indicated by their grey mold disease suppressive properties, making them excellent candidates for alternative, lower-dose, protective fungicides against both foliar and soil/seed-borne plant pathogenic fungi.

2.6 References

1. Aleksandrowicz-Trzcinska, M., Szaniawski, A., Olchowik, J., Drozdowski, S., Effects of copper and silver nanoparticles on growth of selected species of pathogenic and wood-decay fungi *in vitro* (2018) Forestry Chronicle, 94 (2), 109–116.
2. Ashajyothi, C., Prabhurajeshwar, C., Handral, H.K., Kelmani, C.R., Investigation of antifungal and anti-mycelium activities using biogenic nanoparticles: An eco-friendly approach (2016) Environmental Nanotechnology, Monitoring and Management 5, 81–87.
3. Banik, S., Pérez-de-luque, A.; *In vitro* effects of copper nanoparticles on plant pathogens, beneficial microbes and crop plants (2017) Spanish Journal of Agricultural Research, 15 (2), art. no.e1005.

4. Bartnicki-Garcia, Salomon. (1968). Cell Wall Chemistry, Morphogenesis, and Taxonomy of Fungi. Annual review of microbiology. 22. 87-108.

5. Das, R.K., Brar, S.K., Verma, M., Effects of different metallic nanoparticles on germination and morphology of the fungus *Rhizopus oryzae* 1526 and changes in the production of fumaric acid (2015) BioNano Science 5(4), 217–226.
6. FRAC (2013), List of Resistant Plant Pathogenic Organisms http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms---february-2013.pdf?sfvrsn=4 (accessed March 2017).

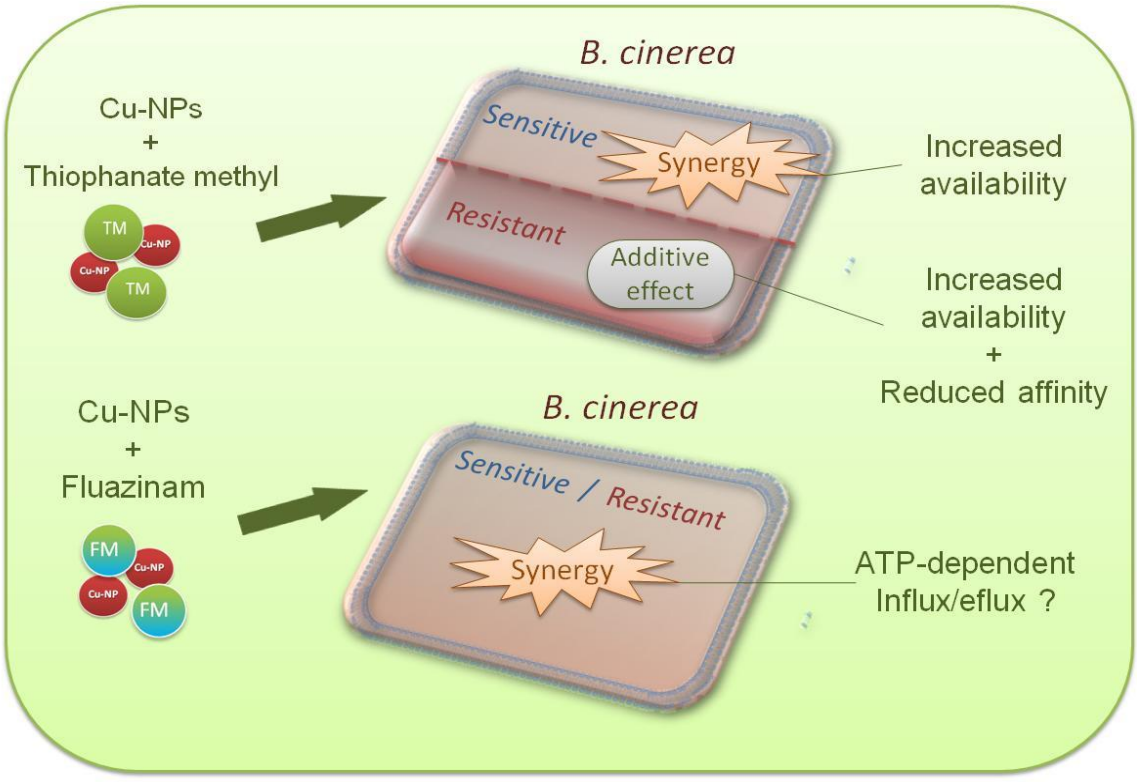
7. Hao, Y., Cao, X., Ma, C., Zhang, Z., Zhao, N., Ali, A., Hou, T., Xiang, Z., Zhuang, J., Wu, S., Xing, B., Zhang, Z., Rui, Y., Potential applications and antifungal activities of engineered nanomaterials against gray mold disease agent *Botrytis cinerea* on rose petals (2017) Frontiers in Plant Science, 8, art. no. 1332.
8. Happy Agarwal, Menon, S., Venkat Kumar, S., Rajeshkumar, S.; Mechanistic study on antibacterial action of zinc oxide nanoparticles synthesized using green route (2018) Chemico-Biological Interactions, 286, 60–70.
9. Hoseinzadeh, E., Makhdoumi, P., Taha, P., Hossini, H., Stelling, J., Kamal, M.A., Ashraf, G.Md. A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms (2017) Current Drug Metabolism, 18 (2), 120–128.
10. Huang, W., Wang, C., Duan, H., Bi, Y., Wu, D., Du, J., Yu, H. Synergistic antifungal effect of biosynthesized silver nanoparticles combined with fungicides (2018) International Journal of Agriculture and Biology, 20 (5), 1225–1229.
11. Jo, Y.-K., Kim, B.H., Jung, G.; Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi (2009) Plant Disease, 93 (10), pp. 1037-1043.

12. Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D.; A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues (2018) Nature Nanotechnology, 13 (8), 677–684.
13. Keller, A.A., Adeleye, A.S., Conway, J.R., et al., Comparative environmental fate and toxicity of copper nanomaterials (2017) NanoImpact 7, 28–40.
14. Kim, S.W., Jung, J.H., Lamsal, K., Kim, Y.S., Min, J.S., Lee, Y.S.; Antifungal effects of silver nanoparticles (AgNPs) against various plant pathogenic fungi (2012) Mycobiology, 40 (1), 53–58.
15. Kovačec, E., Regvar, M., van Elteren, J.T., Arčon, I., Papp, T., Makovec, D., Vogel-Mikuš, K.; Biotransformation of copper oxide nanoparticles by the pathogenic fungus *Botrytis cinerea* (2017) Chemosphere, 180, 178–185.
16. Król, A., Pomastowski, P., Rafińska, K., Railean-Plugaru, V., Buszewski, B., Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism (2017) Advances in Colloid and Interface Science 249, 37–52.
17. Lamsal, K., Kim, S.W., Jung, J.H., Kim, Y.S., Kim, K.S., Lee, Y.S., Application of silver nanoparticles for the control of Colletotrichum species in vitro and pepper anthracnose disease in field (2011) Mycobiology, 39 (3), 194–199.

18. Levard, C., Hotze, E.M., Lowry, G.V., Brown, G.E. Environmental transformations of silver nanoparticles: Impact on stability and toxicity (2012) Environmental Science and Technology, 46 (13), pp. 6900-6914.
19. Li, J., Sang, H., Guo, H., Popko, J.T., He, L., White, J.C., Parkash Dhankher, O., Jung, G., Xing, B.; Antifungal mechanisms of ZnO and Ag nanoparticles to *Sclerotiniahomoeocarpa* (2017) Nanotechnology, 28 (15), art. no. 155101.
20. Malandrakis, A.A., Apostolidou, Z.A., Louka, D., Markoglou, A., Flouri, F.Biological and molecular characterization of field isolates of *Alternariaalternata* with single or double resistance to respiratory complex II and III inhibitors (2018) European Journal of Plant Pathology, 152 (1), 199–211.
21. Malandrakis, A., Koukiasas, N., Veloukas, T., Karaoglanidis, G., Markoglou, A. Baseline sensitivity of *Monilinia laxa* from Greece to fenhexamid and analysis of fenhexamid-resistant mutants (2013) Crop Protection, 46, 13–17.
22. Malkapur, D., Devi, M.S., Rupula, K., Sashidhar, R.B. Biogenic synthesis, characterisation and antifungal activity of gum kondagogu-silver nano bio composite construct: Assessment of its mode of action (2017) IET Nanobiotechnology, 11 (7), 866–873.
23. Maqbool, Q., Iftikhar, S., Nazar, M., Abbas, F., Saleem, A., Hussain, T., Kausar, R., Anwaar, S., Jabeen, N. Green fabricated CuO nanobullets via *Oleaeuropaea* leaf extract shows auspicious antimicrobial potential (2017) IET Nanobiotechnology, 11 (4), 463–468.
24. Matei, P.M., Martín-Gil, J., Iacomi, B.M., Pérez-Lebeña, E., Barrio-Arredondo, M.T., Martín-Ramos, P.; Silver nanoparticles and polyphenol inclusion compounds composites for *Phytophthoracinnamomi* mycelial growth inhibition (2018) Antibiotics, 7 (3), art. no. 76.
25. Narendhran, S., Sivaraj, R., Biogenic ZnO nanoparticles synthesized using *L. aculeata* leaf extract and their antifungal activity against plant fungal pathogens (2016) Bulletin of Materials Science, 39 (1), 1–5.
26. Nemati, A., Shadpour, S., Khalafbeygi, H., Ashraf, S., Barkhi, M., Soudi, M.R.; Efficiency of hydrothermal synthesis of nano/microsized copper and study on *in Vitro* antifungal activity (2015) Materials and Manufacturing Processes, 30 (1), 63–69.
27. Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks (2018) Proceedings of the National Academy of Sciences India Section B - Biological Sciences, 88 (4), 1287–1308.
28. Pařil, P., Baar, J., Čermák, P., Rademacher, P., Prucek, R., Sivera, M., Panáček, A.; Antifungal effects of copper and silver nanoparticles against white and brown-rot fungi (2017) Journal of Materials Science, 52 (5), 2720–2729.
29. Park, J.-W., Lee, I.-C., Shin, N.-R., Jeon, C.-M., Kwon, O.-K., Ko, J.-W., Kim, J.-C., Oh, S.-R., Shin, I.-S., Ahn, K.-S. Copper oxide nanoparticles aggravate airway inflammation and mucus production in asthmatic mice via MAPK signaling (2016) Nanotoxicology, 10 (4), pp. 445-452.
30. Paplomatas, E.J., Tjamos, S.E., Malandrakis, A.A., Kafka, A.L., Zouvelou, S.V. Evaluation of compost amendments for suppressiveness against Verticillium wilt of eggplant and study of mode of action using a novel Arabidopsispatho system (2005) European Journal of Plant Pathology, 112 (2), 183–189.

31. Sun, Q., Li, J., Le, T. Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives (2018) Journal of Agricultural and Food Chemistry, 66 (43), 11209–11220.
32. Venat, O., Iacomi, B., Peticila, A.G., In Vitro studies of antifungal activity of colloidal silver against important plants pathogens (2018) Notulae Botanicae Horti Agrobotanici Cluj-Napoca 46(2), 533–537.
33. Wani, A.H., Shah, M.A. A unique and profound effect of MgO and ZnOnanoparticles on some plant pathogenic fungi (2012) Journal of Applied Pharmaceutical Science, 2 (3), 40–44.
34. Zhang, C., Hu, Z., Deng, B. Silver nanoparticles in aquatic environments: Physiochemical behavior and antimicrobial mechanisms (2016) Water Research, 88, pp. 403-427.
35. Ziogas, B.N., Markoglou, A.N., Malandrakis, A.A. Studies on the inherent resistance risk to fenhexamid in *Botrytis cinerea* (2003) European Journal of Plant Pathology, 109 (4), 311–317.

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



Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Synergy between Cu-NPs and fungicides against Botrytis cinerea (2020) Science of the Total Environment, 703, art. no. 135557, DOI: 10.1016/j.scitotenv.2019.135557.

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

Abstract

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

3.1 Introduction

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

number of fungal pathogens compared to fungicides containing copper (Baker et al., 2002; Winter and Davis, 2006; Park et al., 2016; Keller et al., 2017, Malandrakis et al., 2019).

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

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

This study focuses on the: (a) identification of fungicide resistant phenotypes among *Botrytis cinerea* isolates, (b) evaluation of the control efficacy of Cu-NPs and Cu-NPs/fungicide combinations against sensitive and fungicide resistant *B. cinerea* isolates in *vitro* and *in vivo*, and (c) investigation of possible mechanisms underlying the mode of action of Cu-NPs and the synergistic/additive interactions observed between Cu-NPs and selected

fungicides. To the best of our knowledge, the potential of copper nanoparticles to control fungal isolates resistant to fungicides alone or in combination with conventional fungicides has not been previously explored.

3.2 Materials and Methods

3.2.1 Nanoparticles, reagents and fungicides

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

3.2.2 Fungal isolates and culture conditions

Fifteen fungal *B. cinerea* isolates from the fungal collection of the Pesticide Science Lab (Agricultural University of Athens), originating from various crop fields located in Greece (Table 3.1), were evaluated in terms of their sensitivity against Cu-NPs and selected botrycides. All isolates were grown on Potato Dextrose Agar (PDA) medium in order to obtain inoculum for the fungitoxicity assays and kept in growth chambers in the dark at 25 °C and 70% humidity. For each strain, for long-term storage purposes, four 5-mm mycelial plugs from the margin of rapidly growing fungal colonies were placed in 1.5 mL tubes containing 50% v/v of sterilized glycerol:water, and stored at -20 °C.

Table 3.1. Origin of *Botrytis cinerea* isolates used in this study and spray history of the fields from which the isolates were obtained.

Isolate	Host	Location	Year of collection	Spray history				
				Thiophan-ate methyl	Pyraclo-strobin	Fenhe-xamid	Fludiox-onil	Manco-zeb
BC1	apple	Veroia	2017	+ [a]	+		+	+
BC2	apple	Veroia	2017	+	+		+	+
BC3	peach	Veroia	2017	+	+		+	+
BC4	apple	Veroia	2017	+	+		+	+
BC5	grape	Crete	2017	+	+		+	
BC6	cherry	Crete	2017	+	+		+	
BC7	strawberry	Pelloponisos	2017	+	+	+	+	+
BC8	strawberry	Pelloponisos	2017	+	+	+	+	+
BC9	strawberry	Pelloponisos	2017	+	+	+	+	+
BC10	pepper	Pelloponisos	2017	+	+	+	+	+
BC11	cucumber	Crete	2018	+	+	+	+	
BC12	plum	Crete	2018	+	+		+	
BC13	plum	Crete	2018	+	+		+	
BC14	plum	Crete	2018	+	+		+	
BC15	plum	Crete	2018	+	+		+	

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

3.2.3 *In vitro* fungitoxicity tests

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

In order to evaluate the potential of Cu-NPs to control fungicide-resistant *B. cinerea* isolates as well as sensitive ones, *in vitro* fungitoxicity tests were conducted. The sensitivity of fungal strains to NPs and fungicides was evaluated by measuring radial growth of isolates on PDA. The fungitoxicity was expressed as percent relative growth of isolates grown on PDA

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

3.2.3.2 Synergistic activity of Cu-NPs with fungicides

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

3.2.4 *In vivo* fungitoxicity tests

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

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

Mycelium of selected *B. cinerea* isolates was peeled from the surface of 4-day old cultures grown on fungicide-free PDA at 25 ^{o}C, and ground in liquid nitrogen using a mortar and pestle. TRI reagent (Sigma) was used to isolate the total DNA. A 1.6-kb fragment of the *B. cinerea* *β*-tubulin gene was amplified using the primers BCTubF (5' CTTGAGCGTATGAACGTCTAC 3') and BCTubR (5' TGTACCAATGCAAGAAAGCCTT 3') from the template gDNA. The PCR reactions involved 0.2 mM from each of the primers, 1.5 mM $MgCl_2$, 0.5mM dNTPs, and 1.25 units of HotStar Taq DNA polymerase (Qiagen) in 20 mM TrisHCl and 50 mM KCl. The PCR conditions were as follows: 95 ^{o}C for 15 min followed by 40 cycles of 94 ^{o}C for 30 s, 50 ^{o}C for 30 s, and 72 ^{o}C for 1 min with a final 10 min extension at 72 ^{o}C. A 463-bp fragment of the *B. cinerea cytb* gene was amplified using the primers Bccytb-F1 (5' CGTCGGCCATATAAAAGGTC 3') and Bccytb-R1 (5' CTCCATCCACCATACCTACA 3') from template gDNA under PCR conditions of 95 ^{o}C for 15 min followed by 35 cycles of 94 ^{o}C for 1 min, 65 ^{o}C for 25 s, and 72 ^{o}C for 1 min with a final 10 min extension at 72 ^{o}C. The QIAquick gel extraction kit (Qiagen) was used to purify

the PCR products, which were subsequently ligated to pGEM-Teasy (Promega) vectors and transformed into *Escherichia coli* (DH5a Library Efficiency® Competent Cells, Invitrogen) competent cells. Recombinant plasmids were purified using QIAprep spin miniprep kit plasmid (Qiagen) and then sequenced in both directions. Ten independent clones from each *B. cinerea* isolate were analyzed. Sequence data analysis was performed by use of the Lasergene software (DNAstar, Madison, USA).

3.3 Statistical analysis

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

3.4 Results

3.4.1 Sensitivity of *B. cinerea* isolates to antifungal agents *in vitro*

The sensitivity of *B. cinerea* isolates to selected fungicides against the pathogen was evaluated using discriminatory doses, based on mean EC_{50} values reported in previous studies (Kalamarakis et al., 2000; Malandrakis et al., 2011; Markoglou et al., 2006). The observed mean percent inhibition caused by Cu-NPs and each of the fungicides used in this study are shown in Table 2. Although there was significant variability between the various isolates, in most cases, wild type (baseline) sensitivity to the antifungal agents used was observed. A reduced sensitivity to the benzimidazoles thiophanate-methyl and carbendazim was detected for the isolates BC4, BC5 and BC11 (mean percent inhibition values ranging from 0 to 1.53). Additionally, the isolates BC4, BC5, BC11, and BC13 showed a reduced sensitivity response to the QoI fungicide pyraclostrobin (see Table 3.2). Consequent fungitoxicity tests were conducted to determine the EC_{50} values and to calculate the resistance factors (Rfs) of the resistant phenotypes (see Table 3.3). Based on calculated Rf values, isolates BC4, BC5 and BC11 were classified as highly benzimidazole resistant (BEN-R phenotype), because they were highly resistant to both carbendazim and thiophanate methyl. The calculated Rf values were >100 for thiophanate methyl and >200 for carbendazim in all BEN-R isolates. All BEN-R isolates were more sensitive (Rf values 0.06-0.1) to the benzimide zoxamide than the wild type isolates BC1 and BC2. Pyraclostrobin resistant isolates (PYR-R phenotype) included the BEN-R isolates BC4, BC5, BC11 and the isolate BC13, which was specifically pyraclostrobin-resistant. All PYR-R isolates were highly resistant to pyraclostrobin with Rf values exceeding 100 (see Table 3.3).

Table 3.2 Sensitivity of *Botrytis cinerea* isolates to Cu-NPs and selected fungicides.

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

[a] Calculated as percent inhibition of mycelial growth compared to the untreated control after 4 days incubation at 22 °C (n = 3).
[b] Standard deviation of the means (n = 3).
[c] Numbers in parenthesis indicate fungicide concentrations in µg/mL of active ingredient.

Table 3.3 Cross-resistance profiles of *B. cinerea* isolates sensitive and benzimidazole /pyraclostrobin resistant and respective resistance mutations.

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

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

3.4.2 Detection of target-site resistance mutations

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

3.4.3 Synergistic activity

3.4.3.1 Synergistic activity *in vitro*

The ability of Cu-NPs to control sensitive and resistant isolates in combination with thiophanate methyl, pyraclostrobin, and fluazinam was tested *in vitro*. The calculated synergistic factor (SF) values for Cu-NPs are listed in Table 3.4. An enhanced synergistic effect for Cu-NPs with fluazinam was observed in most cases of both fungicide sensitive and resistant isolates, with estimated SF values ranging from 1.01 to 1.50 (see Table 3.4, Fig. 3.1c). On the contrary, an antagonistic effect was observed (SF: 0.41- 0.97) for the combined use of Cu-NPs and pyraclostrobin in most isolate cases. For the BC4 isolate, synergy was observed (SF=1.22), for the combined use of Cu-NPs and pyraclostrobin mixture; however, inhibition rates were barely statistically significant different from those corresponding to individual use of Cu-NPs (see Fig. 3.1b). The combined use of Cu-NPs with thiophanate methyl resulted in inhibition rates, which were significantly greater than those for the individual antifungal agents in all BEN-S isolates (see Fig. 3.1a). In BEN-R isolates, no significant differences were found between Cu-NPs sensitivity and the combined use of Cu-NPs and thiophanate methyl (see Fig. 3.1a). SF values between Cu-NPs and thiophanate methyl for the BEN-S isolates ranged between 1.03 to 1.59, indicating synergism, while the respective values for BEN-R isolates were close to 1 (0.85 to 0.99) , indicating a slight additive effect (see Table3.4, Fig. 3.2).

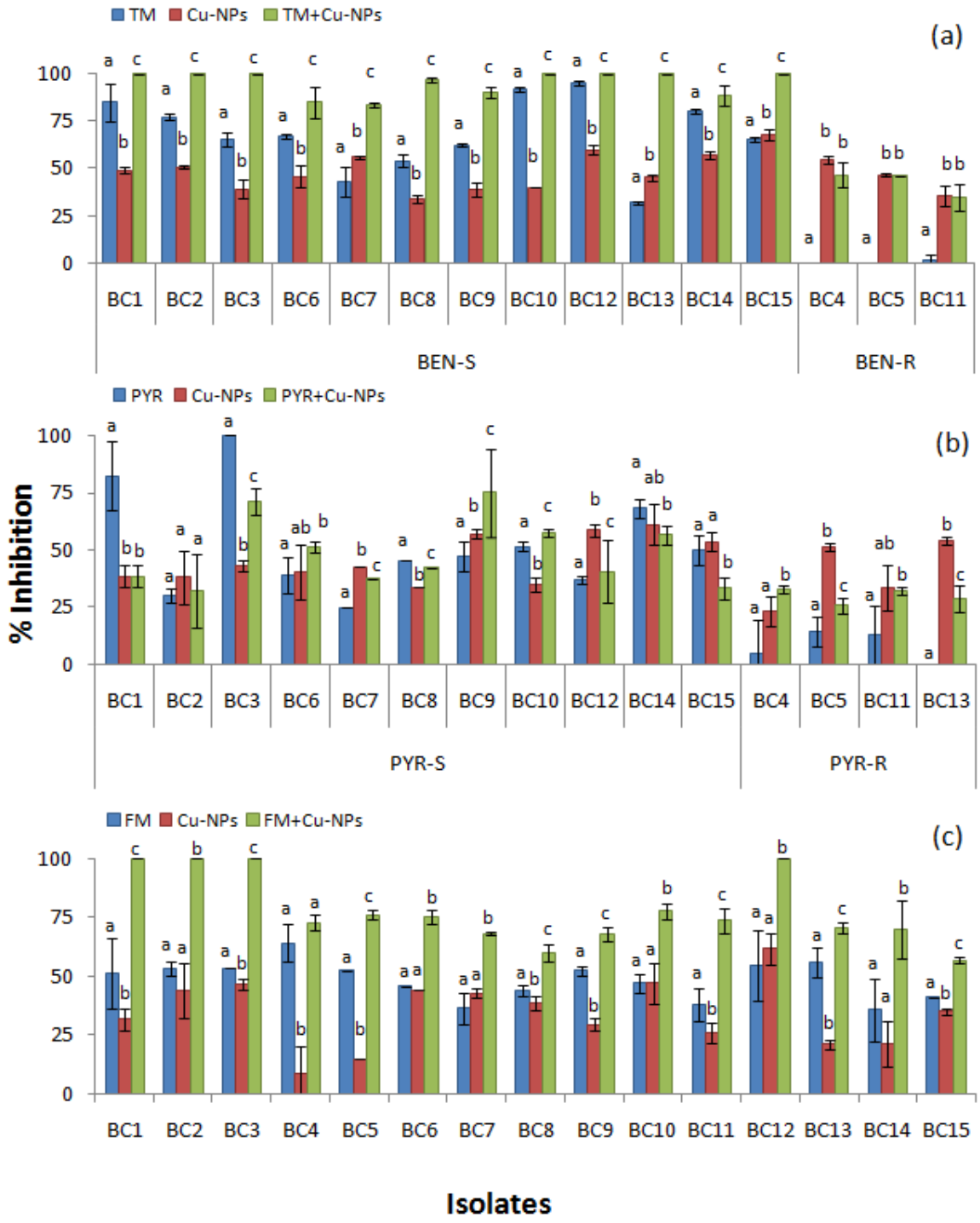


Figure 3.1. Sensitivity of fungicide-sensitive/resistant B. cinerea isolates to Cu-NPs (300 µg/mL) in comparison with (a) thiophanate methyl (0.25 µg/mL) (b) pyraclostrobin (0.15 µg/mL) and (c) fluazinam (0.02 µg/mL) and combinations. BEN-S/R: benzimidazole- Sensitive / Resistant and PYR-S/R: pyraclostrobin-Sensitive / Resistant isolates. TM:thiophanate methyl, FM: fluazinam). Error lines represent the standard deviation of means. Between treatments, bars marked by the same letter do not differ significantly according to Tukey's HSD test (α = 0.05).

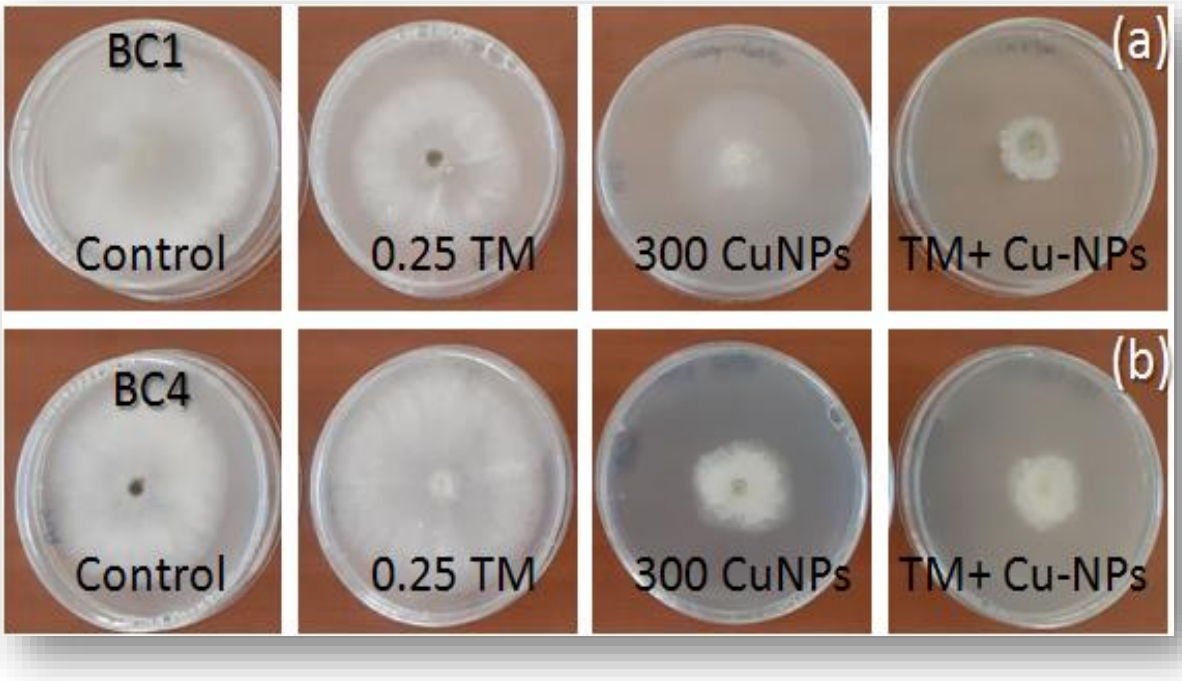


Figure 3.2 Sensitivity of a (a) sensitive (BC1) and a (b) TM-resistant (BC4) B. cinerea isolates to Cu-NPs, TM and their combination. TM: thiophanate methyl.

In an attempt to evaluate the role of nanoparticle nature versus the release of copper ions in the synergistic effect observed between Cu-NPs and fluazinam or thiophanate methyl, a commercial fungicide containing $Cu(OH)_2$ was used in a synergism test *in vitro* with the above fungicides. In contrast to Cu-NPs, the combined use of $Cu(OH)_2$ with fluazinam resulted in an antagonistic effect in almost all the isolate cases with SF values ranging between 0.14 and 1.01 (see Table 3.4), indicating a possible contribution of nanoparticle properties in the observed synergism between Cu-NPs and fluazinam. A strong antagonistic effect was observed between $Cu(OH)_2$ and thiophanate methyl in BEN-R isolates (SF: 0.23-0.72), contrary to BEN-S isolates where a strong synergistic effect (Rf: 1.00-3.97) was observed in most cases (see Table 3.4). These results indicate a possible role of the copper ions released in the observed synergism between Cu-NPs and thiophanate methyl. A possible role of copper ion release in the fungitoxic activity of Cu-NPs against *B. cinerea* isolates was revealed by the synergy tests conducted between Cu-NPs and NaCl. These synergy tests involved the addition of 1% NaCl in PDA containing 300 μg/mL Cu-NPs, which neutralized the fungitoxic effect of Cu-NPs, and yielded very low SF values in the majority of isolate cases (see Table 3.4).

Table 3.4 *In vitro* synergistic activity of Cu-NPs or $Cu(OH)_2$ with selected fungicides against fungicide sensitive and resistant *Botrytis cinerea* isolates. TM: thiophanate methyl, FM: fluazinam.

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

[a] Synergy Factor.

[b] BEN-S/R: Benzimidazole Sensitive/ Resistant and PYR-S/R: Pyraclostrobin sensitive/Resistant isolate.

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

3.4.3.2 Synergistic activity *in vivo*

The synergistic activity between Cu-NPs, thiophanate methyl and fluazinam demonstrated *in vitro* was tested on apple fruit for selected BEN-S and BEN-R/PYR-R *B. cinerea* isolates. Treatment of apple fruit inoculated with fungicide sensitive isolates BC1 and BC2 with 500 µg/mL Cu-NPs resulted in inhibition rates of 30.95 and 27.27%, while 20 µg/mL thiophanate methyl resulted in 54.76 and 43.18% inhibition (SF:1.45 and 1.70, respectively, see Table 5). When the two antifungal agents were applied in combination, disease symptoms caused by BC1 and BC2 BEN-S isolates were almost completely suppressed, demonstrating a strong synergistic effect (see Fig. 3.3). In the case of BEN-R isolates BC4 and BC11, thiophanate methyl could not suppress lesion development even at 2000 µg/mL, while combination with Cu-NPs had a slight additive effect (SF: 0.93 to 0.96, respectively, see Table 3.5). Combination of Cu-NPs with fluazinam resulted in a synergistic effect *in vivo* similar to that observed in the *in vitro* experiments (see Fig. 3.3). A clear synergistic effect was observed in all *B. cinerea* phenotypes with SF values ranging from 1.31 to 1.51, indicating a potential of the above nanoparticles to control both sensitive and resistant isolates in mixtures with FM (see Table 3.5).

Table 3.5 Synergistic activity of Cu-NPs co-applied with thiophanate mehyl or fluazinam on apple fruit against Botrytis cinerea isolates sensitive and resistant to selected fungicides. TM: thiophanate methyl, FM: fluazinam.

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

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

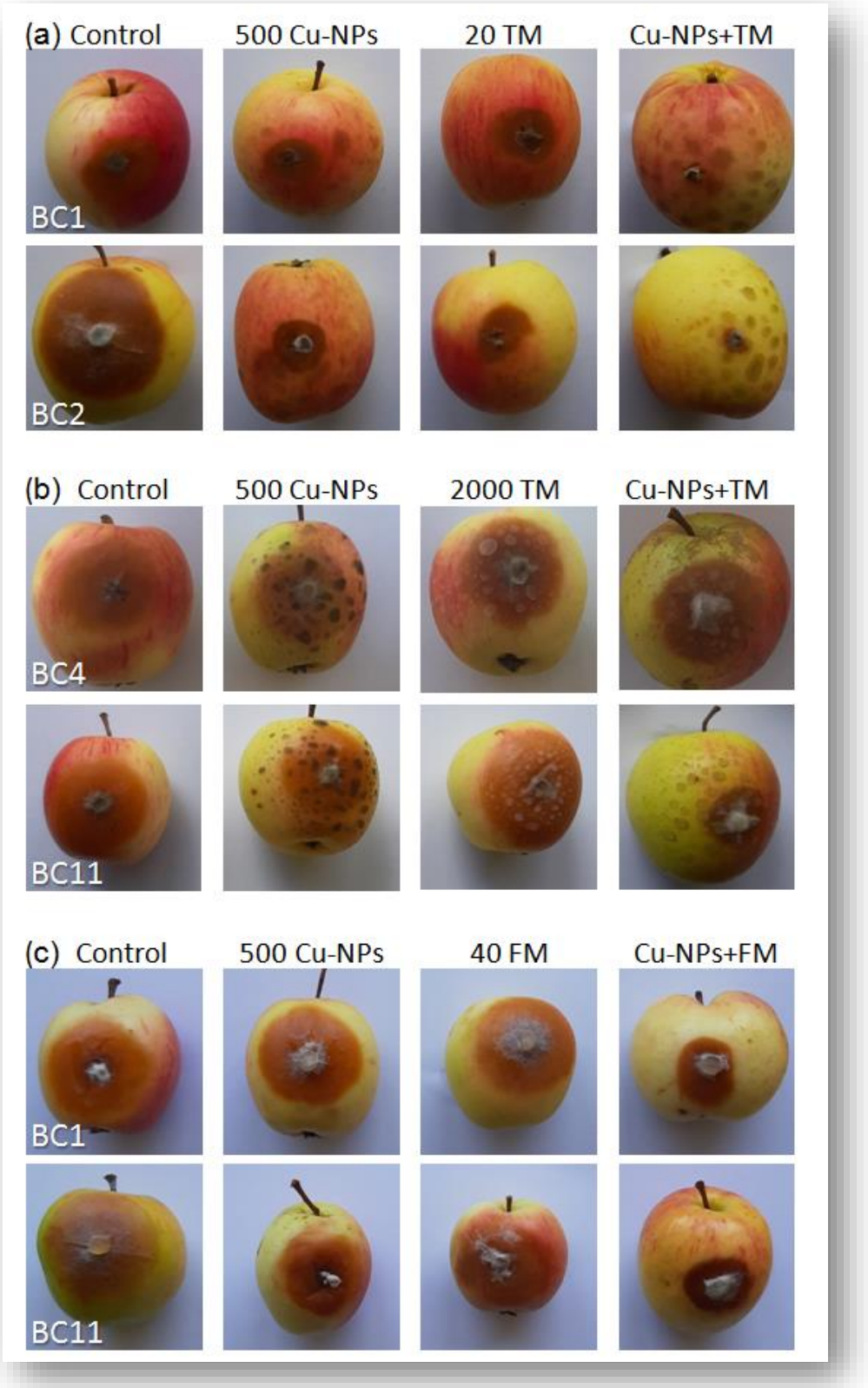


Figure 3.3 Synergistic activity of Cu-NPs (500 µg/mL) in combination with thiophanate methyl (20, 2000 µg/mL) or fluazinam (40 µg/mL) on apple fruit against selected Botrytis cinerea isolates sensitive (BC1, BC2) and resistant (BC4, BC11) to thiophanate methyl. TM: thiophanate methyl, FM: fluazinam.

3.4.4 *B. cinerea* sensitivity correlations

In an attempt to investigate the contribution of Cu-NPs, thiophanate methyl (TM), fluazinam (FM) and $Cu(OH)_2$ in the observed synergistic effect of the respective mixtures, Pearson correlation coefficient values were calculated (see Table 3.6). No significant correlation was found between Cu-NPs and TM, FM or any of their combinations (see Table 3.6). On the contrary, a significant correlation was found between TM, Cu-NPs+TM and $Cu(OH)_2$+TM treatments (see Fig. 4a,b). This result indicates that the observed enhanced inhibitory effect of the above mixtures is probably related with the action of TM rather than that of either Cu-NPs or $Cu(OH)_2$. This was also evident by the positive correlation observed between Sunspot and $Cu(OH)_2$+TM treatments (see Table 3.6). An interesting positive correlation was found between Cu-NPs sensitivity and NaCl, which possibly indicates a common mechanism of fungitoxic action between the two compounds (see Fig. 4c).

Table 3.6. Correlation between sensitivity of *B. cinerea* isolates to Cu-NPs, $Cu(OH)_2$, selected fungicides and their combinations.

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

[a] Pearson correlation coefficient values.

* corresponds to a significance lever of P=0.05.

** corresponds to a significance lever of P=0.01.

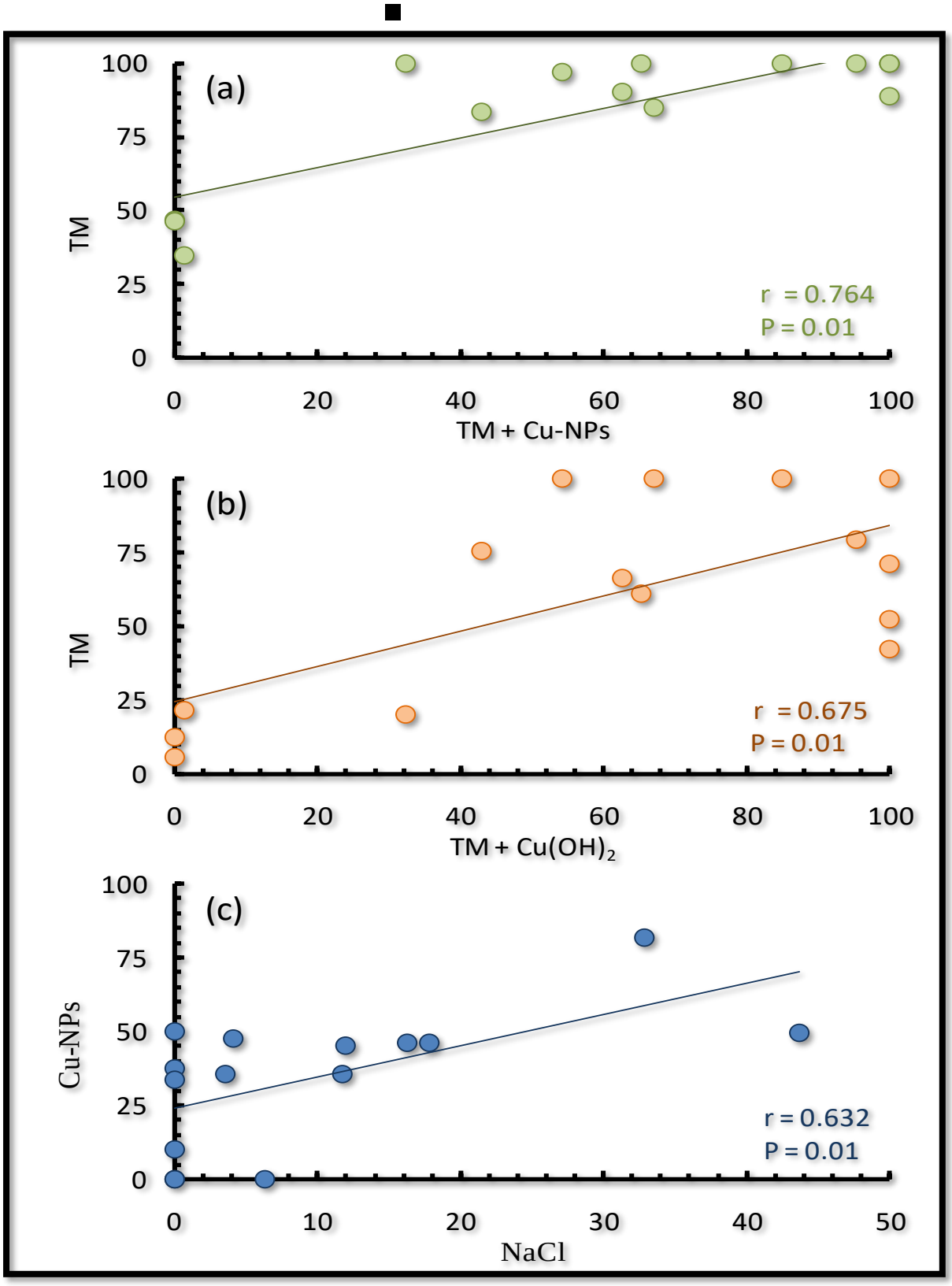


Figure 3.4 Correlation between sensitivities of B. cinerea isolates to thiophanate methyl (TM) (0.25 µg/mL) and (a) Cu-NPs (300 µg/mL), (b) $Cu(OH)_2$ (500 µg/mL) , and between (c) Cu-NPs (300 µg/mL) and NaCl (10^4 µg/mL) in terms of percent inhibition. Here, r is the Pearson correlation coefficient, and P the significance level.

3.4.5 Discussion

In order to evaluate Cu-NPs potential to control fungicide sensitive and resistant isolates, a sensitivity screening of 15 *B. cinerea* isolates to 9 fungicides was undertaken utilizing *in vitro* bioassays. Three of the isolates tested were highly resistant to benzimidazoles thiophanate methyl and carbendazim (BEN-R) due to a well-known target site mutation (E198A) in the *B. cinerea β*-tubulin gene, as revealed by DNA sequencing. The above mutation has been related to high levels of benzimidazole resistance in many plant pathogenic fungi, including *B. cinerea* (Leroux et al. 2002; Ziogas et al., 2009; Ma Michailides 2005; FRAC 2013). The above isolates as well as an additional isolate (BC13) were also highly resistant to the QoI fungicide pyraclostrobin. All pyraclostrobin resistant isolates (PYR-R) harbored the G143A resistance mutation in their mitochondrial *cytb* gene. An extensive list of plant pathogens that developed high resistance to QoI fungicides due to the G143A mutation have been explored over the last decade by a large number of studies (FRAC 2013; Malandrakis et al., 2011; Avenot and Michailides 2015; Malandrakis et al., 2018). The effect of Cu-NPs, selected fungicides and their combinations against the above resistant phenotypes were investigated.

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

In the present study, copper nanoparticles were equally effective against sensitive and BEN-R or PYR-R *B. cinerea* isolates. The combination of Cu-NPs with TM *in vitro* resulted in an enhanced inhibition of BEN-S *B. cinerea* isolates, compared to individual treatments. This observation indicates a synergistic interaction, which was more profound in *in vivo* experiments where even 50-fold decrease in the recommended TM dose in combination with Cu-NPs could fully suppress disease symptoms. Inhibition of BEN-R isolates by the Cu-NPs/TM mixture was not statistically different from that of the individual Cu-NPs treatment *in vitro*. This difference in synergistic interaction of Cu-NPs and TM between BEN-S and BEN-R isolates and the positive correlation found between TM and TM+Cu-NPs treatments indicate that sensitivity to TM is a key factor behind the observed synergism. These results lead to the hypothesis that in Cu-NPs+TM treatments a higher dose of the active ingredient of TM is

available, in contact with its target site inside the fungal cell. A number of mechanisms could accomplish such an increased availability of the fungicide to its target site, including increased uptake, decreased efflux activity or faster transformation of the fungicide to a more toxic form inside the fungal cell (Jampilek, 2016). Even though the role of Cu-NPs in the above mechanisms is not certain, we speculate that a potential involvement of fungal influx/efflux pumps could contribute to a higher accumulation of TM inside the fungal cell. The involvement of the ABC transporter BcmfsM2 in efflux regulation of carbendazim leading to resistance in *B. cinerea* has been demonstrated by Leroux et al. (2013). Increased availability of the fungicide is consistent with the lack of synergism in the case of BEN-R isolates where additional TM concentration would not lead to enhanced toxicity, because of the reduced affinity of *β*-tubulin with TM resulting from the target site mutation E198A. An indication that the fungitoxic activity of Cu-NPs is associated with ATP-dependent metabolism is the enhanced inhibition rates observed in all isolate cases when the Cu-NPs/fluazinam mixture was used, compared to the inhibition caused by the individual antifungal agents. Fluazinam is a known ATP-synthetase inhibitor preventing oxidative phosphorylation and thus resulting in energy starvation and subsequent impairment of energy-dependent efflux pumps (Kalamarakis et al., 2000; Leroux et al. 2013). A decreased efflux pump activity regulating metal ion homeostasis caused by fluazinam could result in an increased accumulation of Cu-NPs inside the fungal cell and account for the observed increased fungitoxic effect of the Cu-NPs/FM mixture. Certainly, further studies are needed to validate such a hypothesis. A decrease in multi-drug resistance transporter activity of the marine organism *Mytilus galloprovincialis* following treatment with CuO-NPs or copper ions, as well as dysregulation of efflux pumps associated with the ability of Ag-NPs to control *Candida albicans* MDR strains in combination with antibiotics has been reported in the literature (Torres-Duarte et al., 2019; Sun et al., 2016).

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

Concluding, Cu-NPs were effective against *B. cinerea* isolates, sensitive and resistant to benzimidazoles and pyraclostrobin while their antifungal activity was in most cases enhanced when applied in combination with fluazinam or thiophanate methyl both *in vitro* and *in vivo.* Indications that [Cu^{+2}] cations and ATP-dependent metabolism are involved in the

fungitoxic action and the synergistic interactions of Cu-NPs with tested fungicides were found. The results of this study suggest that Cu-NPs are promising antifungal alternatives, suitable both for effective anti-resistance strategies and a means for reducing environmental pollution caused by synthetic fungicides.

3.4.6 References

Avenot, H. F., & Michailides, T. J. (2015). Detection of isolates of *Alternaria alternata* with multiple-resistance to fludioxonil, cyprodinil, boscalid and pyraclostrobin in California pistachio orchards. Crop Protection, 78, 214–221.

Baker, B.P., Benbrook, C.M., Groth, E., Benbrook, K.L., Pesticide residues in conventional, integrated pest management (IPM)-grown and organic foods: Insights from three US data sets (2002) Food Additives and Contaminants, 19 (5), pp. 427-446.

Brent, K. J., & Hollomon, D. W. (1998). Fungicide Resistance: The assessment of risk. FRAC Monograph No 2, pp. 1–48. Brussels: Global Crop Protection Federation.

FRAC (2013), List of Resistant Plant Pathogenic Organisms http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms---february-2013.pdf?sfvrsn=4 (accessed March 2017).

Gabrielyan, L., Hakobyan, L., Hovhannisyan, A., Trchounian, A., Effects of iron oxide (Fe_3O_4) nanoparticles on *Escherichia coli* antibiotic-resistant strains (2019). Journal of Applied Microbiology, 126 (4), pp. 1108-1116.

Gisi, U., Synergistic interaction of fungicides in mixtures (1996) Phytopathology, 86 (11), pp. 1273-1279.

Hamed, S., Emara, M., Shawky, R.M., El-domany, R.A., Youssef, T. ,Silver nanoparticles: Antimicrobial activity, cytotoxicity, and synergism with N-acetyl cysteine (2017). Journal of Basic Microbiology, 57 (8), pp. 659-668.

Hoseinzadeh, E., Makhdoumi, P., Taha, P., Hossini, H., Stelling, J., Kamal, M.A., Ashraf, G.Md. A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms (2017) Current Drug Metabolism, 18 (2), 120–128.

Huang, W., Wang, C., Duan, H., Bi, Y., Wu, D., Du, J., Yu, H., Synergistic antifungal effect of biosynthesized silver nanoparticles combined with fungicides (2018). International Journal of Agriculture and Biology, 20 (5), 1225–1229.

Jamdagni, P., Rana, J.S., Khatri, P., Comparative study of antifungal effect of green and chemically synthesised silver nanoparticles in combination with carbendazim, mancozeb, and thiram (2018) IET Nanobiotechnology, 12 (8), pp. 1102-1107.

Jampilek, J., How can we bolster the antifungal drug discovery pipeline? (2016) Future Medicinal Chemistry, 8 (12), pp. 1393-1397.

Jo, Y.-K., Kim, B.H., Jung, G.; Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi (2009) Plant Disease, 93 (10), pp. 1037-1043.

Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D.; A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues (2018) Nature Nanotechnology, 13 (8), 677–684.

Kalamarakis, A.E., Petsikos-Panagiotarou, N., Mavroidis, B., Ziogas, B.N., Activity of fluazinam against strains of *Botrytis cinerea* resistant to benzimidazoles and/or dicarboximides and to a benzimidazole-phenylcarbamate Mixture (2000). Journal of Phytopathology, 148 (7-8), pp. 449-455.

Keller, A.A., Adeleye, A.S., Conway, J.R., et al., Comparative environmental fate and toxicity of copper nanomaterials (2017) NanoImpact 7, 28–40.

Król, A., Pomastowski, P., Rafińska, K., Railean-Plugaru, V., Buszewski, B., Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism (2017) Advances in Colloid and Interface Science 249, 37–52.

Leroux, P., Walker, A.-S.,Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance (2013) European Journal of Plant Pathology, 135 (4), pp. 683-693.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. (2002). Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Management Science, 58, 876–888

Ma, Z., Michailides, T.J., Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi (2005). Crop Protection, 24 (10), pp. 853-863.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019). Science of the Total Environment, 670, pp. 292-299.

Malandrakis, A.A., Apostolidou, Z.A., Louka, D., Markoglou, A., Flouri, F., Biological and molecular characterization of field isolates of *Alternaria alternata* with single or double resistance to respiratory complex II and III inhibitors (2018). European Journal of Plant Pathology, 152 (1), 199–211.

Malandrakis, A., Markoglou, A., Ziogas, B., Molecular characterization of benzimidazole-resistant *B. cinerea* field isolates with reduced or enhanced sensitivity to zoxamide and diethofencarb (2011). Pesticide Biochemistry and Physiology, 99 (1), pp. 118-124.

Markoglou, A.N., Malandrakis, A.A., Vitoratos, A.G., Ziogas, B.N., Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides (2006). European Journal of Plant Pathology, 115 (2), pp. 149-162.

Nejabatdoust, A., Salehzadeh, A., Zamani, H., Moradi-Shoeili, Z., Synthesis, Characterization and Functionalization of ZnO Nanoparticles by Glutamic Acid (Glu) and Conjugation of ZnO@Glu by Thiosemicarbazide and Its Synergistic Activity with Ciprofloxacin Against Multi-drug Resistant *Staphylococcus aureus* (2019). Journal of Cluster Science, 30 (2), pp. 329-336.

Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks (2018) Proceedings of the National Academy of Sciences India Section B - Biological Sciences, 88 (4), 1287–1308.

Paralikar, P., Ingle, A.P., Tiwari, V., Golinska, P., Dahm, H., Rai, M., Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria (2019). Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 54 (5), pp. 381-390.

Park, J.-W., Lee, I.-C., Shin, N.-R., Jeon, C.-M., Kwon, O.-K., Ko, J.-W., Kim, J.-C., Oh, S.-R., Shin, I.-S., Ahn, K.-S., Copper oxide nanoparticles aggravate airway inflammation and mucus production in asthmatic mice via MAPK signaling (2016). Nanotoxicology, 10 (4), pp. 445-452.

Punjabi, K., Mehta, S., Chavan, R., Chitalia, V., Deogharkar, D., Deshpande, S., Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens (2018). Frontiers in Microbiology, 9 (SEP), art. no. 2207, DOI: 10.3389/fmicb.2018.02207.

Stehmann, C., de Waard, M.A., Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl, and vinclozolin, Eur. J. Plant Pathol. 102 (1996) 171–180.

Sun, Q., Li, J., Le, T., Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives (2018). Journal of Agricultural and Food Chemistry, 66 (43), 11209–11220.

Sun, L., Liao, K., Li, Y., Zhao, L., Liang, S., Guo, D., Hu, J., Wang, D., Synergy between polyvinylpyrrolidone-coated silver nanoparticles and azole antifungal against drug-resistant *Candida albicans* (2016). Journal of Nanoscience and Nanotechnology, 16 (3), pp. 2325-2335.

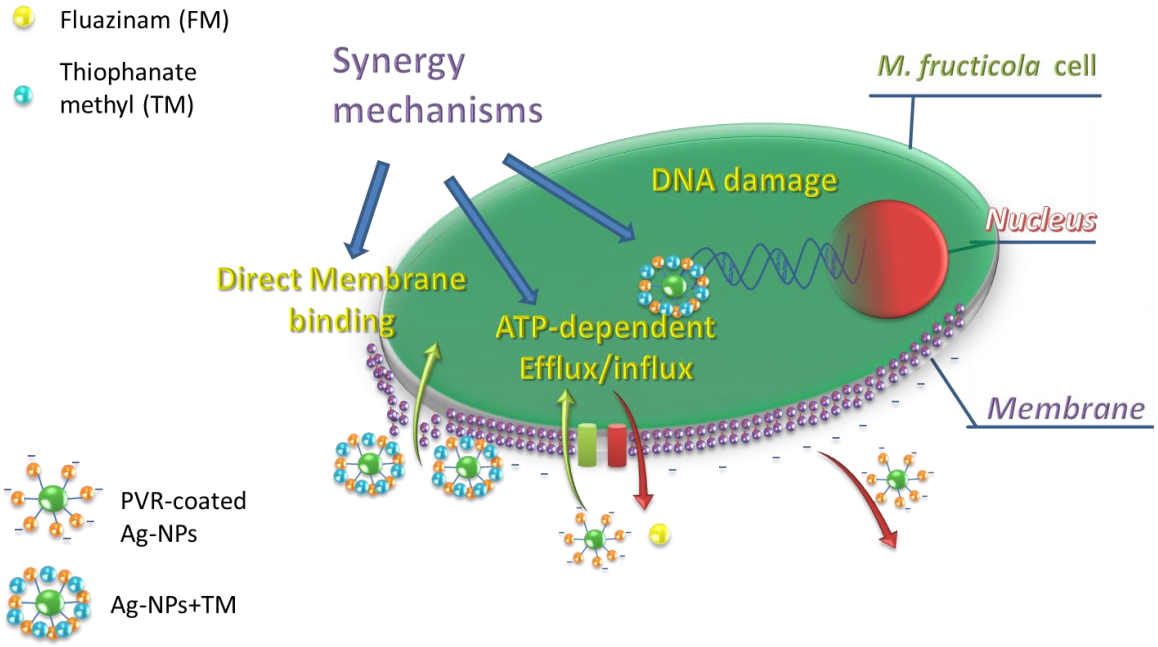
Torres-Duarte, C., Hutton, S., Vines, C., Moore, J., Cherr, G.N.; Effects of soluble copper and copper oxide nanoparticle exposure on the immune system of mussels, *Mytilus galloprovincialis* (2019) Environmental Toxicology, 34 (3), pp. 294-302.

Winter, C.K., Davis, S.F.,Organic foods (2006). Journal of Food Science, 71 (9), pp. R117-R124.

Xue, J., Luo, Z., Li, P., Ding, Y., Cui, Y., Wu, Q., A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles (2014). Scientific Reports, 4, art. no. 5408, DOI: 10.1038/srep05408.

Ziogas, B.N., Nikou, D., Markoglou, A.N., Malandrakis, A.A., Vontas, J., Identification of a novel point mutation in the *β*-tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay (2009). European Journal of Plant Pathology, 125 (1), pp. 97-107.

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



Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of silver nanoparticles to counter fungicide-resistance in Monilinia fructicola (2020) Science of the Total Environment, 747, art. no. 141287, DOI: 10.1016/j.scitotenv.2020.141287.

4. Use of silver nanoparticles to counter fungicide-resistance in Monilinia fructicola

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

4.1 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 (Pantley 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 utilised 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.

4.2 Materials and Methods

4.2.1 Nanoparticles, reagents and fungicides

Polyvinylpyrrolidone (PVP)-coated silver nanoparticles [Ag-NPs] (<100nm 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 4.S1) were measured in triplicate with a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA).

Table 4. S1. Zeta potential and diameter size of Ag-NPs (50 mg/mL), TM (0.5 mg/mL) and mixtures Ag-NPs+ NaCl (50 mg/mL + 1%) and Ag-NPs+TM (50+0.5 mg/mL)

	Zeta potential (mV) (mean ± SD[b])	Size (d.nm) (mean ± SD)
Ag-NPs	-29.6 ± 0.5	82.8 ± 12.6
Ag-NPs+NaCl	-19.3 ± 2.1	262.6 ± 35.8
TM	3.65 ± 0.4	346.7 ± 0.4
Ag-NPs+TM	-19.5 ± 0.2	79.6 ± 0.7

4.2.2 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 fruits 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 ^{o}C with 14h 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.

4.2.3 *In vitro* bioassays

4.2.3.1 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: 500μg/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 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:

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

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

4.2.3.2 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:

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

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

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

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.

4.2.4 *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 hr 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.

4.2.5 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 ^{o}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 $MgCl_2$, 0.5mM dNTPs, and 1.25 units of HotStar Taq DNA polymerase (Qiagen) in 20 mM TrisHCl and 50 mM KCl. The PCR conditions were: 95 ^{o}C for 15 min followed by 40 cycles of 94 ^{o}C for 30 s, 50 ^{o}C for 30 s, and 72 ^{o}C for 1 min with a final 10 min extension at 72 ^{o}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.

4.3 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 The SPSS v20 software (SPSS Inc., Chicago, IL, USA).

4.4 Results

4.4.1 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. 4.1. Sensitivity to Ag-NPs was widely distributed with EC_{50} values ranging between 7 and 870 μg mL^{-1} with a median value of 50 μg mL^{-1}. 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 4.1). 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 4.1). Resistance factors (Rfs) of the resistant phenotypes were calculated based on EC_{50} values determined by additional fungitoxicity tests (see Table 4.2). 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 4.2).

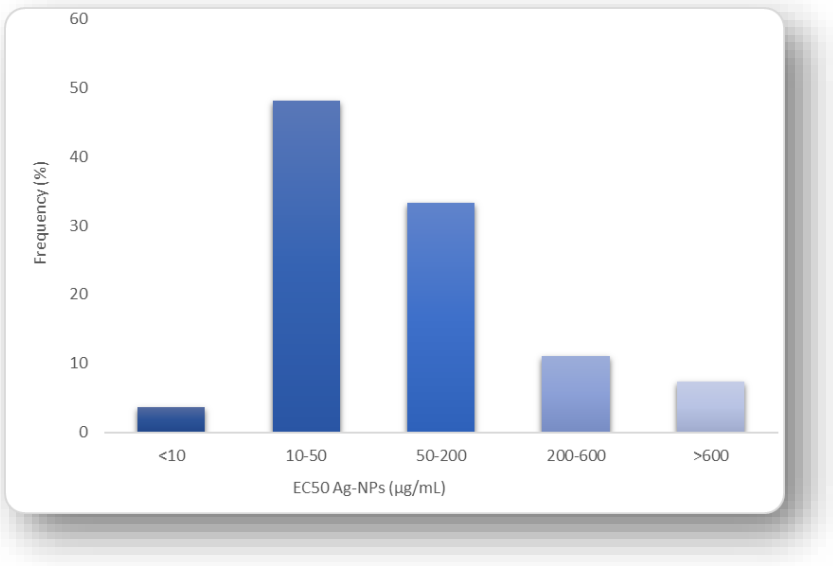


Figure 4.1. Sensitivity distribution of Monilia fructicola field isolates based on EC50 values to Ag-NPs.

Table 4.1 Sensitivity of *Monilia fructicola* isolates to Ag-NPs and selected fungicides.

	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

[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

4.4.2 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 4.2). 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.

Table 4.2 Cross-resistance profiles of representative M. fructicola isolates sensitive (BEN-S) and benzimidazole resistant (BEN-R) and respective resistance mutations.

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] 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).

4.4.3 Synergy between Ag-NPs and fungicides

4.4.3.1 *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. 4.2c). Estimated SF values between Ag-NPs and fluazinam ranged between 1.11 to 1.53 (see Table 4.3). A similar synergistic profile (SF: 0.99 - 5.20) was observed between Ag-NPs and thiophanate methyl (see Table 4.3). The above combination completely inhibited BEN-S isolates while it significantly enhanced Ag-NPs effectiveness in most of the BEN-R isolates (Fig. 4.2a, Fig. 4.3). 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. 4.2b). 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 4.3), 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 4.3). 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 4.3). 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 4.3).

Table 4.3. *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
MF28	BEN-R	1.29	1.16	1.22	1.09	1.93	0.60	1.08

[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.

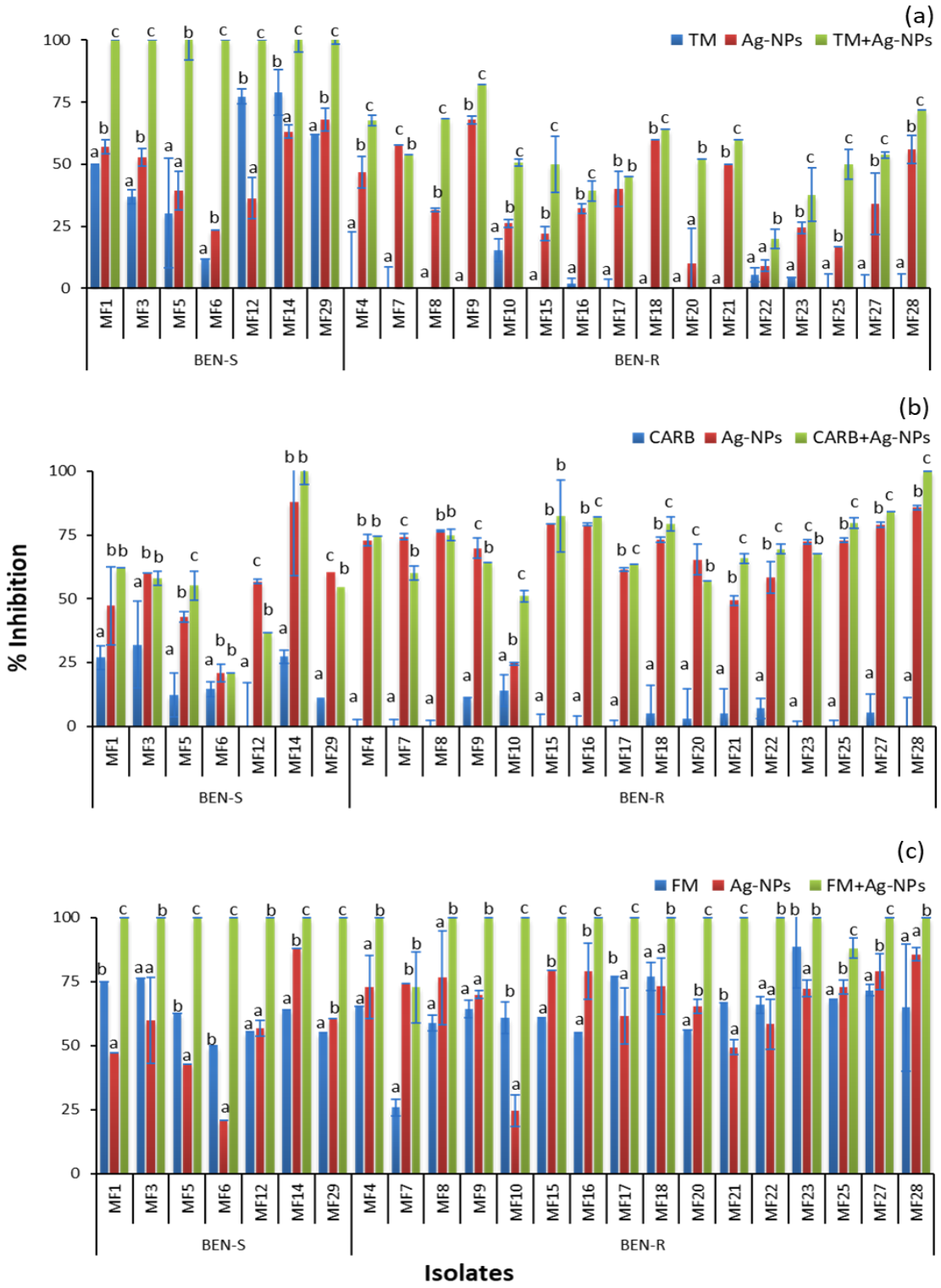


Figure 4.2 Sensitivity of fungicide-sensitive/resistant M. fructicola isolates to Ag-NPs (50 µg/mL) in comparison with: (a) thiophanate methyl (0.5 µg/mL), (b) carbendazim (0.15 µg/mL), and (c) fluazinam (0.2 µg/mL), and combinations. BEN-S/R: benzimidazole- Sensitive/Resistant isolates (TM: thiophanate 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 ($\alpha = 0.05$).

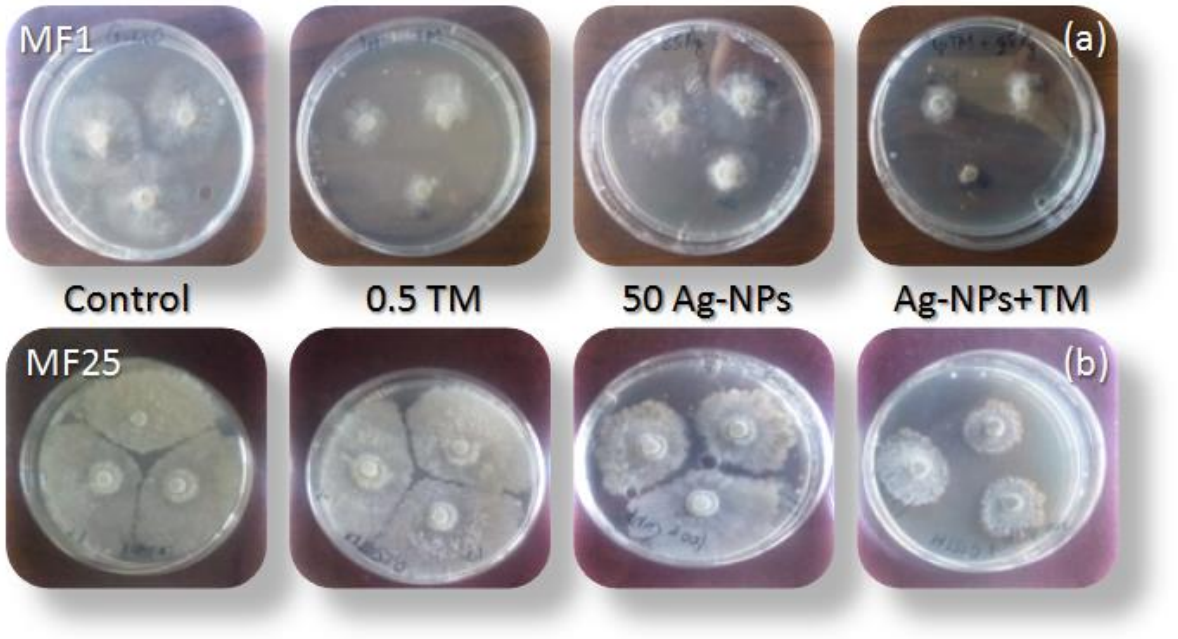


Figure 4.3 Fungitoxic activity of Ag-NPs, TM and their combination in (a) sensitive (MF1), and (b) TM-resistant (MF25) M. fructicola isolates (TM: thiophanate methyl).

4.4.3.2 *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. 4.4a). 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 4.5, Fig. 4.4b). The synergy between Ag-NPs and fluazinam demonstrated *in vitro* was also observed in the *in vivo* experiments (see Fig. 4.4c). 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 4.4).

Table 4.4 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	Pheno-type	Percent inhibition [a] (mean±SD[b])			SF[f]	Percent inhibition (mean±SD)			SF
		Ag-NPs (100) [d]	TM (50/1000) [e]	Ag-NPs +TM		Ag-NPs (100)	FM (500)	Ag-NPs +FM	
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 ^{o}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.

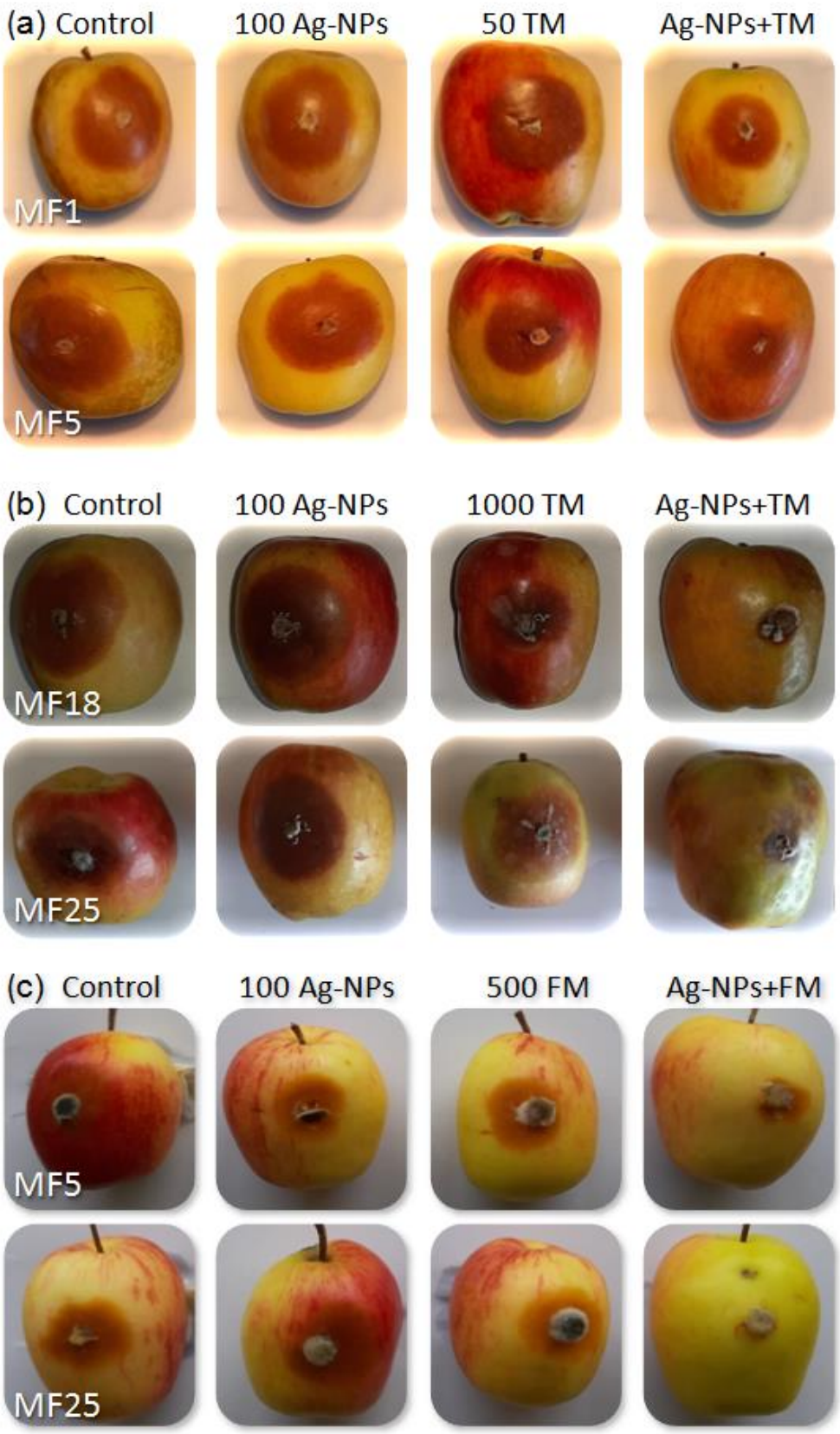


Figure 4.4 Synergistic activity of Ag-NPs (100 µg/mL) in combination with a,b) thiophanate methyl (50,) and c) fluazinam (500 µg/mL) on apple fruit against selected Botrytis cinerea isolates sensitive (MF1, MF5) and resistant (MF18, MF25) to thiophanate methyl (TM: thiophanate methyl, FM: fluazinam).

4.4.4 Sensitivity correlations between Ag-NPs, AgNO3 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 4.5). No significant correlation was found between TM and Ag-NPs, FM and their respective combinations (see Table 4.5). On the contrary, a significant correlation was found between Ag-NP and Ag-NPs+TM treatments (see Fig. 4.5a) 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 fungitoxic effect of their mixture (see Table 4. 5, Fig. 4.5b). No correlation was found between Ag-NPs and NaCl (see Table 4.5).

Table 4.5 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	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

[a] Pearson correlation coefficient values.

TM: thiophanate methyl, FM:fluazinam,TEB:tebuconazole.

* corresponds to a significance lever of P=0.05.

** corresponds to a significance lever of P=0.01.

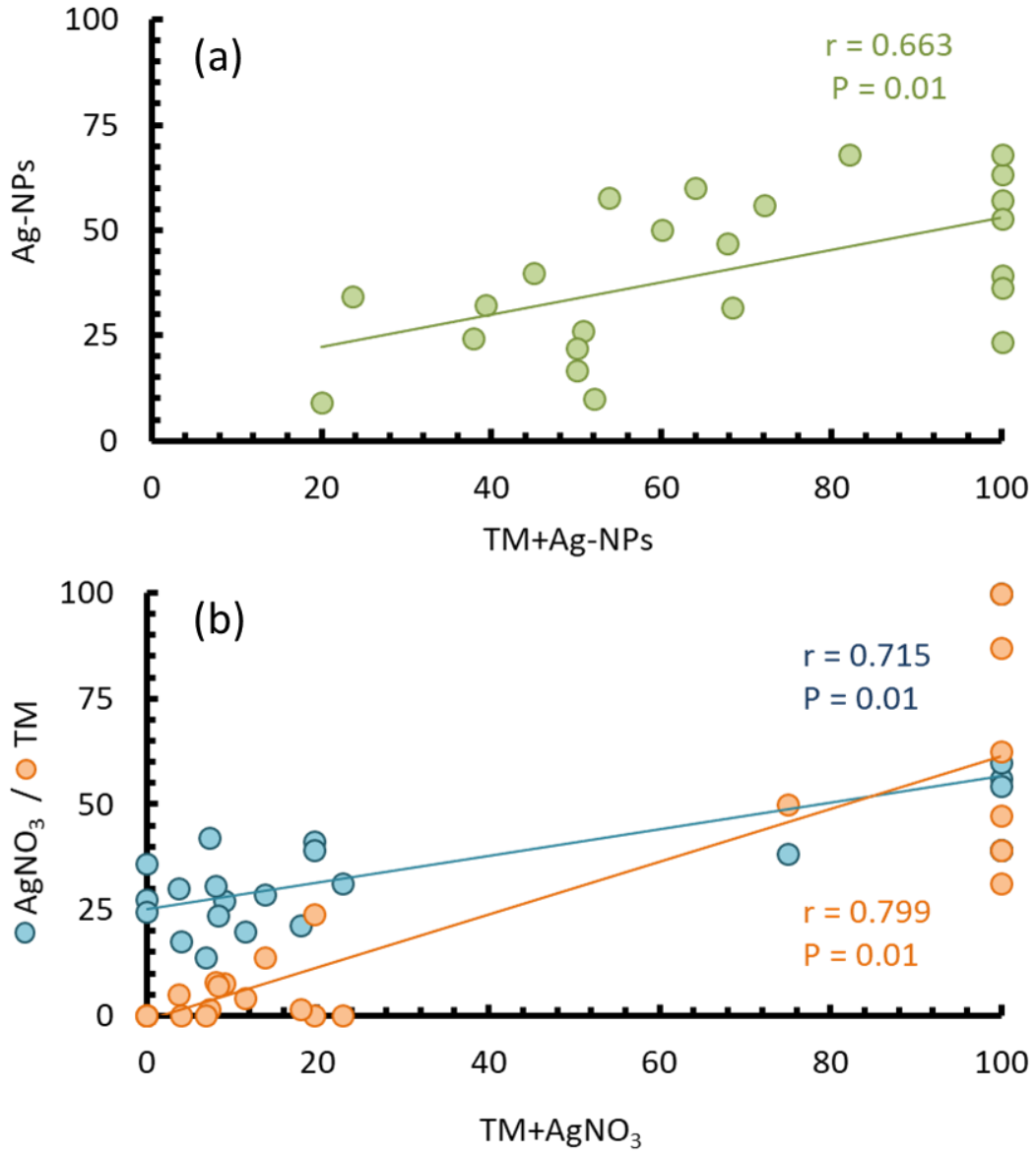


Figure 4.5. Correlation between sensitivities of M. fructicola isolates to: (a) Ag-NPs (50 µg/mL) and its combination with thiophanate methyl (TM) (0.5 µg/mL) and, (b) $AgNO_3$ (3 µg/mL), TM (0. 5 µg/mL) and their combination. Here, r is the Pearson correlation coefficient, and P the significance level.

4.5 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 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 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 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., 2019). 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., 2019). PVP-coated Ag-NPs used in this study were negatively charged and, as a consequence, are expected to be repelled by the also negatively 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.

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 et al. 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., 2019; 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.

4.6 References

Avenot, H. F., & Michailides, T. J., Detection of isolates of *Alternaria alternata* with multiple-resistance to fludioxonil, cyprodinil, boscalid and pyraclostrobin in California pistachio orchards (2015). Crop Protection, 78, 214–221.

Batarseh, K.I., Anomaly and correlation of killing in the therapeutic properties of silver (I) chelation with glutamic and tartaric acids (2004) Journal of Antimicrobial Chemotherapy, 54 (2), pp. 546-548.

Brent, K. J., & Hollomon, D. W. (1998). Fungicide Resistance: The assessment of risk. FRAC Monograph No 2, pp. 1–48. Brussels: Global Crop Protection Federation.

Chen, F., Liu, X., Chen, S., Schnabel, E., Schnabel, G. (2013). Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. Plant Disease (2013), 97, Number 5, pp. 645-651.

Chen, S.N., Shang, Y., Wang, Y., Schnabel, G., Yin, L.F., Luo, C.X., Sensitivity of *Monilinia fructicola* from peach farms in China to four fungicides and characterization of isolates resistant to carbendazim and azoxystrobin (2014). Plant Disease, 98 (11), pp. 1555-1560.

FRAC (2018), List of Plant Pathogenic Organisms Resistant to Disease Control Agents https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2 (accessed April 2020).

Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G., Galdiero, M., Silver nanoparticles as potential antibacterial agents (2015). Molecules, 20 (5), pp. 8856-8874.

Gabrielyan, L., Hakobyan, L., Hovhannisyan, A., Trchounian, A., Effects of iron oxide (Fe_3O_4) nanoparticles on *Escherichia coli* antibiotic-resistant strains (2019). Journal of Applied Microbiology, 126 (4), pp. 1108-1116.

Gisi, U., Synergistic interaction of fungicides in mixtures (1996) Phytopathology, 86 (11), pp. 1273-1279.

Hamed, S., Emara, M., Shawky, R.M., El-domany, R.A., Youssef, T., Silver nanoparticles: Antimicrobial activity, cytotoxicity, and synergism with N-acetyl cysteine (2017). Journal of Basic Microbiology, 57 (8), pp. 659-668.

Hily, J.M., Singer, S.D., Vilani, S.M., Cox, K.D., Characterization of the cytochrome b (cyt b) gene from Monilinia species causing brown rot of stone and pome fruit and its significance in the development of QoI resistance (2011). Pest Management Science, 67, pp. 385-396.

Hoseinzadeh, E., Makhdoumi, P., Taha, P., Hossini, H., Stelling, J., Kamal, M.A., Ashraf, G.Md. A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms (2017). Current Drug Metabolism, 18 (2), 120–128.

Huang, W., Wang, C., Duan, H., Bi, Y., Wu, D., Du, J., Yu, H., Synergistic antifungal effect of biosynthesized silver nanoparticles combined with fungicides (2018). International Journal of Agriculture and Biology, 20 (5), 1225–1229.

Jamdagni, P., Rana, J.S., Khatri, P., Comparative study of antifungal effect of green and chemically synthesised silver nanoparticles in combination with carbendazim, mancozeb, and thiram (2018) IET Nanobiotechnology, 12 (8), pp. 1102-1107.

Jampilek, J., How can we bolster the antifungal drug discovery pipeline? (2016) Future Medicinal Chemistry, 8 (12), pp. 1393-1397.

Jo, Y.-K., Kim, B.H., Jung, G.; Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi (2009). Plant Disease, 93 (10), pp. 1037-1043.

Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D.; A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues (2018). Nature Nanotechnology, 13 (8), 677–684.

Kalamarakis, A.E., Petsikos-Panagiotarou, N., Mavroidis, B., Ziogas, B.N., Activity of fluazinam against strains of *Botrytis cinerea* resistant to benzimidazoles and/or dicarboximides and to a benzimidazole-phenylcarbamate Mixture (2000). Journal of Phytopathology, 148 (7-8), pp. 449-455.

Khan, S.T., Musarrat, J., Al-Khedhairy, A.A., Countering drug resistance, infectious diseases, and sepsis using metal and metal oxides nanoparticles: Current status (2016). Colloids and Surfaces B: Biointerfaces, 146, pp. 70-83.

Król, A., Pomastowski, P., Rafińska, K., Railean-Plugaru, V., Buszewski, B., Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism (2017). Advances in Colloid and Interface Science 249, 37–52.

Lane, C.R., 2002. A synoptic key for differentiation of *Monilinia fructicola, M. fructigena* and *M. laxa*, based on examination of cultural characters. EPPO Bull. 32, 489-493.

Leroux, P., Walker, A.-S., Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance (2013). European Journal of Plant Pathology, 135 (4), pp. 683-693.

Luo, Y., Ma, Z., Michailides, T.J.,Quantification of allele E198A in beta-tubulin conferring benzimidazole resistance in *Monilinia fructicola* using real-time PCR (2007). Pest Management Science, 63 (12), pp. 1178-1184.

Ma, Z., Yoshimura, M.A., Michailides, T.J., Identification and Characterization of Benzimidazole Resistance in *Monilinia fructicola* from Stone Fruit Orchards in California (2003). Applied and Environmental Microbiology, 69 (12), pp. 7145-7152.

Ma, Z., Michailides, T.J., Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi (2005). Crop Protection, 24 (10), pp. 853-863.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Synergy between Cu-NPs and fungicides against *Botrytis cinerea* (2020). Science of the Total Environment, 703, art. no. 135557, DOI: 10.1016/j.scitotenv.2019.135557.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019). Science of the Total Environment, 670, pp. 292-299.

Malandrakis, A., Markoglou, A., Ziogas, B., Molecular characterization of benzimidazole-resistant *B. cinerea* field isolates with reduced or enhanced sensitivity to zoxamide and diethofencarb (2011). Pesticide Biochemistry and Physiology, 99 (1), pp. 118-124.

Markoglou, A.N., Malandrakis, A.A., Vitoratos, A.G., Ziogas, B.N., Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides (2006). European Journal of Plant Pathology, 115 (2), pp. 149-162.

May-De Mio, L., Luo, Y., Michailides, T., Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management (2011). Plant Disease, 95, pp. 821-827.

Miessner, S., Stammler, G., *Monilinia laxa, M. fructigena* and *M. fructicola*: Risk estimation of resistance to Qoi fungicides and identification of species with cytochrome b gene sequences (2010.) Journal of Plant Diseases and Protection, 117 (4), pp. 162-167.

Nejabatdoust, A., Salehzadeh, A., Zamani, H., Moradi-Shoeili, Z., Synthesis, Characterization and Functionalization of ZnO Nanoparticles by Glutamic Acid (Glu) and Conjugation of ZnO@Glu by Thiosemicarbazide and Its Synergistic Activity with Ciprofloxacin Against Multi-drug Resistant *Staphylococcus aureus* (2019). Journal of Cluster Science, 30 (2), pp. 329-336.

Nisar, P., Ali, N., Rahman, L., Ali, M., Shinwari, Z.K., Antimicrobial activities of biologically synthesized metal nanoparticles: an insight into the mechanism of action (2019). Journal of Biological Inorganic Chemistry, 24 (7), pp. 929-941.

Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks (2018) Proceedings of the National Academy of Sciences India Section B - Biological Sciences, 88 (4), 1287–1308.

Paralikar, P., Ingle, A.P., Tiwari, V., Golinska, P., Dahm, H., Rai, M., Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria (2019). Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 54 (5), pp. 381-390.

Prasher, P., Singh, M., Mudila, H., Silver nanoparticles as antimicrobial therapeutics: current perspectives and future challenges (2018). 3 Biotech, 8 (10), art. no. 411, DOI: 10.1007/s13205-018-1436-3.

Penrose L. J., Koffmann W., Nicholls M. R., Field occurrence of vinclozolin resistance in *Monilinia fructicola* (1985). Plant Pathology 34, 228-234.

Punjabi, K., Mehta, S., Chavan, R., Chitalia, V., Deogharkar, D., Deshpande, S., Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens (2018). Frontiers in Microbiology, 9 (SEP), art. no. 2207, DOI: 10.3389/fmicb.2018.02207.

Rai, M., Ingle, A.P., Pandit, R., Paralikar, P., Gupta, I., Chaud, M.V., dos Santos, C.A., Broadening the spectrum of small-molecule antibacterials by metallic nanoparticles to overcome microbial resistance (2017). International Journal of Pharmaceutics, 532 (1), pp. 139-148.

Ruddaraju, L.K., Pammi, S.V.N., Guntuku, G.S., Padavala, V.S., Kolapalli, V.R.M., A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present and future perspectives of green nano technological combinations (2020). Asian Journal of Pharmaceutical Sciences, 15 (1), pp. 42-59.

Rudramurthy, G.R., Swamy, M.K., Sinniah, U.R., Ghasemzadeh, A., Nanoparticles: Alternatives against drug-resistant pathogenic microbes (2016) Molecules, 21 (7), art. no. 836, DOI: 10.3390/molecules21070836.

Stehmann, C., de Waard, M.A., Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl, and vinclozolin, Eur. J. Plant Pathol. 102 (1996) 171–180.

Sun, Q., Li, J., Le, T., Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives (2018). Journal of Agricultural and Food Chemistry, 66 (43), 11209–11220.

Sun, L., Liao, K., Li, Y., Zhao, L., Liang, S., Guo, D., Hu, J., Wang, D., Synergy between polyvinylpyrrolidone-coated silver nanoparticles and azole antifungal against drug-resistant *Candida albicans* (2016). Journal of Nanoscience and Nanotechnology, 16 (3), pp. 2325-2335.

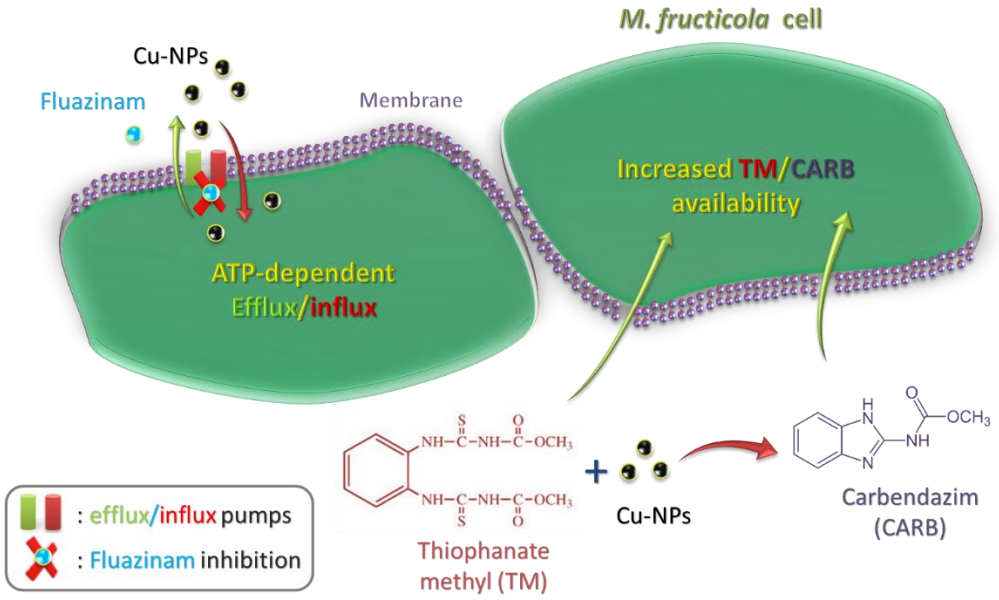
Torres-Duarte, C., Hutton, S., Vines, C., Moore, J., Cherr, G.N.; Effects of soluble copper and copper oxide nanoparticle exposure on the immune system of mussels, *Mytilus galloprovincialis* (2019) Environmental Toxicology, 34 (3), pp. 294-302.

Xue, J., Luo, Z., Li, P., Ding, Y., Cui, Y., Wu, Q., A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles (2014). Scientific Reports, 4, art. no. 5408, DOI: 10.1038/srep05408.

Zheng, M., Wang, Y., Wang, C., Wei, W., Ma, S., Sun, X., He, J., Silver nanoparticles-based colorimetric array for the detection of Thiophanate-methyl (2018). Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy, 198, pp. 315-321.

Ziogas, B.N., Nikou, D., Markoglou, A.N., Malandrakis, A.A., Vontas, J., Identification of a novel point mutation in the *β*-tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay (2009). European Journal of Plant Pathology, 125 (1), pp. 97-107.

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



Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Copper nanoparticles against benzimidazole-resistant Monilinia fructicola field isolates (2021) Pesticide Biochemistry and Physiology, 173, art. no. 104796, DOI: 10.1016/j.pestbp.2021.104796.

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

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 harbouring 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.

5.1 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-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 fueled 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 2018). 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., 2020). 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.

5.2 Materials and Methods

5.2.1 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.

5.2.2 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 ^{o}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.

5.2.3 *In vitro* bioassays

5.2.3.1 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.

5.2.3.2 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.

5.2.4 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 hr. 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 hrs 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.

5.2.5 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.

5.2.6 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).

5.3 Results

5.3.1 *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.5.1. 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 5.1). 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 5.1, 5.S1).

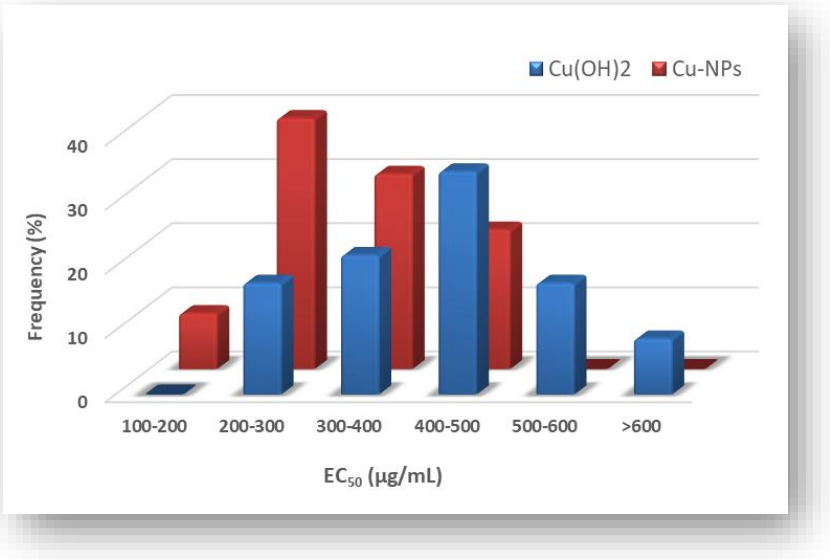


Figure 5.1. Sensitivity distribution of Monilia fructicola field isolates to Cu-NPs and $Cu(OH)_2$ based on EC_{50} values.

Table 5.1 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)	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).

Table 5.S1. Sensitivity of *Monilia fructicola* isolates to copper nanoparticles. their bulk counterparts and benzimidazole-resistance phenotypes.

	Percent Inhibition [a] (mean±SD [b])					**Resistance phenotypes/mutations in the *β*-tubulin gene**	
Isolate	Cu-NPs (250)[c]	CuO-NPs (500)	$Cu(OH)_2$ (500)	$CuSO_4$ (250)	EDTA (100)	Phenotype	Amino acid substitution
MF1	27.14 ± 2.01	8.23 ± 0.33	42.50 ± 2.23	25.15 ± 2.23	33.33 ± 1.04	BEN-S [d]	E198
MF3	52.63 ± 0.25	4.54 ± 0.14	45.45 ± 4.00	12.23 ± 5.05	22.22 ± 0.54	BEN-S	E198
MF4	46.77 ± 1.30	7.40 ± 2.00	49.06 ± 1.09	12.12 ± 2.27	11.11 ± 1.10	BEN-R	E198A
MF5	39.39 ± 0.02	12.76± 1.02	55.00 ± 1.98	54.35 ± 4.06	17.95 ± 0.78	BEN-S	E198
MF6	23.53 ± 4.40	26.19± 3.10	56.41 ± 0.99	2.22 ± 0.01	1.00 ± 0.03	BEN-S	E198
MF7	57.69 ± 2.15	0.00	55.00 ± 0.73	29.33 ± 2.82	0.00	BEN-R	E198A
MF8	31.75 ± 0.07	3.63± 0.22	40.00 ± 7.15	16.00 ± 2.22	36.92 ± 1.55	BEN-R	E198A
MF9	67.86 ± 5.63	16.00 ± 0.11	47.73 ± 3.93	17.74 ± 1.55	6.98 ± 0.44	BEN-R	E198A
MF10	26.15 ± 3.00	21.73 ± 0.98	46.55 ± 5.01	0.00	13.23 ± 0.98	BEN-R	E198A
MF12	36.36 ± 0.39	23.53 ± 0.18	40.00 ± 6.00	0.00	4.00 ± 1.00	BEN-S	E198
MF14	63.16 ± 0.85	0.00	6.25 ± 0.14	18.64 ± 1.38	2.56 ± 1.12	BEN-S	E198
MF15	22.00 ± 5.11	0.00	64.29 ± 2.25	15.79 ± 4.00	23.40 ± 1.90	BEN-R	E198A
MF16	32.14 ± 0.98	5.12 ± 0.18	35.42 ± 5.09	3.28 ± 1.48	17.07 ± 2.00	BEN-R	E198A
MF17	40.00 ± 0.83	7.05 ± 1.00	57.14 ± 1.11	12.23 ± 3.30	15.27 ± 1.15	BEN-R	E198A
MF18	60.00 ± 3.33	6.75 ± 0.10	40.00 ± 7.88	5.75 ± 1.04	12.10 ± 1.67	BEN-R	E198A
MF20	10.00 ± 2.32	7.81 ± 0.20	51.72 ± 1.72	10.67 ± 0.89	16.95 ± 0.26	BEN-R	E198A
MF21	50.00 ± 5.45	0.00	70.00 ± 6.32	12.28 ± 2.90	41.18 ± 2.68	BEN-R	E198A
MF22	9.09 ± 1.33	3.85 ± 0.05	59.02 ± 4.45	5.45 ± 1.01	29.09 ± 0.03	BEN-R	E198A
MF23	24.44 ± 2.00	25.92 ± 2.02	78.18 ± 0.15	8.89 ± 1.85	33.90 ± 2.08	BEN-R	E198A
MF25	16.67 ± 1.74	8.77± 2.81	48.98 ± 3.14	21.88 ± 2.40	38.98 ± 1.99	BEN-R	E198A
MF27	34.21 ± 1.00	0.00	55.56 ± 0.89	14.49 ± 1.56	6.52 ± 0.18	BEN-R	E198A
MF28	56.00 ± 2.62	8.20 ± 0.19	54.00 ± 9.00	1.25 ± 0.04	20.45 ± 2.23	BEN-R	E198A
MF29	68.00 ± 4.24	4.76 ± 1.04	57.45 ± 5.34	7.55 ± 0.18	13.89 ± 0.82	BEN-S	E198

[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

5.3.2 Synergy between copper NPs and fungicides

5.3.2.1 *In vitro* bioassays

Synergy factors (SF) calculated between copper nanoparticles and combinations with fungicides in *in vitro* fungitoxicity tests are listed in Table 5.2. 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. 5.2c). Respective SF values ranged between 0.99 to 1.61 (see Table5. 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 5.3). 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 5.2b). 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 5.2, Fig. 5.2b, Fig.5.3). A similar synergistic profile, although to a lesser extend, was observed between CuO-NPs and thiophanate methyl (see Table 5.2, Fig. 5.2a). 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. 5.2a). 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 5.3, Fig. 5.2d).

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 5.2). 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 5.2), 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 5.2).

Table 5.2. *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).

		SF[a]								
		Cu-NPs (250)					$Cu(OH)_2$ (500)		CuO-NPs (500)	
Isolate	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/**	**0.89 b/A**	**0.92 b/A**	**0.63 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 (α = 0.05). Small letters correspond to statistical differences between rows (treatments) while capitals to columns (phenotypic groups).

Figure 5.2. 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 (α = 0.05).

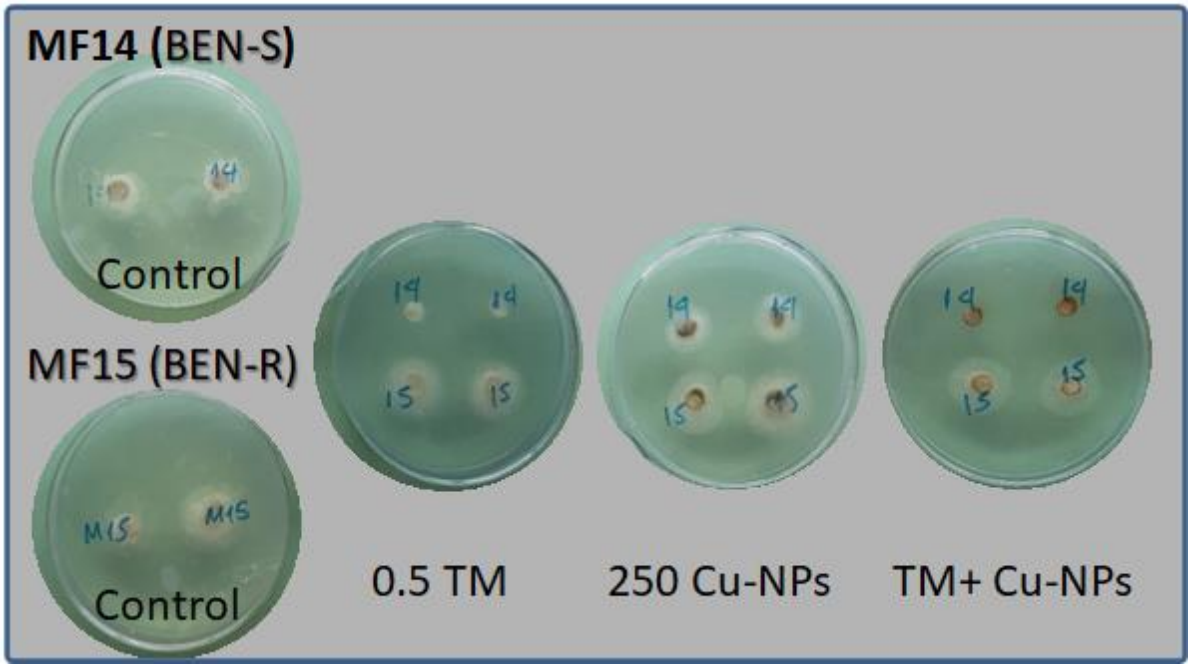


Figure 5.3. Fungitoxic activity of Cu-NPs. TM and their combination in sensitive (MF14) and TM-resistant (MF15) M. fructicola isolates (TM: thiophanate methyl. BEN-S/R: Benzimidazole Sensitive/ Resistant).

5.3.2.2 *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 a strong synergistic effect (SF:1.48 and 2.40, respectively, Table 5.3, Fig. 5.4a). 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 5.4, Fig. 5.4b). The synergy profiles observed *in vitro* between Cu-NPs and fluazinam were consistent with that observed *in vivo* (see Fig. 5.4c). 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 5.3).

Table 5.3. 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	Phenotype	Percent inhibition [a] (mean±SD[b])				Percent inhibition (mean±SD)			
		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	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.

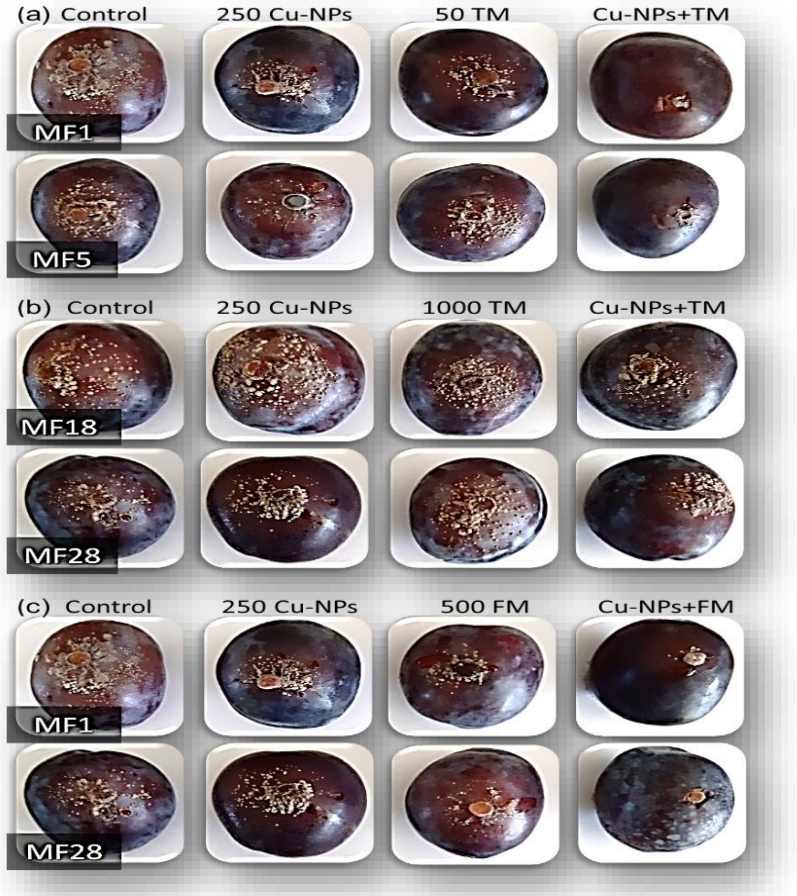


Figure 5.4. Synergistic activity of Cu-NPs (250 µg/mL) in combination with a,b) thiophanate methyl (50,1000 µg/mL) and c) fluazinam (500 µg/mL) on plum fruit against selected Monilia fructicola isolates sensitive (MF1, MF5) and resistant (MF18, MF28) to thiophanate methyl (TM: thiophanate methyl. FM: fluazinam).

5.3.3 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 found between TM and Cu-NPs, FM or their respective combinations while, the correlation found between TM and Cu-NPs+TM treatments (see Fig. 5.5a) 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 5.5). 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 5.5) while CuO-NPs toxicity was positively (r=0.43, P=0.038) correlated with the toxicity exerted by the commercial fungicide containing $Cu(OH)_2$.

Table 5.4. 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	-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

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

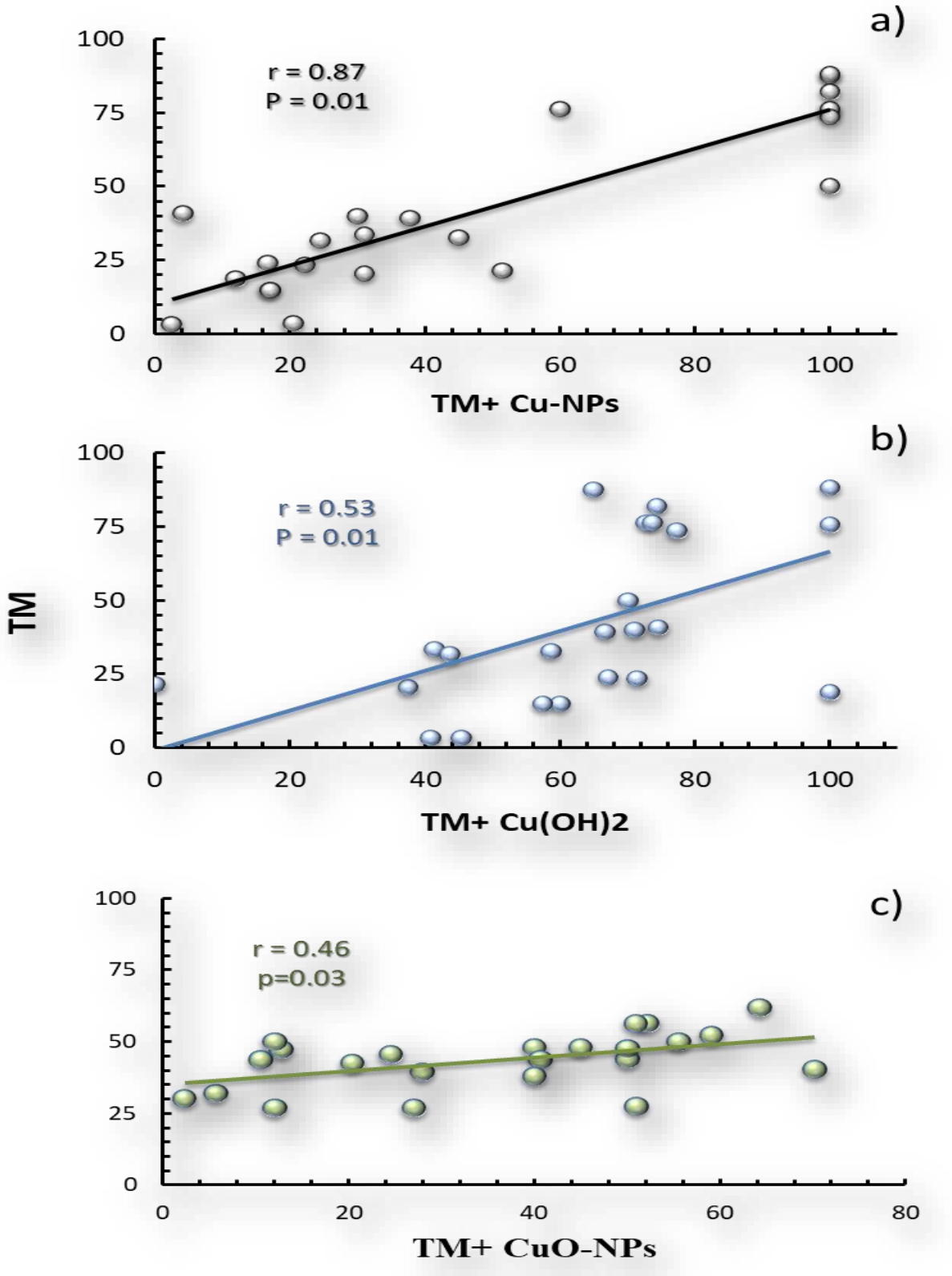


Figure 5.5. Correlation between sensitivities of M. fructicola isolates to TM (0.5 µg/mL) and its combination with (a) Cu-NPs (250 µg/mL). (b) $Cu(OH)_2$ (500 µg/mL) and (c) and CuO-NPs (500 µg/mL). r is the Pearson correlation coefficient. and p the significance level.

5.3.4 Copper mediated TM transformation 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.

5.4. 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 (Ma et al., 2003; Stehmann and de Waard 1996; Malandrakis et al., 2011; Ziogas et al., 2009; Ma & Michailides 2005; FRAC 2018). 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 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 et al. 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 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 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., 2020). 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 et al. (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.

5.5 References

Abed-Alwahed, F.R.; Abed Al-Baqi, N.J. Effect of the silver nanoparticles on ERG11 expression in fluconazole-resistant *Candida albicans* isolates. *Plant Archives* 2020, **20,** 616-620.

Agrios, G. Brown rot of stone fruit. In: Plant Pathology, fifth ed. Elsevier Academic, UK, 2005, pp. 509-510.

Ammar, H.A.; Rabie, G.H.; Mohamed, E. Novel fabrication of gelatin-encapsulated copper nanoparticles using *Aspergillus versicolor* and their application in controlling of rotting plant pathogens. *Bioprocess and Biosystems Engineering* 2019, **42**, 1947-1961.

Antsotegi-Uskola, M.; Markina-Iñarrairaegui, A.; Ugalde, U. New insights into copper homeostasis in filamentous fungi. *International Microbiology* 2020, **23**, 65-73.

Assadi, Z.; Emtiazi, G.; Zarrabi, A. Novel synergistic activities of tetracycline copper oxide nanoparticles integrated into chitosan micro particles for delivery against multiple drug resistant strains: Generation of reactive oxygen species (ROS) and cell death. *Journal of Drug Delivery Science and Technology* 2018,**44**, 65-70.

Brent, K. J. & Hollomon, D. W.. Fungicide Resistance: The assessment of risk. Brussels: Global Crop Protection Federation, FRAC Monograph No 2, 1998, pp. 1–48.

Chen, F.; Liu, X.; Chen, S.; Schnabel, E.; Schnabel, G. Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. *Plant Disease* 2013,**97**,645-651.

Chen, S.N.; Shang, Y.; Wang, Y.; Schnabel, G.; Yin, L.F.; Luo, C.X. Sensitivity of *Monilinia fructicola* from peach farms in China to four fungicides and characterization of isolates resistant to carbendazim and azoxystrobin. *Plant Disease* 2014,**98,** 1555-1560.

El-Abeid, S.E.; Ahmed, Y.; Daròs, J.-A.; Mohamed, M.A. Reduced graphene oxide nanosheet-decorated copper oxide nanoparticles: A potent antifungal nanocomposite against fusarium root rot and wilt diseases of tomato and pepper plants. *Nanomaterials* 2020, DOI: 10.3390/nano10051001.

FRAC, List of Plant Pathogenic Organisms Resistant to Disease Control Agents, https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2 (accessed April 2020) .

Franci, G.; Falanga, A.; Galdiero, S.; Palomba, L.; Rai, M.; Morelli, G.; Galdiero, M. Silver nanoparticles as potential antibacterial agents. *Molecules* 2015,**20,** 8856-8874.

Gabrielyan, L; Hakobyan, L; Hovhannisyan, A; Trchounian, A. Effects of iron oxide (Fe_3O_4) nanoparticles on *Escherichia coli* antibiotic-resistant strains. *Journal of Applied Microbiology* 2019,**126,**1108-1116.

Gisi, U. Synergistic interaction of fungicides in mixtures. *Phytopathology* 1996, **86,** 1273-1279.

Hamed, S.; Emara, M.; Shawky, R.M.; El-domany, R.A.; Youssef, T. Silver nanoparticles: Antimicrobial activity, cytotoxicity, and synergism with N-acetyl cysteine. *Journal of Basic Microbiology* 2017,**57,** 659-668.

Hoseinzadeh, E.; Makhdoumi, P.; Taha, P.; Hossini, H.; Stelling, J.; Kamal, M.A.; Ashraf, G.Md., A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms. *Current Drug Metabolism* 2017, **18,** 120–128.

Huang, W; Wang, C; Duan, H; Bi, Y; Wu, D; Du, J; Yu, H. Synergistic antifungal effect of biosynthesized silver nanoparticles combined with fungicides. *International Journal of Agriculture and Biology* 2018, **20,** 1225–1229.

Jamdagni, P.; Rana, J.S.; Khatri, P. Comparative study of antifungal effect of green and chemically synthesized silver nanoparticles in combination with carbendazim, mancozeb, and thiram. *IET Nanobiotechnology* 2018,**12,** 1102-1107.

Jampilek, J. How can we bolster the antifungal drug discovery pipeline? *Future Medicinal Chemistry* 2016, **8,** 1393-1397.

Jankauskaitė, V.; Vitkauskienė, A.; Lazauskas, A.; Baltrusaitis, J.; Prosyčevas, I.; Andrulevičius, M. Bactericidal effect of graphene oxide/Cu/Ag nanoderivatives against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus. International Journal of Pharmaceutics* 2016, **511,** 90-97.

Kah, M.; Kookana, R.S.; Gogos, A.; Bucheli, T.D. A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. *Nature Nanotechnology* 2018,**13**, 677–684.

Keller, A.A.; Adeleye, A.S.; Conway, J.R., et al. Comparative environmental fate and toxicity of copper nanomaterials. *NanoImpact* 2017,**7,** 28–40.

Khamis, Y.; Hashim, A.F.; Margarita, R.; Alghuthaymi, M.A.; Abd-Elsalam, K.A. Fungicidal efficacy of chemically-produced copper nanoparticles against *Penicillium digitatum* and *Fusarium solani* on citrus fruit. *Philippine Agricultural Scientist* 2017, **100,** 69-78.

Khan, S.T.; Musarrat, J.; Al-Khedhairy, A.A. Countering drug resistance, infectious diseases, and sepsis using metal and metal oxides nanoparticles: Current status. *Colloids and Surfaces B: Biointerfaces* 2016,**146**, 70-83.

Khurana, C.; Sharma, P.; Pandey, O.P.; Chudasama, B. Synergistic Effect of Metal Nanoparticles on the Antimicrobial Activities of Antibiotics against Biorecycling Microbes. *Journal of Materials Science and Technology* 2016, **32,** 524-532.

Król, A.; Pomastowski, P.; Rafińska, K.; Railean-Plugaru, V.; Buszewski, B. Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism. *Advances in Colloid and Interface Science* 2017,**249**, 37–52.

Leroux, P.; Walker, A.-S. Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance. *European Journal of Plant Pathology* 2013,**135,** 683-693.

Ma, Z.; Michailides, T.J., Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection* 2005,**24,** 853-863.

Ma, Z.; Yoshimura, M.A.; Michailides, T.J. Identification and Characterization of Benzimidazole Resistance in *Monilinia fructicola* from Stone Fruit Orchards in California. *Applied and Environmental Microbiology* 2003,**69,**7145-7152.

Malandrakis, A.; Markoglou, A.; Ziogas, B. Molecular characterization of benzimidazole-resistant *B. cinerea* field isolates with reduced or enhanced sensitivity to zoxamide and diethofencarb. *Pesticide Biochemistry and Physiology* 2011, **99**,118-124.

Malandrakis, A.A.; Kavroulakis, N.; Chrysikopoulos, C.V. Synergy between Cu-NPs and fungicides against *Botrytis cinerea*. *Science of the Total Environment* 2020a, DOI: 10.1016/j.scitotenv.2019.135557.

Malandrakis, A.A.; Kavroulakis, N.; Chrysikopoulos, C.V. Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens. *Science of the Total Environment* 2019,**670,** 292-299.

Malandrakis, A.A; Kavroulakis, N; Chrysikopoulos, C.V., Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola.* Science of the Total Environment 2020b, 747, art. no. 141287, DOI: 10.1016/j.scitotenv.2020.141287.

Marathe, K.; Naik, J.; Maheshwari, V. Biogenic Synthesis of Silver Nanoparticles Using *Streptomyces* spp. and their Antifungal Activity Against *Fusarium verticillioides. Journal of Cluster Science* 2020, DOI: 10.1007/s10876-020-01894-5.

Miessner, S.; Stammler, G. *Monilinia laxa*, *M. fructigena* and *M. fructicola*: Risk estimation of resistance to Qoi fungicides and identification of species with cytochrome b gene sequences. *Journal of Plant Diseases and Protection* 2010, **117,** 162-167.

Muthuchamy, M.; Muneeswaran, T.; Rajivgandhi, G.; Franck, Q.; Muthusamy, A.; Ji-Ming, S. Biologically synthesized copper and zinc oxide nanoparticles for important biomolecules detection and antimicrobial applications. *Materials Today Communications* 2020, DOI: 10.1016/j.mtcomm.2019.100766.

Nejabatdoust, A; Salehzadeh, A; Zamani, H; Moradi-Shoeili, Z. Synthesis, Characterization and Functionalization of ZnO Nanoparticles by Glutamic Acid (Glu) and Conjugation of ZnO@Glu by Thiosemicarbazide and Its Synergistic Activity with Ciprofloxacin Against Multi-drug Resistant *Staphylococcus aureus*. *Journal of Cluster Science* 2019,**30,** 329-336.

Nisar, P.; Ali, N.; Rahman, L.; Ali, M.; Shinwari, Z.K. Antimicrobial activities of biologically synthesized metal nanoparticles: an insight into the mechanism of action. *Journal of Biological Inorganic Chemistry* 2019, **24**, 929-941.

Pandey, S.; Giri, K.; Kumar, R.; Mishra, G.; Raja Rishi, R. Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks. Proceedings of the National Academy of Sciences India Section B - Biological Sciences 2018,**88**, 1287–1308.

Paralikar, P; Ingle, A.P; Tiwari, V; Golinska, P; Dahm, H; Rai, M. Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering* 2019,**54,** 381-390.

Penrose L. J.; Koffmann W.; Nicholls M. R. Field occurrence of vinclozolin resistance in *Monilinia fructicola*. *Plant Pathology* 1985, **34**, 228-234.

Prasher, P.; Singh, M.; Mudila, H. Silver nanoparticles as antimicrobial therapeutics: current perspectives and future challenges. *3 Biotech* 2018, DOI: 10.1007/s13205-018-1436-3.

Punjabi, K.; Mehta, S; Chavan, R; Chitalia, V; Deogharkar, D; Deshpande, S. Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens. *Frontiers in Microbiology* 2018, DOI: 10.3389/fmicb.2018.02207.

Rai, M.; Ingle, A.P.; Pandit, R.; Paralikar, P.; Gupta, I.; Chaud, M.V.; dos Santos, C.A. Broadening the spectrum of small-molecule antibacterials by metallic nanoparticles to overcome microbial resistance. *International Journal of Pharmaceutics* 2017, **532,**139-148.

Ruddaraju, L.K.; Pammi, S.V.N.; Guntuku, G.S.; Padavala, V.S.; Kolapalli, V.R.M. A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present and future perspectives of green nano technological combinations. *Asian Journal of Pharmaceutical Sciences* 2020,**15,** 42-59.

Rudramurthy, G.R.; Swamy, M.K.; Sinniah, U.R.; Ghasemzadeh, A. Nanoparticles: Alternatives against drug-resistant pathogenic microbes. *Molecules* 2016,DOI: 10.3390/molecules21070836.

Stehmann, C.; de Waard, M.A. Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl, and vinclozolin. *Eur. J. Plant Pathol.* 1996,**102,** 171–180.

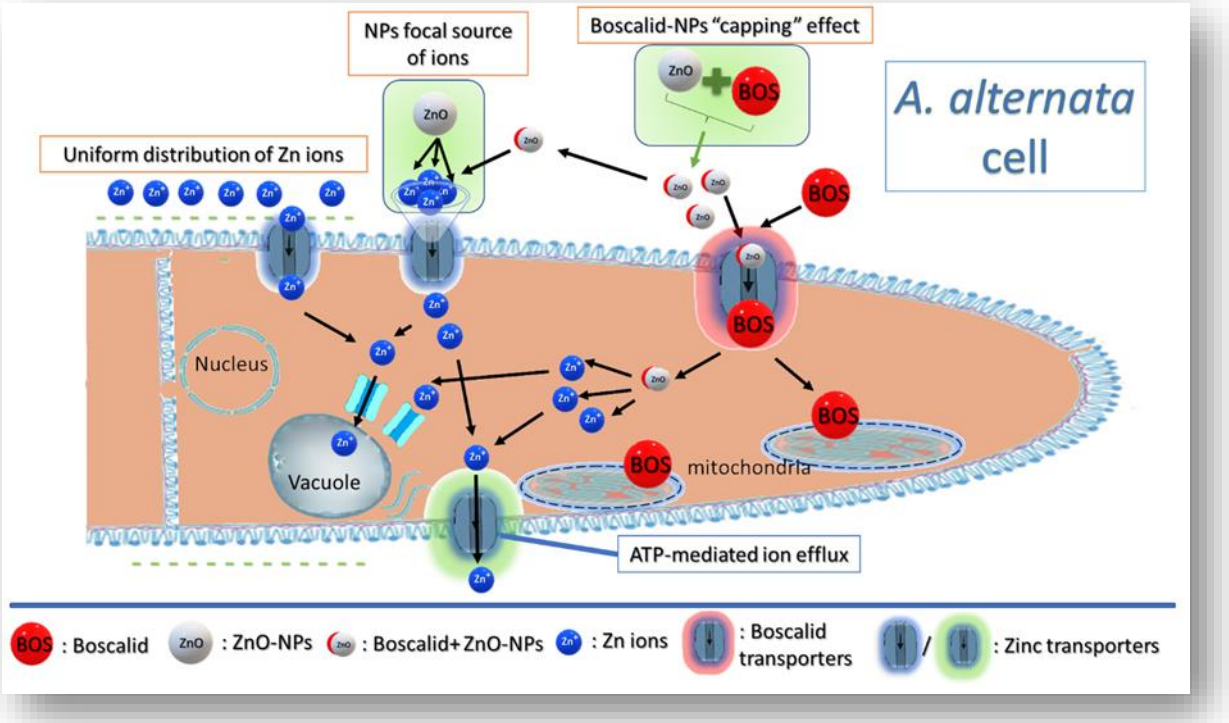
Sun, Q.; Li, J.; Le, T. Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives. *Journal of Agricultural and Food Chemistr* 2018,**66**, 11209–11220.

Torres-Duarte, C.; Hutton, S.; Vines, C.; Moore, J.; Cherr, G.N. Effects of soluble copper and copper oxide nanoparticle exposure on the immune system of mussels, *Mytilus galloprovincialis. Environmental Toxicology* 2019,**34,** 294-302.

Xue, J; Luo, Z; Li, P; Ding, Y; Cui, Y; Wu, Q. A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles. *Scientific Reports* 2014, DOI: 10.1038/srep05408.

Ziogas, B.N.; Nikou, D.; Markoglou, A.N.; Malandrakis, A.A.; Vontas, J., Identification of a novel point mutation in the *β*-tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay. *European Journal of Plant Pathology* 2009, 125, 97-107.

6 Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant *Alternaria alternata* isolates



Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant Alternaria alternata isolates (2022) Science of the Total Environment, 829, art. no. 154638, DOI: 10.1016/j.scitotenv.2022.154638.

6. Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant *Alternaria alternata* isolates

Abstract

The antifungal potential of ZnO-NPs against *Alternaria alternata* isolates with reduced sensitivity to the succinate dehydrogenase inhibitor (SDHI) boscalid, resulting from target site modifications, was evaluated *in vitro* and *in vivo.* ZnO-NPs could effectively inhibit mycelial growth in a dose-dependent way in both boscalid (BOSC) sensitive (BOSC-S) and resistant (BOSC-R) isolates. The fungitoxic effect of ZnO-NPs against the pathogen was significantly enhanced when combined with boscalid compared to the individual treatments in all phenotype cases (BOSC-S/R) both *in vitro* and *in vivo*. Fungitoxic effect of ZnO-NPs could be –at least partly– attributed to zinc ion release as indicated by the positive correlation between sensitivities to the nanoparticles and their ionic counterpart $ZnSO_4$ and the alleviation of the ZnO-NPs fungitoxic action in the presence of the strong chelating agent EDTA. The superior effectiveness of ZnO-NPs against *A. alternata* compared to $ZnSO_4$ could be due to nanoparticle properties interfering with cellular ion homeostasis mechanisms. The observed additive action of the oxidative phosphorylation-uncoupler fluazinam (FM) against all phenotypes indicates a possible role of ATP-dependent ion efflux mechanism in the mode of action of ZnO-NPs. A potential role of ROS production in the fungitoxic action of ZnO-NPs was evident by the additive/synergistic action of Salicylhydroxamate (SHAM), which blocks the alternative oxidase antioxidant action. Mixture of ZnO-NPs and boscalid resulting in a "capping" effect for the nanoparticles significantly reducing their mean size probably accounted for the synergistic effect of the mixture against both sensitive and resistant *A. alternata* isolates. Summarizing, results indicated that ZnO-NPs can be effectively used against *A. alternata* while combination with boscalid optimizes their performance and provides an effective tool for combating SDHI-resistance and reducing the environmental fingerprint of synthetic fungicides by reducing recommended doses for the control of the pathogen.

6.1 Introduction

All alternative measures to combat plant diseases considered, chemical control utilizing protective or systemic synthetic fungicides still remains the most efficient and economically feasible disease management strategy so far, ensuring food sustainability and safety (Pandey et al., 2018; Malandrakis et al., 2018). Nevertheless, certain drawbacks of conventional fungicides such as side effects to non-target organisms and environmental pollution –especially aquifer contamination– have caused the withdrawal of a great number of fungicide active ingredients enforced by implementation of strict EU regulations (Malandrakis et al., 2021). This fact in combination with the compromise of fungicide efficacy due to the emergence of fungicide-resistant pathogens underline the necessity of alternative disease control measures capable of reducing disease incidence and, at the same time, mitigating environmental risks. Nanofungicides, containing nanoparticles (NPs) as active ingredients, are promising environmentally compatible fungicide alternatives due to their unique properties that include high antifungal effectiveness, enhanced residual action and drug delivery and low resistance

risk (Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). Zinc oxide NPs have a number of advantages including low cost, increased stability in extreme conditions compared to their bulk counterparts and substantial antimicrobial activity at low concentrations while being relatively safe to humans (Król et al., 2017; Sardella et al., 2017; Malandrakis et al., 2019; Sun et al., 2018). The fungitoxic potential of ZnO-NPs against fungal plant pathogens including *Fusarium* sp., *Sclerotinia homoeocarpa*, *Botrytis cinerea*, *Aspergillus niger*, *Penicillium expansum* and *Rhizopus stolonifer* has been evaluated in a number of studies (Li et al., 2017; Król et al., 2017; Ashajyothi et al., 2016; Sardella et al., 2017). Several biochemical mechanisms responsible for their mycotoxic mode of action have been proposed even though further studies are needed to acquire conclusive evidence in order to elucidate/validate these potential mechanisms (Sardella et al., 2017). DNA recombination, membrane/transmembrane transport, protein synthesis, ion homeostasis, and ROS generation/induction of oxidative stress are some of the proposed physiological processes that ZnO-NPs interfere with causing the toxic action against plant pathogens (Márquez et al., 2018; Rai et al., 2017; Khan et al., 2016; Malandrakis et al., 2019).

Alternaria alternata is a cosmopolitan fungal pathogen with a wide range of hosts causing economically significant yield losses in cucurbitaceae, brassicaceae and solanaceae vegetables (Strandberg, 1992). Symptom severity depends on weather conditions, host and cultivar susceptibility and pathogen pathotype and vary from preharvest leaf blight, brown leaf spot, stem canker to postharvest fruit rot (Morris et al., 2000; Strandberg, 1992). On tomato plants, the pathogen is mostly responsible for post-harvest damage of ripe tomato especially affecting the tomato processing industry (Morris et al., 2000; Davis et al., 1997). On top of that, food safety issues arise from the ability of *A. alternata* to produce a number of mycotoxins (secondary metabolites that exert genotoxic, mutagenic or even acute toxic effects on humans) including tenuazonic acid (TeA), alternariol (AOH) and alternariol monomethyl ether (AME), highlighting the importance of effective control of the fungus (Logrieco et al., 2009).

Chemical control represents the main method for reducing incidence of *A. alternata* utilizing both protective or systemic fungicides such as DeMethylation Inhibitors (DMIs), Succinate DeHydrogenase Inhibitors (SDHIs) and Quinone outside Inhibitors (QoIs) (Sierotzki and Scalliet, 2013; Malandrakis et al., 2018). The SDHI, carboxamide boscalid is a very effective fungicide used against a wide range of plant pathogens including *A. alternata* (Malandrakis et al., 2018; Xiao and Boal, 2009; Avenot and Michailides, 2015). SDHIs target complex II of the respiration pathway by specifically binding with the succinate dehydrogenase enzyme blocking the electron transport chain and eventually resulting cell death due to ATP starvation (Bartlett et al., 2002; Sierotzki and Scalliet, 2013). Unfortunately, due to the intensity of usage and their site-specific mode of action, efficacy of these fungicides has been compromised due to the emergence of SDHI-resistant strains (Avenot et al., 2008; Veloukas et al., 2013; Avenot and Michailides, 2015; Fan et al., 2015; Malandrakis et al., 2017). Isolates of *A. alternata* resistant to SDHIs bearing mutations in 3 out of 4 SDH subunits were reported shortly after their introduction (Ma et al., 2003; Avenot and Michailides, 2015; Fan et al., 2015). Specifically, several substitutions in positions 277 of the *sdh*B subunit, 134 and 135 of the *sdh*C, and 47, 123 or 133 of the *sdh*D subunits of the SDH enzyme were associated with resistance to SDHIs in *A. alternata* (Sierotzki and Scalliet, 2013; Sang and Lee, 2020).

A novel, recently proposed, environmentally compatible countermeasure against drug/fungicide resistance concerns the use of metal nanoparticles alone or in combination with conventional drugs (Malandrakis et al., 2019; 2020a, b; 2021b). Ag-NPs, ZnO-NP and Fe-NPs have been reported to be effective (alone or in combination with antibiotics) against drug-resistant pathogenic bacteria including *Proteus mirabilis, Klebsiella pneumonia, Pseudomonas aeruginosa, Eschericia coli, Enterococcus faecalis,* and *Staphylococcus aureus* (Punjabi et al., 2018; Gabrielyan et al., 2019; Nejabatdoust et al., 2019; Paralikar et al., 2019). Furthermore, Ag-NPs, Cu-NPs and ZnO-NPs used individually or in combination with conventional fungicides, have also been reported to be effective against fungicide resistant fungal pathogens in a limited number of cases (Malandrakis et al., 2020; 2021a, b; Huang et al., 2018; Jamdagni et al., 2018; Xue et al., 2014).

Under this light, this study aimed to: (a) evaluate the effectiveness of ZnO-NPs against sensitive and boscalid resistant *A. altarnata* isolates, (b) investigate the potential of ZnO-NPs used in combinations with fungicides against sensitive/resistant *A. alternata* phenotypes both *in vitro* and on fruit, and (c) elucidate mechanisms underlying the mode of fungitoxic action of ZnO-NPs, and the synergy observed between ZnO-NPs and boscalid.

6.2. Materials and Methods

6.2.1 Nanoparticles, fungicides and reagents

Zinc oxide nanoparticles [ZnO-NPs] (<100nm particle size), zinc sulphate [$ZnSO_4$], Ethylenediaminetetraacetic acid (EDTA) and Salicylhydroxamate [SHAM], were purchased from Sigma Aldrich, MO, USA. Commercial fungicides containing active ingredients boscalid (Cantus 50 WG), fluazinam (Azzuro 50 SC), mancozeb (Trimanoc 75 WG) and $Cu(OH)_2$ (Copperblau-N 50 WP), were purchased from their respective manufacturers. Analytical grade active ingredients of remaining fungicides: tebuconazole and fenhexamid were supplied by Bayer CropScience AG (Leverkusen, Germany), pyraclostrobin and boscalid by BASF AG (Limburgerhof, Germany) and fludioxonil by Syngenta Crop Protection AG (Basle, Switzerland). All analytical grade stock solutions contained ethanol as a solvent in except for boscalid, and pyraclostrobin which were dispersed in methanol. In *in vitro* bioassays, active ingredients were added aseptically to sterilized growth medium prior to inoculation. Antifungal agent concentrations were prepared by adding appropriate quantities of the active ingredients making sure that the solvent never exceeded 1 % (v:v) of the total volume. An appropriate volume of solvent was also added in the control treatments. Stock solutions of nanoparticles and commercial fungicides were prepared in distilled-sterilized water. In order to deter particle aggregation, nanoparticle suspensions were sonicated for 30 min using a Transonic 420 (Elma, Germany) sonicator prior to their incorporation in growth media. Zeta potential and hydrodynamic diameter measurements for the ZnO-NPs were measured in triplicate with a Zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA).

6.2.2 Fungal isolates and culture conditions

Alternaria alternata isolates originated from infected tomato fruits collected from greenhouses located in Crete (southern Greece) during a survey conducted in 2020. Most greenhouses of collection had a prior history of frequent spaying with SDHIs (boscalid and fluopyram) and QoIs (pyraclostrobin, famoxadone) for at least 2 years. Conidia from infected fruit were scrapped using a sterilized needle under aseptic conditions, transferred in glass tubes containing sterilized water and plated on acidified PDA medium (containing 1mL/L of lactic acid) in order to acquire single spore isolates and avoid bacterial infections. Single spore isolates were identified to be *A. alternata* by morphological examination under a microscope, a fact subsequently validated by sequencing of the *sdh* gene subunits evaluated in this study. Ten isolates were selected for further assessment. Isolates used in fungitoxicity assays were kept on Potato Dextrose Agar (PDA) medium in growth chambers at 25 ^{o}C with 14h day^{-1} light and 70% RH. Once every two months, isolates were transferred in PDA-containing glass tubes and stored at 4 °C in the dark for long-term storage.

6.2.3 *In vitro* bioassays

6.2.3.1 Sensitivity of *A. alternata* to ZnO-NPs and selected fungicides

The potential of zinc oxide NPs to control sensitive and fungicide-resistant *A. alternata* isolates, was evaluated by fungitoxicity tests *in vitro* utilizing the poison agar assay. Fungitoxicity was expressed as percent relative inhibition of isolates grown on PDA (except in the cases of treatments containing fluopyram and boscalid where water agar was used) amended with appropriate concentrations of the antifungal active ingredients. In order to assess the sensitivity of *A. alternata* isolates to fungicides, discriminatory concentrations equal to mean EC_{50} values (effective concentration causing 50% inhibition of mycelial growth) reported previously were used in the *in vitro* bioassays. Specifically, concentrations used were: 500μg/mL for $Cu(OH)_2$, 0.05 μg/mL for fluazinam, 0.2 μg/mL for fludioxonil, 2 μg/mL for tebuconazole and 2.5 μg/mL for boscalid (Avenot and Michailides, 2015; Kalamarakis et al., 2000; Malandrakis et al., 2019; Markoglou et al., 2006). Sensitivity of *A. alternata* to ZnO-NPs was evaluated by applying concentrations of 10, 25, 50, 100, 250, 500 and 1000 μg/mL ZnO-NPs in order to obtain fungitoxicity-curves and calculate a mean EC_{50} value. EC_{50} values and respective resistance factors (Rf: EC_{50} of the resistant isolate over mean EC_{50} of sensitive isolates) of isolates with reduced sensitivity to boscalid were calculated by subjecting them to additional fungicide doses. Specifically, concentrations of 0, 0.1, 1, 2.5, 5, 10, 50, 100 and 150 μg/mL boscalid and 0, 0.01, 0.05, 1, 2.5, 5, 10 μg/mL fluopyram were used to obtain fungitoxicity-curves for *A. alternata* SDHI-resistant isolates. All fungicide concentrations were applied in triplicate. Growth media treated with antifungal agents or not (control) were inoculated with a 5-mm mycelial plug cut from the edge of a 4-day old colony of each isolate placed the center of each plate. Cultures were then incubated in a growth chamber at 25 °C with 70% RH in the dark for 4 days. The following formula: 100-(mean diameter of the colony

on the fungicide-treated plates divided by the mean diameter of the untreated control) ×100 was used to calculate percent inhibition rates. Tests were repeated twice for each isolate and antifungal agent concentration.

6.2.3.2 Synergy between ZnO-NPs and antifungal agents

Potential synergy activity of ZnO-NPs or their bulk/ionic counterpart $ZnSO_4$ when applied in mixtures with fungicides or other reagents was assessed *in vitro* by poison agar assays. Appropriate volumes from stock solutions were added to PDA medium (or WA in the case of treatments containing boscalid or fluopyram) in order to obtain concentrations of 10 μg/mL boscalid, 2 μg/mL fluopyram and tebuconazole, 0.05 μg/mL fluazinam and 0.2 μg/mL fludioxonil individually or in combination with 300 μg/mL ZnO-NPs or 500 μg/mL $ZnSO_4$. The effect of EDTA (100μg/mL) and SHAM (100μg/mL) on the sensitivity of isolates to ZnO-NPs or $ZnSO_4$ was also evaluated. Plates inoculated with each *A. alternata* isolate were incubated for 4 days at 25 °C in the dark. Subsequently, mycelial growth was measured in terms of colony diameter and percent inhibition rates were calculated. Synergistic interaction of ZnO-NPs/$ZnSO_4$ with tested antifungal agents was evaluated according to the Abbortt method (Gisi, 1996). Briefly, the expected combined percent inhibition (% CI_{exp}) was calculated as: % $CI_{exp} = I_A+I_B–(I_A×I_B/100)$, where I_A and I_B are the percent inhibition of each antifungal agent. Synergy factors (SFs) were determined according to the formula $SF=I_{AB}/(\% CI_{exp})$, 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.

6.2.4 Characterization of ZnO-NPs/boscalid mixtures

In an attempt to elucidate the possible mechanism underlying the synergy observed between ZnO-NPs and boscalid against *A. alternata* isolates, suspensions containing 10 μg/mL boscalid, 300 μg/mL ZnO-NPs and their mixture were prepared. All suspensions were subjected to sonication for 30 mins to prevent nanoparticle aggregation. Subsequently, zeta potential and hydrodynamic diameter measurements for the ZnO-NPs, boscalid and mixtures were measured in triplicate with a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA). pH values were also measured for all suspensions using a WTW pH-meter (inoLab® pH 7110).

6.2.5 Fungitoxicity tests *in vivo*

The effectiveness of ZnO-NPs alone or in combination with boscalid to control sensitive and fungicide-resistant *A. alternata* isolates *in vivo* was tested using wound-free tomato fruit (*Lycopersicon esculentum* cv mojito) with uniform maturity, shape and size. Four

tomato fruits per isolate were treated with ZnO-NPs, boscalid and their combinations and then inoculated with two sensitive (AA236, AA239), one medium (AA203) and one highly (AA171) boscalid-resistant isolates. Control treatment consisted of four tomato fruits spayed with distilled water. Fruits were immersed in a 1% sodium hypochlorite water solution for 10 min in order to be surface-disinfected. Following disinfection, the fruit were rinsed three times with distilled-sterilized water and left to dry before treatment with boscalid or ZnO-NPs. Dry fruits were then sprayed with solutions of 1000 μg/mL ZnO-NPs, 500 and 1000 μg/mL boscalid (1/2, 1× of the maximum recommended dose) and their combinations. Fruits were left to air-dry for an additional a period of 2 hr and then wounded with 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 *A. alternata* isolate was placed on top of each wound. Inoculated fruits were placed inside plastic boxes 24×34×10 cm [length×width×height] on top of a wet sterilized paper and covered by a lid to ensure moist conditions before incubation at 25 °C in the dark for 4 days. Percent symptom severity was calculated by dividing mean lesion diameter around each wound of treated fruit by the respective lesion diameter of the water-treated control. All experiments were conducted in triplicate.

6.2.6 DNA extraction and sequence analysis of SDH gene subunits from A. alternata isolates

In an attempt to investigate the resistance mechanism of boscalid-resistant *A. altarnata* isolates, 3 subunits (*sdh*B, *sdh*C, *sdh*D) of the gene encoding the succinate dehydrogenase (SDH), target of the SDH-inhibitor fungicides, were amplified and sequenced. Specifically, fungal cultures were grown on fungicide-free PDA at 25 ^{o}C for ten days. Subsequently, mycelia were collected by scraping and ground using a mortar and pestle in the presence of liquid nitrogen. Total DNA was isolated using TRI reagent (Sigma) following the manufacturer's instructions. Primer pairs AaSDHB-F (5' ATACGCGCTTTCACTCGTCT 3') and AaSDHB-R (5' GCATGTCCTTGAGCAGTTGA 3'), AaSDHC-F (5' ATGGCTTCTCAGCGGGTATTTC 3') and AaSDHC-R (5' CATCCGAGGAAGGTGTAGTA 3'), AaSDHD-F (5' GCCTCCGTCATGCGTCCCGG 3') and AaSDHD-R (5' CTATGCGTGCCACAACCTC 3') were used for the amplification of the respective *sdh*B, *sdh*C and *sdh*D gene fragments from each *A. alternata* isolate using gDNA as template. PCR reactions comprised of 0.2 mM from each of the primers, 1.5 mM $MgCl_2$, 0.5mM dNTPs, and 1.25 units of HotStar Taq DNA polymerase (Qiagen) in 20 mM TrisHCl and 50 mM KCl. The PCR conditions were: 95 ^{o}C for 15 min followed by 40 cycles of 94 ^{o}C for 30 s, 58 ^{o}C for 30 s, and 72 ^{o}C for 1 min with a final 10 min extension at 72 ^{o}C. QIAquick gel extraction kit (Qiagen)was used to purify PCR products which were subsequently ligated to pGEM-Teasy (Promega) vectors and transformed into *E. coli* competent cells (DH5a Library Efficiency® Competent Cells, Invitrogen). Plasmids containing the *sdh*B, *sdh*C *sdh*D respective gene fragments were purified using QIAprep spin miniprep kit plasmid (Qiagen) and then sequenced in both directions. Ten independent clones from each *A. alternata* tested isolate were analyzed. Sequence data analysis was performed using the Lasergene (DNAstar, Madison, USA) software.

6.3. Statistical analysis

The EC_{50} values for each isolate and fungicide were estimated by regression of the relative inhibition of mycelial growth against the Log_{10} of the compound concentrations. Pearson correlation coefficients were used to evaluate correlation between isolate sensitivities to tested NPs/fungicides. Inhibition rates caused by ZnO-NPs and fungicides were subjected to analysis of variance (ANOVA) 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).

6.4. Results

6.4.1 Sensitivity screening of *A. alternata* isolates *in vitro*

Sensitivity of *A. alternata* to ZnO-NPs in terms of EC_{50} values ranged between 250 and 388 μg mL^{-1} with a median value of 303 μg mL^{-1}. Fungitoxicity tests showed that ZnO-NPs were significantly ($P<0.01$) more effective against *A. alternata* than its ionic counterpart $ZnSO_4$ or the reference protective fungicide containing $Cu(OH)_2$ *in vitro* (see Table 6.1). Screening fungicide resistant isolates using discriminatory doses revealed that *A. alternata* isolates exhibited baseline sensitivity to most fungicides tested (see Table 6.1). This was not the case for boscalid, where all but 2 isolates tested exhibited reduced sensitivity to the SDHI boscalid (see table 6.1,6.2), probably due to the extensive use of this class of fungicides against the pathogen during the last years. Resistance factors (Rfs) for boscalid and fluopyram, of SHHI-resistant isolates were calculated based on EC_{50} values determined by additional fungitoxicity tests (see Table 6.2). Analysis revealed one *A. alternata* isolate (AA171) highly resistant (BOSC-HR) to SDHIs boscalid and fluopyram (Rf: >96 and 145 respectively), seven isolates with medium resistance (BOSC-MR) to boscalid but not to fluopyram (Rf: 28.44-36.76) and two isolates (AA236, AA239) sensitive to both boscalid (BOSC-S) and fluopyram (see Table 6.2).

Table 6.1 Sensitivity of *Monilia fructicola* isolates to Ag-NPs and selected fungicides.

	Percent Inhibition [a] (mean±SD [b])								
Isolate	ZnO-NPs (500)[c]	$ZnSO_4$ (500)	$Cu(OH)_2$ (500)	mancozeb (100)	boscalid (10)	Fluopyram (2)	Tebucona-zole (2)	fluazinam (0.05)	Fludiox-onil (0.2)
AA201	64.41 ± 0.35	53.33 ± 0.11	51.32 ± 1.05	25.35 ± 0.71	42.55 ± 0.71	100	64.79 ± 1.41	40.84 ± 0.29	93.65 ± 0.24
AA234	53.65 ± 1.51	35.56 ± 0.18	38.50 ± 3.02	24.24 ± 2.83	55.58 ± 1.41	100	53.03 ± 0.74	30.11 ± 1.32	61.82 ± 2.57
AA191	65.59 ± 0.96	66.67 ± 1.01	40.89 ± 2.10	35.29 ± 2.83	53.33 ± 1.41	100	70.59 ± 0.28	26.31 ± 1.04	93.22 ± 2.30
AA203	64.18 ± 2.15	71.67 ± 0.50	41.12 ± 3.05	22.86 ± 0.05	46.67 ±0.02	100	65.71 ± 0.83	32.69 ± 0.17	64.71 ± 2.28
AA202	77.86 ± 1.10	30.23 ± 1.00	39.14 ± 2.85	32.26 ± 0.12	57.78 ± 0.10	100	66.13 ±0.04	59.78 ± 0.67	72.31 ± 0.75
AA236	63.70 ± 3.10	49.23 ± 0.71	28.96 ± 3.54	28.57 ± 0.71	95.1 ±0 .09	100	50.79 ± 0.00	25.05 ± 1.09	53.85 ± 5.44
AA204	67.41 ± 2.55	60.32 ± 0.71	37.10 ± 0.10	32.43 ± 0.06	48.94 ± 0.05	100	66.22 ± 1.25	19.51 ± 0.25	73.53 ± 4.16
AA239	66.22 ± 1.68	38.46 ± 1.41	50.12 ± 1.65	34.29 ± 4.24	93.81 ± 0.16	100	60.05 ± 0.92	28.03 ± 2.10	47.54 ± 3.45
AA171	65.72 ± 1.54	73.08 ±0.17	54.19± 6.19	13.85 ± 2.83	1.00 ± 0.03	58.52 ± 3.54	69.23 ± 0.06	23.68 ± 2.14	100. ± 0.00
AA235	62.08 ± 3.13	35.82 ±0.68	12.32 ± 1.53	28.99 ± 0.71	41.88 ± 0.71	100	55.07 ± 0.77	28.04 ± 1.22	65.52 ± 5.22

[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 6.2 Sensitivity profiles of representative *A. alternata* isolates to SDHI fungicides boscalid and fluopyram and respective resistance mutations in the target site gene subunits (*sdh*B, *sdh*C, *sdh*D).

	Fungicides				Succinate Dehydrogenase gene subunits amino acid substitutions		
	Boscalid		fluopyram				
Isolate	EC_{50}[a] (mean ± SD[b])	Rf[c]	EC_{50} (mean ± SD)	Rf	*sdh*B	*sdh*C	*sdh*D
AA236	1.55 ± 0.25	1.00	0.04 ± 0.01	1.33	-	-	-
AA239	1.65 ± 0.30	1.06	0.03 ± 0.00	1.00	-	-	-
AA201	56.37 ± 3.36	36.37	0.05 ± 0.02	2.50	-	-	A47T
AA234	44.09 ± 2.51	28.44	0.10 ± 0.05	3.33	-	`-	A47T
AA191	45.62 ± 3.70	29.32	0.15 ± 0.10	5.00	-	-	A47T
AA203	52.60 ± 5.18	33.93	0.08 ± 0.02	2.66	-	-	A47T
AA202	48.29 ± 4.07	31.15	0.11 ± 0.04	3.67	-	-	A47T
AA204	54.22 ± 4.05	34,98	0.09 ± 0.02	3.00	-	-	A47T
AA235	56.98 ± 1.88	36.76	0.12 ± 0.04	4.00	-	-	A47T
AA171	>150	>96.77	4.35 ± 0.05	145.0	-	H134R	A47T

[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 the most sensitive isolate).

6.4.2 Identification of target-site resistance mutations

The existence of resistance mutations in the gene encoding the target site of SDHI fungicides in the BOSC-MR/HR isolates was validated by sequencing *sdh*B, *sdh*C and *sdh*D gene fragments isolated from sensitive and resistant isolates.

Sequencing analysis and comparison of the deduced amino-acid sequence between BOSC-M/HR and BOSC-S isolates revealed an alanine (A: GCC) substitution by threonine (T: ACC) at position 47 of the *sdh*D protein leading to the A47T resistance mutation in all boscalid resistant isolates. An additional resistance mutation was found in the highly boscalid and fluopyram resistant AA171 isolate resulting from the replacement of histidine (H: CAC) with arginine (CGC) at position 134 (H134R) of the *sdh*C protein (see Table 6.2). The H134R amino

acid substitution is a well-documented SDHI resistance mutation, known to confer high resistance levels in *A. alternata* and *A. solani* (Sang and Lee, 2020). These results indicated that target-site modification reducing the affinity between boscalid and their succinate dehydrogenase (SDH) target was the mechanism responsible for the observed BOSC-MR and BOSC-HR resistant phenotypes.

6.4.3 Synergy between ZnO-NPs and fungicides

6.4.3.1 *In vitro* bioassays

ZnO-NPs were tested *in vitro* against sensitive and boscalid-resistant *A. alternata* isolates in combination with boscalid, fluopyram, fludioxonil, tebuconazole, SHAM, EDTA and fluazinam. Similar tests were carried out to evaluate potential synergism between $ZnSO_4$ and boscalid, fluopyram, EDTA and SHAM. Synergy factors (SF) were calculated for ZnO-NPs/$ZnSO_4$ and combinations and the respective values are shown in Table 6.4. ZnO-NPs significantly enhanced the fungitoxic effect of boscalid against both BOSC-S and BOSC-M/HR *A. alt*ernata isolates, resulting in almost complete inhibition of mycelial growth in most cases (see Fig. 6.1a, 6.2). SF values between ZnO-NPs and boscalid ranged between 1.08 to 2.57 (see Table 6.3). Contrary to the profound synergistic profile exhibited by ZnO-NPs, the combined use of its ionic counterpart $ZnSO_4$ with boscalid resulted in a mostly additive effect with SF values ranging between 0.85 and 1.06 (see Table 6.3, Fig. 6.1b), probably indicating an additional role of nanoparticle properties to the observed synergism between ZnO-NPs and boscalid other than zinc ion release. An additive or synergistic effect (SF: 0.91 - 1.34) was observed between ZnO-NPs and fluazinam (see Table 3). In most cases, the addition of fluazinam enhanced toxicity of ZnO-NPs while, in some BOSC-MR cases, it led to complete inhibition of mycelial growth (Fig. 6.1c). A slight additive effect was observed in the cases of ZnO-NPs/fludioxonil (SF: 0.80 - 1.17) and ZnO-NPs/tebuconazole (SF: 0.80 - 0.89) combinations (see Table 6.3).

The possible role of zinc ions release on the fungitoxic mode of action of ZnO-NPs was investigated by combining them with the strong chelating agent EDTA in a synergism bioassay *in vitro.* Addition of 100 μg/mL EDTA in growth medium containing 300 μg/mL ZnO-NPs resulted in the alleviation of the fungitoxic effects of the zinc oxide nanoparticles against *A. alternata* indicating that zinc ion release contributes -at least partly- on the nanoparticle inhibitory action. This was evident on the synergy factor values between ZnO-NPs and EDTA that ranged between 0.16 and 0.60 suggesting a strong antagonistic effect (see Table 6.3). A similar antagonistic effect was observed between $ZnSO_4$ and EDTA in most isolate cases.

In a similar context, aiming to investigate the involvement of the ROS generation on the fungitoxic mode of action of ZnO-NPs, the alternative oxidase (AOX) specific inhibitor SHAM was employed. Inhibiting the AOX pathway with SHAM resulted in an enhanced fungitoxic effect of both ZnO-NPs and its ionic counterpart $ZnSO_4$. Synergy factor values recorded were close to 1 in both cases indicating an additive effect (see Table 6.3).

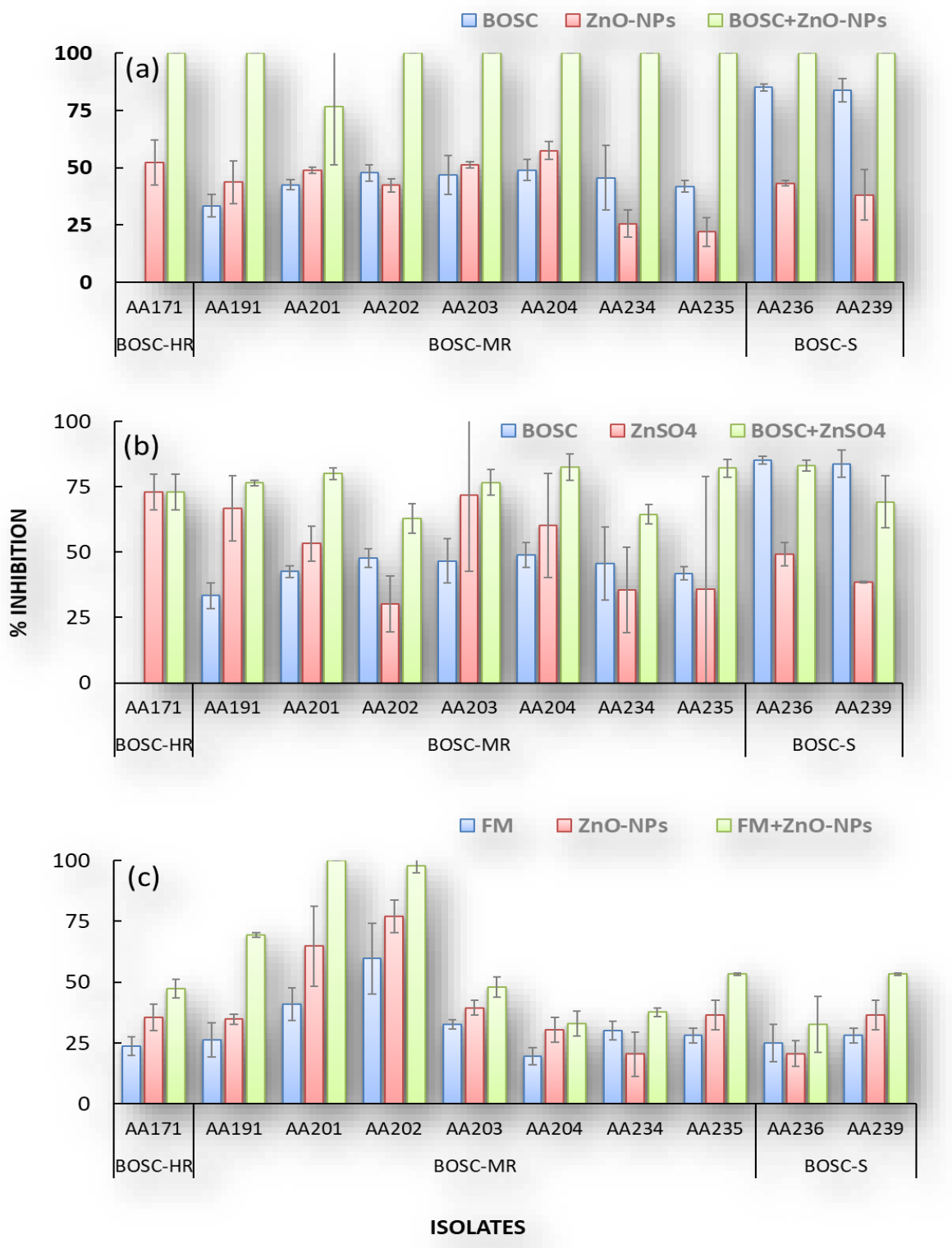


Figure 6.1. Sensitivity of fungicide-sensitive/resistant *A. alternata* isolates to boscalid (10 µg/mL) in comparison with: (a) ZnO-NPs (300 µg/mL), (b) $ZnSO_4$ (500 µg/mL), and fluazinam (0.2 µg/mL) with (c) ZnO-NPs (300 µg/mL) and their combinations. BOSC-S/MR/HR: boscalid-Sensitive/Moderately or Highly Resistant isolates (BOSC: boscalid, FM: fluazinam). Error bars 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 6.3. *In vitro* synergistic activity of ZnO-NPs or $ZnSO_4$ with selected fungicides against boscalid sensitive and resistant *Alternaria alternata* isolates (Bosc: boscalid, Fluo: fluopyram, FM: fluazinam, TEB: tebuconazole, FLUD: fludioxonil).

Isolate		SF[a]										
		ZnO-NPs (300)							$ZnSO_4$ (500)			
	Resistance Phenotype	BOSC (75) [c]	FLUO (2)	FM (0.05)	EDTA (100)	SHAM (100)	TEB (2)	FLUD (0.2)	BOSC (75)	FLUO (2)	EDTA (100)	SHAM (100)
AA236	BOSC-S	1.45	1.04	0.91	0.38	1.35	0.86	0.87	1.03	0.91	0.52	0.93
AA239	BOSC-S	1.89	0.91	0.98	0.44	0.98	0.80	0.80	0.85	0.81	1.80	1.05
AA201	BOSC-MR	1.08	0.76	1.26	0.51	0.99	0.88	0.98	1.04	1.01	0.42	1.09
AA234	BOSC-MR	2.24	0.69	0.95	0.52	0.84	0.89	0.81	0.94	0.96	2.25	0.84
AA191	BOSC-MR	1.60	1.60	1.34	0.16	0.94	0.80	0.98	0.86	0.91	1.02	0.82
AA203	BOSC-MR	1.35	2.11	0.91	0.52	0.96	0.88	0.90	0.87	1.05	0.20	1.09
AA202	BOSC-MR	1.15	1.21	1.08	0.35	1.75	0.89	1.17	1.06	0.97	0.49	1.02
AA204	BOSC-MR	1.28	0.99	0.95	0.60	1.17	0.81	0.90	1.00	0.89	0.38	1.28
AA235	BOSC-MR	2.57	0.97	0.98	0.51	0.86	0.88	1.02	1.03	0.86	0.55	0.89
AA171	BOSC-HR	1.89	1.46	0.93	0.61	0.99	0.83	1.02	0.92	2.23	0.54	1.01

[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

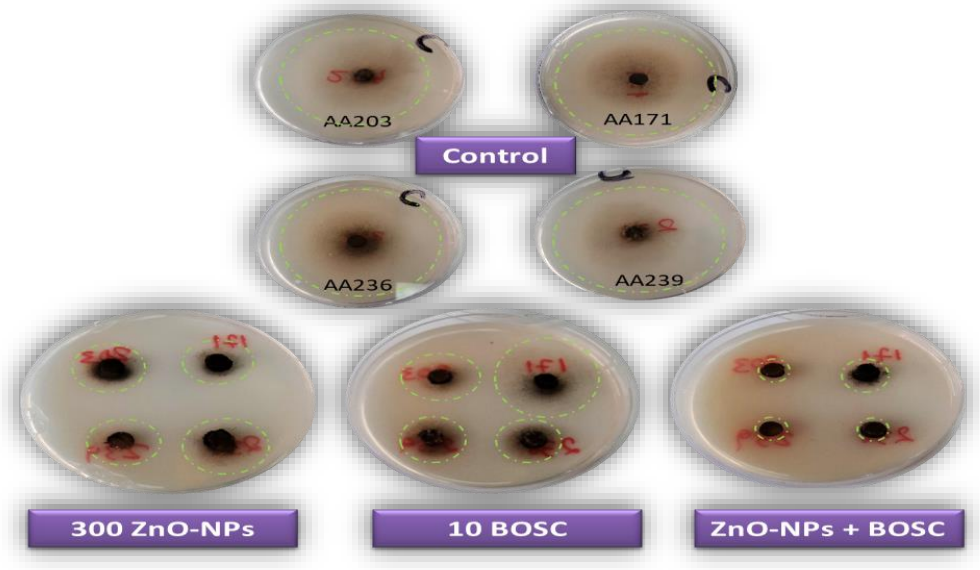


Figure 6.2. Fungitoxic activity of ZnO-NPs, boscalid (BOSC) and their combination in sensitive (AA236, AA239, moderately (AA203) and highly (AA171) boscalid-resistant *Alternaria alternata* isolates. Green dashed circles indicate colony margins.

6.4.3.2 *In vivo* bioassays

The synergistic activity between ZnO-NPs and boscalid against both sensitive and boscalid-resistant *A. alternata* isolates observed *in vitro*, was tested on tomato fruit inoculated with representative isolates from each phenotypic category. The addition of boscalid significantly enhanced percent disease suppression caused by ZnO-NPs (see Fig. 6.3). Specifically, percent inhibition of lesion development in ZnO-NPs/boscalid combinations ranged between 34.21 and 82.32% while the respective values for ZnO-NPs alone ranged between 0 and 32.25% (see Table 6.4). Synergy factors (SF:1.18 to 1.57) between ZnO-NPs and boscalid clearly indicated a strong synergistic effect between the two antifungals confirming the findings of the *in vitro* experiments.

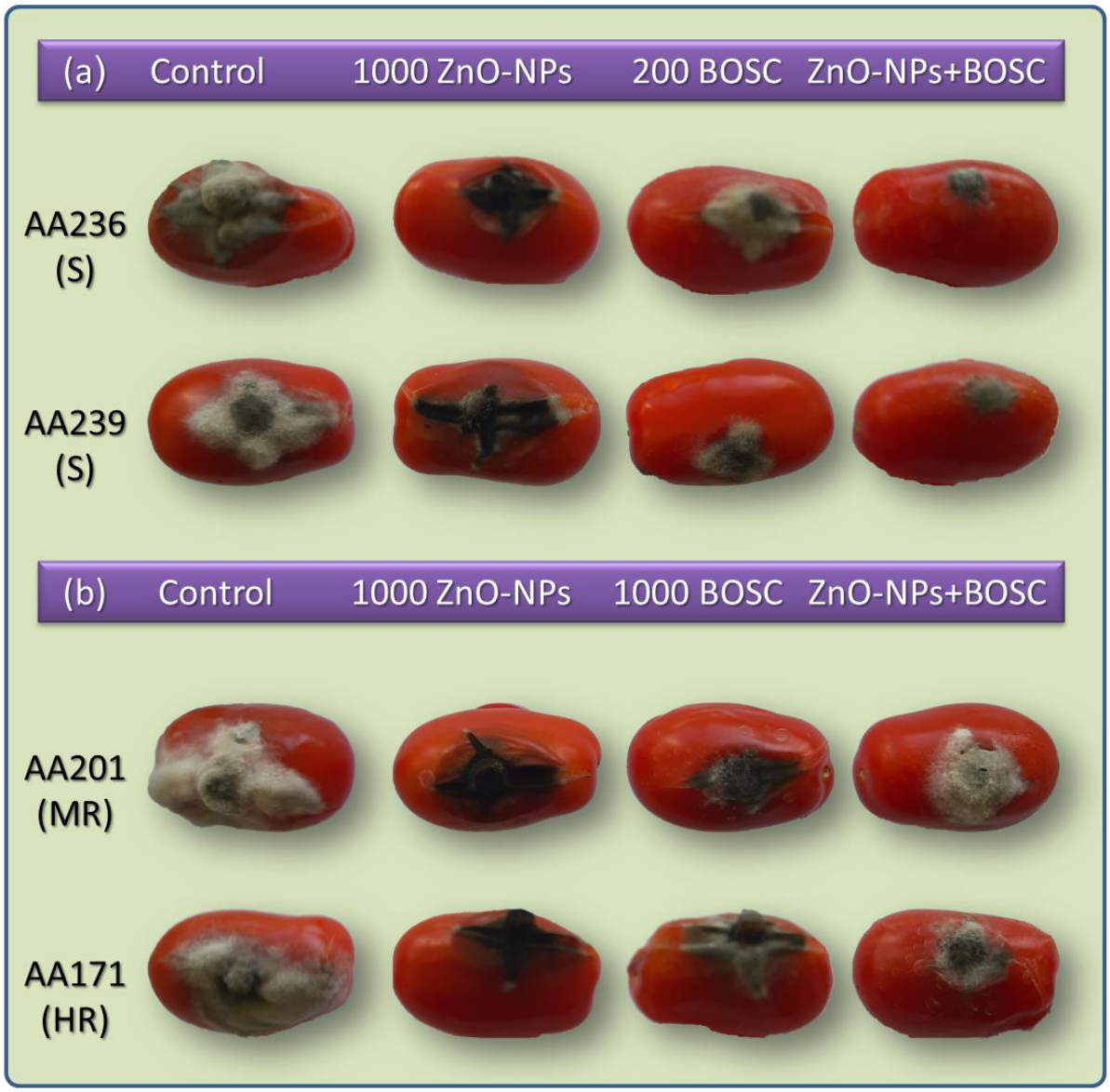


Figure 6.3 Synergistic activity of ZnO-NPs (1000 µg/mL) in combination with boscalid (200/1000 µg/mL) against representative (a) sensitive and (b) medium (AA201) or highly (AA171) boscalid-resistant *Alternaria alternata* isolates (BOSC: boscalid).

Table 6.4 Synergistic activity of ZnO-NPs co-applied with boscalid on tomato fruit against *Alternaria alternata* isolates sensitive and resistant to boscalid (BOSC) fungicide.

Isolate	Phenotype	Percent inhibition [a] (mean±SD[b])			Expected Inhibition (%)	SF[f]
		ZnO-NPs (1000) [d]	BOSC (500/1000[e])	ZnO-NPs + BOSC		
AA236	BOSC-S	14.24 ± 3.19	44.42 ± 2.35	82.32 ± 0.12	52.33	1.57
AA239	BOSC-S	2.05 ± 1.52	50.35 ± 3.08	74.56 ± 1.05	51.37	1.45
AA191	BOSC-MR	32.50 ± 0.13	30.25 ± 0.75	80.34 ± 3.42	52.92	1.52
AA201	BOSC-MR	16.98 ± 2.45	19.06 ± 1.17	43.40 ± 0.88	32.80	1.32
AA203	BOSC-MR	0.00	28.95 ± 2.90	34.21 ± 1.45	28.95	1.18
AA171	BOSC-HR	22.81 ± 2.06	10.05 ± 0.05	52.63 ± 3.19	30.57	1.72

[a] Calculated as percent inhibition of lesion development on apple fruit sprayed with ZnO-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] BOSC-S: Boscalid Sensitive, BOSC-MR/HR: boscalid Medium/Highly Resistant isolate.
[d] Numbers in parenthesis indicate fungicide concentrations in μg/mL of active ingredient.
[e] Boscalid concentrations applied on tomato fruit were 500 μg/mL for the BOSC-S and 1000 μg/mL for the BOSC-MR/HR isolates.
[f] Synergy Factor calculated as observed inhibition caused by the ZnO-NPs+BOSC mixture over the expected inhibition by the individual treatments.

6.4.3.3 Characterization of ZnO-NPs, boscalid and mixture

Size, charge and pH values of 10 μg/mL boscalid, 300 μg/mL ZnO-NPs and their mixture are shown in Table 6.5. Mean hydrodynamic diameter of ZnO-NPs were measured to be 237.3 nm while boscalid had a respective value of 360.2 nm. Interestingly, their mixture resulted in a homogenous suspension of particles with a mean size of 169.6 nm and a similar to boscalid distribution (see Table 6.5, Fig. 6.4). Furthermore, the addition of the negatively charged boscalid (-42.4 mV) to positively charged ZnO-NPs (25.9 mV) resulted in an also negatively charged (-35.6 mV) suspension of ZnO-NPs-boscalid conjugates. This negative charge probably acted as a dispersive factor resulting to a homogenous suspension with lower hydrodynamic particle sizes. This reduction in nanoparticle size could be a reason for the enhanced fungitoxic effect of the ZnO-NPs+boscalid mixture compared to ZnO-NPs alone.

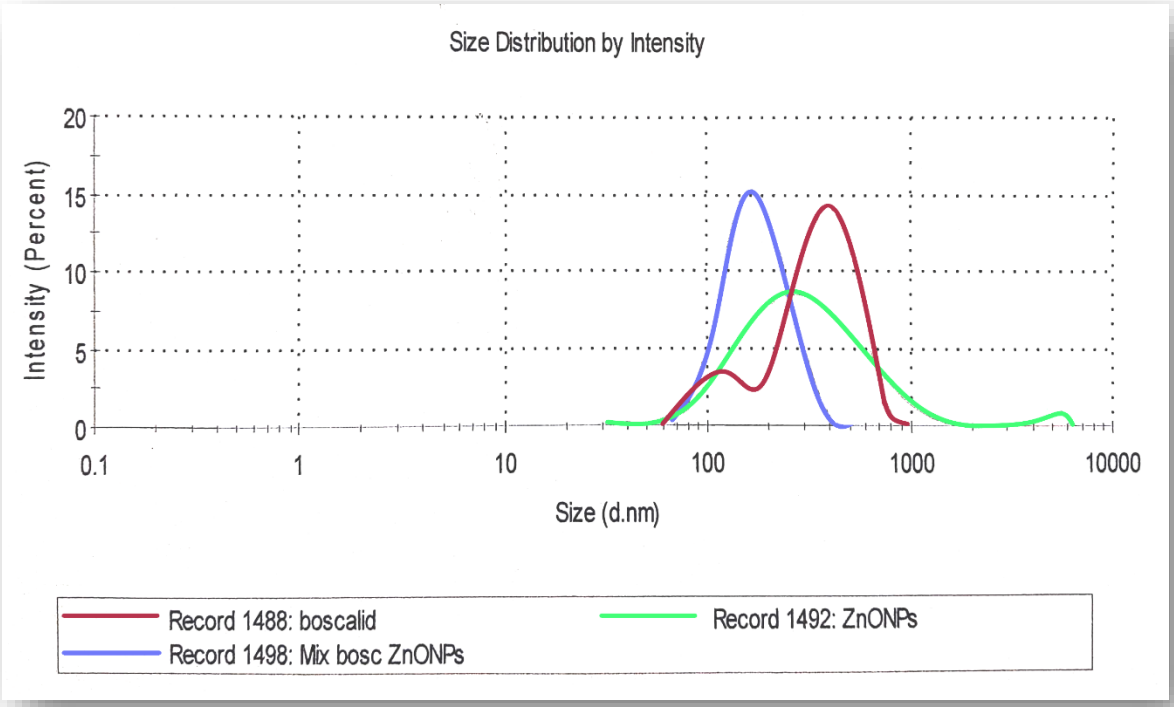


Figure 6.4 Mean size distribution of boscalid (10 µg/mL), ZnO-NPs (300 µg/mL) particles and their mixture measured by Dynamic Light Scattering.

Table 6.5 Zeta potential, diameter size and pH values of ZnO-NPs (300 mg/mL), boscalid (10 mg/mL) and mixture.

	pH	Zeta potential (mV) (mean ± SD[a])	Size (d.nm) (mean ± SD)
ZnO-NPs	7.0	25.9 ± 0.2	237.3 ± 2.7
boscalid	5.7	-42.4 ± 5.5	360.2 ± 4.1
ZnO-NPs + boscalid	6.7	-35.6 ± 1.5	169.6 ± 1.0

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

6.4.4 *S*ensitivity correlations between ZnO-NPs, $ZnSO_4$, antifungal agents and combinations

In order to investigate the possible mode of action of ZnO-NPs or its contribution in the observed synergistic relationship with fungicides or reagents tested, correlations were calculated using Pearson correlation coefficients (see Table 6.6, Fig. 6.5). The significant correlation (r =0.73, P=0.05) found between ZnO-NP and $ZnSO_4$ indicated a similar mode of action between the counterparts suggesting that zinc ion release plays an important role in the observed antifungal action of ZnO-NPs (See Fig 5a). Both ZnO-NPs and fluazinam seem to contribute on the observed additive fungitoxic effect of their combination as indicated by the positive correlation of each of both antifungals with ZnO-NPs+fluazinam in terms of sensitivity (see Fig. 6.5d). On the contrary, no statistically significant correlation was observed between ZnO-NPs, boscalid and their combination (see Table 6.6). A positive correlation (r = 0.67, P=0.05) was found between boscalid and fluazinam while a negative one between boscalid and SHAM (see Table 6.6, Fig. 6.5c). Sensitivity of *A. alternata* to ZnO-NPs was positively correlated with their respective sensitivity to EDTA as shown in Fig. 5b.

Table 6.6 Correlation between sensitivity of *A. alternata* isolates to ZnO-NPs, $ZnSO_4$, boscalid (BOSC), fluazinam (FM) and their combinations.

	ZnO-NPs	$ZnSO_4$	BOSC	FM	ZnO-NPs + BOSC	ZnO-NPs + FM
ZnO-NPs	1.0 [a]	0.73*	0.23	0.88**	0.32	0.92**
$ZnSO_4$	-	1.0	-0.27	-0.46	-0.04	-0.20
BOSC	-	-	1.0	0.672*	-0.10	0.43
FM	-	-	-	1.0	-0.28	0.81**
ZnO-NPs + BOSC	-	-	-	-	1.0	-0.61
ZnO-NPs + FM	-	-	-	-	-	1.0

[a] Pearson correlation coefficient values.
* Corresponds to a significance lever of P=0.05.
** Corresponds to a significance lever of P=0.01

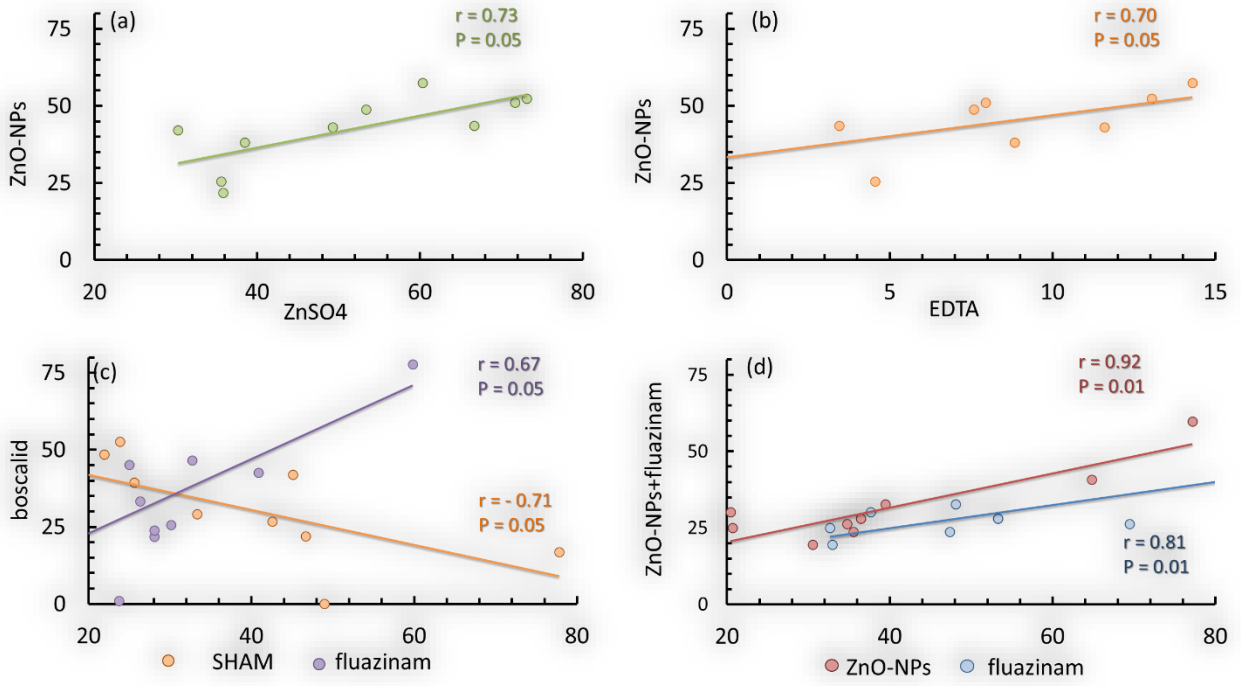


Figure 6.5 Correlation between sensitivities of A. alternata isolates to ZnO-NPs (300 µg/mL) and (a) ZnSO4 (500 µg/mL), (b) EDTA (100 µg/mL), (c) boscalid (10 µg/mL) and SHAM (100 µg/mL) or fluazinam (0.05 µg/mL), and (d) fluazinam (0.05 µg/mL) and ZnO-NPs alone or in combination with fluazinam. Here, r is the Pearson correlation coefficient, and P the significance level.

6.5. Discussion

The potential of ZnO-NPs to control sensitive and boscalid resistant *A. alternata* isolates collected from Greek tomato-growing greenhouses was evaluated by bioassays conducted both *in vitro* and *in vivo*. Sensitivity screening of *A. alternata* isolates revealed 3 boscalid-sensitivity phenotypes: sensitive (S), moderately (MR) and Highly (HR) boscalid resistant. Sequencing analysis of the succinate dehydrogenase (SDH) gene, encoding the target enzyme of the SHDI boscalid revealed one resistance mutation (A47T) in the *sdh*D subunit of the gene, present in all resistant phenotypes. An additional mutation (H134R) in the *sdh*C subunit was found in the case of the AA171 HR isolate. The H134R mutation has been implicated with high-level SDHI-resistance in various plant pathogens while the A47T has only been reported in *A. alternata* (Sang and Lee, 2020; Sierotzki and Scalliet, 2013; Malandrakis et al., 2018). These results confirmed that target site modification was the main resistance mechanism of MR and HR isolates. Subsequently, characterized sensitive and boscalid resistant isolates were utilized to evaluate the effectiveness of ZnO-NPs as antifungal agents and their potential as fungicide-partners in the combat against drug-resistance.

Research on metal nanoparticles used as antibiotic alternatives/partners is gaining ground following their successful use against life-threatening clinical drug or multi drug-

resistant (MDR) pathogens (Jampilek, 2016; Punjabi et al., 2018). The majority of these studies are about bacterial infections due to their importance in human health while limited are the reports available on the respectful interaction of metal nanoparticles with fungicides controlling sensitive or, even less, with drug-resistant fungal strains (Malandrakis et al., 2021b). Polyvinylpyrrolidone (PVP)-coated Ag-NPs were shown to be effective against the drug-resistant strains of the human fungal pathogen *Candida albicans* alone or in combination with sterol biosynthesis inhibiting fungicides (Sun et al., 2016). In the case of plant pathogenic fungi, an enhanced toxicity of. Ag-NPs and ZnO-NPs combined with fungicides carbendazim, mancozeb and thiram, was reported against *B. cinerea, A. alternata, Fusarium oxysporum*, *Aspergilus niger and, Penicillium expansum* (Jamdagni et al., 2018). Synergy as well as photo-degradation properties of ZnO-NPs when applied with the fungicide thiram was also reported *Phytophthora capsici* (Xue et al., 2014). Recent studies have revealed successful antifungal potential of silver and copper containing NPs against sensitive or benzimidazole-resistant strains of *B. cinerea* and *M. fructicola*, which was enhanced when combined with fungicides thiophanate methyl or fluazinam (Malandrakis et al. 2020a, b, 2021b).

ZnO nanoparticles tested in this study, were effective against both sensitive and boscalid resistant *A. alternata* isolates and even more effective in comparison with their ionic counterpart $ZnSO_4$ or the reference protective fungicide containing $Cu(OH)_2$. When used in combination with boscalid, a profound enhancement of the fungitoxic effect of ZnO-NPs against both sensitive and boscalid-resistant *A. alternata* isolates was observed *in vitro*. No synergy was observed between boscalid and $ZnSO_4$ indicating that nanoparticle properties probably account for enhanced toxicity of boscalid+ZnO-NPs treatment. This synergistic effect was also demonstrated *in vivo*, where the treatment of tomato fruits with the boscalid + ZnO-NPs combination significantly increased the suppression of disease symptoms compared to the individual treatments. The similar ZnO-NPs/boscalid synergy factor values between isolates belonging to different boscalid sensitivity phenotypes suggest that the enhanced toxicity observed could more likely be attributed to ZnO-NPs rather than boscalid. This is in agreement with a previous study where synergy observed between Ag-NPs and thiophanate methyl (TM) against *M. fructicola* was also attributed to an enhanced NP toxicity (Malandrakis et al., 2020b). On the contrary, when Cu-NPs and TM mixtures were utilized against *B. cinerea* or *M. fructicola*, synergy observed most likely resulted from a higher fungicide bioavailability (Malandrakis et al., 2020a; 2021b). In the present study, mixture of ZnO-NPs with boscalid resulted in a significantly reduced NP size as assessed by dynamic light scattering (DLS) using a Zetasizer. This reduction in nanoparticle size could be responsible for the increased toxicity exerted by the mixture compared with the ZnO-NPs alone. Several studies have indicated that smaller size of NPs leads to a more favorable surface area-to-volume ratio that promotes their antifungal activity although size is not the only major factor affecting NP toxicity (Kalia et al., 2021; Cruz-Luna et al., 2021). Another plausible explanation for the enhanced ZnO-NPs toxicity when combined with boscalid could involve a drug-facilitated mechanism that modulate delivery of the NPs inside the fungal cell. A recent study by Kalampokis et al. (2018) utilizing *A. nidulans* genetically inactivated mutants, has demonstrated that boscalid uptake is mediated by several nucleobase membrane transporters. It is possible that boscalid particles decorated with ZnO-NPs are "recognized" by those nucleobase transporters and are transferred

inside the fungal cell where they can exert an additional toxic action by generating reactive oxygen species (ROS) or interacting with proteins and enzymes crucial for fungal metabolism. Typically, direct contact of the positive charged ZnO-NPs with the fungal membrane is mediated by electrostatic attraction with the negative charged cell surface (Ruddaraju et al., 2020). On the contrary, ZnO-NPs + boscalid conjugates were negatively charged and thus are expected to be repelled by the also negatively charged fungal membrane. Internalization of the NP-drug conjugate by the above-mentioned transporters could bypass this obstacle and allow ZnO-NPs to exert their fungitoxic action internally and more effectively.

The fungitoxic action of metal NPs has been attributed to mechanisms that lead to disruption of cell walls or membrane integrity, generation of ROS, protein inactivation, disruption of the electron transport chain, damage to DNA and, eventually, cell death (Rudramurthy et al., 2016; Ruddaraju et al., 2020; Nisar et al, 2019; Cruz-Luna et al., 2021; Márquez et al., 2018). Metallic counterparts of NPs in their ionic form share the same or similar mechanisms of toxic action against pathogens giving rise to the question whether toxicity is caused by the metal ions escaping nanoparticle surfaces or by the unique NP properties (Sun et al., 2018; Hoseinzadeh et al., 2017; Król et al., 2017; Robinson et al., 2021; Slavin et al., 2017). In an attempt to resolve this debate, several approaches have been utilized involving biochemical, genomic, transcriptomic and metabolomic methods (Márquez et al., 2018; Cruz-Luna et al., 2021; Kumari et al., 2019; Sardella et al., 2017). Zinc ion accumulation facilitated by zinc transporters was reported to be responsible for the fungitoxic action of ZnO-NPs in *Sclerotinia homeocarpa* (Li et al., 2017). High throughput Gene Deletion Analysis (GDA) of *C. serevisiae* mutants highly sensitive to ZnO-NPs revealed that transmembrane transport and cellular ion homeostasis play a pivotal role in ZnO-NPs antifungal activity (Márquez et al., 2018).

In the present study, a strong chelating agent (EDTA) was added in ZnO-NPs containing treatments in order to arrest Zn^{+2} cations dissolving from NPs. This resulted in a significant alleviation of the toxic effect of ZnO-NPs against *A. alternata* suggesting that ion release is mainly responsible for the fungitoxic action of ZnO-NPs. This was also confirmed by the positive correlation found between $ZnSO_4$ and ZnO-NPs sensitivities in the respective *A. alternata* fungitoxicity bioassays. This is in agreement with a previous study by Sardella et al. (2017) where, EDTA caused inactivation of ZnO-NPs toxic activity against the postharvest plant pathogen *P. expansum*. The same antagonistic effect between EDTA and Cu-NPs was observed in the case of *M. fructicola* but not when Ag-NPs were used instead of copper NPs indicating that ion release role in toxicity could vary between different metal NPs (Malandrakis et al, 2020b; 2021b). Another possible mechanism of fungitoxic action of ZnO-NPs against *A. alternata* involving ROS was evaluated using SHAM, a specific inhibitor which blocks the alternative oxidase antioxidant action. When the ROS scavenging function by AOX was inhibited with SHAM, the fungitoxic effect of ZnO-NPs was enhanced indicating that ROS generation could contribute to augmenting the action of NPs. Some ROS such as H_2O_2, produced by the interaction of NPs with the cell membrane proteins, can penetrate cell membrane and cause damage to proteins, enzymes or DNA (Cruz-Luna et al., 2021; Slavin et al., 2017; Arciniegas-Grijalba et al., 2019). NPs properties that affect their size and shape such

as the presence of edges or defects could enhance their ability to generate ROS (Slavin et al., 2017). Nevertheless, synergy factor values between SHAM and ZnO-NPs or its ionic counterpart $ZnSO_4$ in *A. alternata* were similar indicating no quantitative differences between Zn-ion and NPs mediated ROS generation mechanism. However, given that ZnO-NPs were more toxic compared to their ionic counterpart, an additional potential mechanism must be responsible for the observed fungitoxic action of the NPs to *A. alternata*.

Fungi utilize specific zinc transporters that are responsible for Zn^{+2} ion influx, cytosol and vacuole transport and ion efflux as a part of their cellular ion homeostasis mechanism (Li et al., 2017; Robinson et al., 2021). Zinc toxicity results from the excessive number of ions present outside or inside the fungal cell and the inability of the ion homeostatic mechanism to cope with the accumulated ion load. Cellular defense mechanisms include extracellular inhibition of ion uptake/internalization and intracellular compartmentalization, binding with biomolecules or active efflux of metal ions (Priyadarshini et al., 2021). Differences in the level of toxicity of NPs compared to their ionic counterparts could result from the way they are distributed around the fungal cell. Metal ions are uniformly distributed around fungal cells without specific localization in contrast with the larger NPs, which are unevenly distributed around cells creating focal sources of continuously released ions. This large NP-generated ion concentration induces ion penetration and impairs ion homeostatic mechanisms that cannot cope with the ion release rates produced, thus causing a more profound toxic effect than individual metal ions. This could explain the superior fungitoxic effect of ZnO-NPs against *A. alternata* compared to $ZnSO_4$. The synergistic/additive effect observed between fluazinam (an ATP-synthetase inhibitor) and ZnO-NPs could also be an indication that an efflux mechanism regulating zinc ion homeostasis is involved in the toxicity of ZnO-NPs (Kalamarakis et al., 2000; Leroux and Walker, 2013).

Concluding, ZnO-NPs were effective against *A. alternata* sensitive and resistant to boscalid phenotypes and exhibited an enhanced antifungal activity when applied in combination with boscalid or fluazinam. Zinc ion release is probably the main mechanism of ZnO-NPs mode of fungitoxic action, which is superior to its ionic counterpart $ZnSO_4$ probably due to NPs ROS production or the inability of the fungal ion homeostasis mechanism to cope with the excessive ion dissolution rate of the NPs. Synergy between boscalid and ZnO-NPs probably results from a potential capping effect that reduces ZnO nanoparticle size. These results highlight the promising potential of ZnO-NPs to be used as antifungal agents for both combating fungicide-resistance and reducing environmental impact of synthetic fungicides.

6.6 References

Arciniegas-Grijalba, P.A., Patiño-Portela, M.C., Mosquera-Sánchez, L.P., Guerra Sierra, B.E., Muñoz-Florez, J.E., Erazo-Castillo, L.A., Rodríguez-Páez, J.E. ZnO-based nanofungicides: Synthesis, characterization and their effect on the coffee fungi *Mycena citricolor* and Colletotrichum sp. (2019) Materials Science and Engineering C, 98, pp. 808-825.

Ashajyothi, C., Prabhurajeshwar, C., Handral, H.K., Kelmani, C.R. Investigation of antifungal and anti-mycelium activities using biogenic nanoparticles: An eco-friendly approach (2016) Environmental Nanotechnology, Monitoring and Management, 5, pp. 81-87.

Avenot, H.,Morgan, D. P., & Michailides, T. J. (2008). Resistance to pyraclostrobin, boscalid and multiple resistance to pristine ® (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. Plant Pathology, 57, 135–140.

Avenot, H. F., & Michailides, T. J., Detection of isolates of *Alternaria alternata* with multiple-resistance to fludioxonil, cyprodinil, boscalid and pyraclostrobin in California pistachio orchards (2015). Crop Protection, 78, 214–221.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. Pest Management Science, 58, 649–662.

Cruz-Luna, A.R., Cruz-Martínez, H., Vásquez-López, A., Medina, D.I. Metal nanoparticles as novel antifungal agents for sustainable agriculture: Current advances and future directions (2021) Journal of Fungi, 7 (12), art. no. 1033, DOI: 10.3390/jof7121033.

Davis, R., Miyao, E., Mullen, R., Valencia, J., May, D., & Gwynne, B. (1997). Benefits of applications of chlorothalonil for the control of black mold of tomato. Plant Disease, 81, 601–603.

Fan, Z., Yang, J.-H., Fan, F., Luo, C.-X., & Schnabel, G. (2015). Fitness and competitive ability of *Alternaria alternata* field isolates with resistance to SDHI, QoI, and MBC fungicides. Plant Disease, 99, 1744–1750.

Gabrielyan, L., Hakobyan, L., Hovhannisyan, A., Trchounian, A., Effects of iron oxide (Fe_3O_4) nanoparticles on *Escherichia coli* antibiotic-resistant strains (2019). Journal of Applied Microbiology, 126 (4), pp. 1108-1116.

Gisi, U., Synergistic interaction of fungicides in mixtures (1996) Phytopathology, 86 (11), pp. 1273-1279.

Hoseinzadeh, E., Makhdoumi, P., Taha, P., Hossini, H., Stelling, J., Kamal, M.A., Ashraf, G.Md. A review on nano-antimicrobials: Metal nanoparticles, methods and mechanisms (2017). Current Drug Metabolism, 18 (2), 120–128.

Huang, W., Wang, C., Duan, H., Bi, Y., Wu, D., Du, J., Yu, H., Synergistic antifungal effect of biosynthesized silver nanoparticles combined with fungicides (2018). International Journal of Agriculture and Biology, 20 (5), 1225–1229.

Jamdagni, P., Rana, J.S., Khatri, P., Comparative study of antifungal effect of green and chemically synthesised silver nanoparticles in combination with carbendazim, mancozeb, and thiram (2018) IET Nanobiotechnology, 12 (8), pp. 1102-1107.

Jampilek, J., How can we bolster the antifungal drug discovery pipeline? (2016) Future Medicinal Chemistry, 8 (12), pp. 1393-1397.

Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D.; A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues (2018). Nature Nanotechnology, 13 (8), 677–684.

Kalampokis, I.F., Kapetanakis, G.C., Aliferis, K.A., Diallinas, G. Multiple nucleobase transporters contribute to boscalid sensitivity in *Aspergillus nidulans* (2018) Fungal Genetics and Biology, 115, pp. 52-63.

Kalamarakis, A.E., Petsikos-Panagiotarou, N., Mavroidis, B., Ziogas, B.N., Activity of fluazinam against strains of *Botrytis cinerea* resistant to benzimidazoles and/or dicarboximides and to a benzimidazole-phenylcarbamate Mixture (2000). Journal of Phytopathology, 148 (7-8), pp. 449-455.

Kalia, A., Kaur, J., Tondey, M., Manchanda, P., Bindra, P., Alghuthaymi, M.A., Shami, A., Abd-Elsalam, K.A. Differential antimycotic and antioxidant potentials of chemically synthesized zinc-based nanoparticles derived from different reducing/complexing agents against pathogenic fungi of maize crop (2021) Journal of Fungi, 7 (3), art. no. 223, DOI: 10.3390/jof7030223

Khan, S.T., Musarrat, J., Al-Khedhairy, A.A., Countering drug resistance, infectious diseases, and sepsis using metal and metal oxides nanoparticles: Current status (2016). Colloids and Surfaces B: Biointerfaces, 146, pp. 70-83.

Król, A., Pomastowski, P., Rafińska, K., Railean-Plugaru, V., Buszewski, B., Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism (2017). Advances in Colloid and Interface Science 249, 37–52.

Kumari, M., Giri, V.P., Pandey, S., Kumar, M., Katiyar, R., Nautiyal, C.S., Mishra, A. An insight into the mechanism of antifungal activity of biogenic nanoparticles than their chemical counterparts (2019) Pesticide Biochemistry and Physiology, 157, pp. 45-52.

Leroux, P., Walker, A.-S., Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance (2013). European Journal of Plant Pathology, 135 (4), pp. 683-693.

Li, J., Sang, H., Guo, H., Popko, J.T., He, L., White, J.C., Parkash Dhankher, O., Jung, G., Xing, B. Antifungal mechanisms of ZnO and Ag nanoparticles to *Sclerotinia homoeocarpa* (2017) Nanotechnology, 28 (15), art. no. 155101, DOI: 10.1088/1361-6528/aa61f3.

Logrieco, A., Moretti, A., & Solfrizzo, M. (2009). Alternaria toxins and plant diseases: An overview of origin, occurrence and risks. World Mycotoxin Journal, 2, 129–140.

Malandrakis, A.A., Vattis, K.N., Markoglou, A.N., Karaoglanidis, G.S. Characterization of boscalid-resistance conferring mutations in the SdhB subunit of respiratory complex II and impact on fitness and mycotoxin production in *Penicillium expansum* laboratory strains (2017) Pesticide Biochemistry and Physiology, 138, pp. 97-103.

Malandrakis, A.A., Apostolidou, Z.A., Louka, D., Markoglou, A., Flouri, F. Biological and molecular characterization of field isolates of *Alternaria alternata* with single or double resistance to respiratory complex II and III inhibitors (2018) European Journal of Plant Pathology, 152 (1), pp. 199-211.

Malandrakis, A.A., Kavroulakis, N., Avramidou, M., Papadopoulou, K.K., Tsaniklidis, G., Chrysikopoulos, C.V. Metal nanoparticles: Phytotoxicity on tomato and effect on symbiosis with the *Fusarium solani* FsK strain (2021a) Science of the Total Environment, 787, art. no. 147606, DOI: 10.1016/j.scitotenv.2021.147606.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates (2021b) Pesticide Biochemistry and Physiology, 173, art. no. 104796,DOI: 10.1016/j.pestbp.2021.104796.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Synergy between Cu-NPs and fungicides against *Botrytis cinerea* (2020a). Science of the Total Environment, 703, art. no. 135557, DOI: 10.1016/j.scitotenv.2019.135557.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola* (2020b) Science of the Total Environment, 747, art. no. 141287, DOI: 10.1016/j.scitotenv.2020.141287

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019). Science of the Total Environment, 670, pp. 292-299.

Márquez, I.G., Ghiyasvand, M., Massarsky, A., Babu, M., Samanfar, B., Omidi, K., Moon, T.W., Smith, M.L., Golshani, A. Zinc oxide and silver nanoparticles toxicity in the baker's yeast, *Saccharomyces cerevisiae* (2018) PLoS ONE, 13 (3), art. no. e0193111, DOI: 10.1371/journal.pone.019311

Markoglou, A.N., Malandrakis, A.A., Vitoratos, A.G., Ziogas, B.N., Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides (2006). European Journal of Plant Pathology, 115 (2), pp. 149-162.

Morris, P. F., Connolly, M. S., & St Clair, D. A. (2000). Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs. Mycological Research, 104, 286–292.

Nejabatdoust, A., Salehzadeh, A., Zamani, H., Moradi-Shoeili, Z., Synthesis, Characterization and Functionalization of ZnO Nanoparticles by Glutamic Acid (Glu) and Conjugation of ZnO@Glu by Thiosemicarbazide and Its Synergistic Activity with Ciprofloxacin Against Multi-drug Resistant *Staphylococcus aureus* (2019). Journal of Cluster Science, 30 (2), pp. 329-336.

Nisar, P., Ali, N., Rahman, L., Ali, M., Shinwari, Z.K., Antimicrobial activities of biologically synthesized metal nanoparticles: an insight into the mechanism of action (2019). Journal of Biological Inorganic Chemistry, 24 (7), pp. 929-941.

Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks (2018) Proceedings of the National Academy of Sciences India Section B - Biological Sciences, 88 (4), 1287–1308.

Paralikar, P., Ingle, A.P., Tiwari, V., Golinska, P., Dahm, H., Rai, M., Evaluation of antibacterial efficacy of sulfur nanoparticles alone and in combination with antibiotics against multidrug-resistant uropathogenic bacteria (2019). Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 54 (5), pp. 381-390.

Priyadarshini, E., Priyadarshini, S.S., Cousins, B.G., Pradhan, N. Metal-Fungus interaction: Review on cellular processes underlying heavy metal detoxification and synthesis of metal nanoparticles (2021) Chemosphere, 274, art. no. 129976. DOI: 10.1016/j.chemosphere.2021.129976.

Punjabi, K., Mehta, S., Chavan, R., Chitalia, V., Deogharkar, D., Deshpande, S., Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens (2018). Frontiers in Microbiology, 9 (SEP), art. no. 2207, DOI: 10.3389/fmicb.2018.02207.

Rai, M., Ingle, A.P., Pandit, R., Paralikar, P., Gupta, I., Chaud, M.V., dos Santos, C.A., Broadening the spectrum of small-molecule antibacterials by metallic nanoparticles to overcome microbial resistance (2017). International Journal of Pharmaceutics, 532 (1), pp. 139-148.

Robinson, J.R., Isikhuemhen, O.S., Anike, F.N. Fungal–metal interactions: A review of toxicity and homeostasis (2021) Journal of Fungi, 7 (3), art. no. 225, DOI: 10.3390/jof7030225.

Ruddaraju, L.K., Pammi, S.V.N., Guntuku, G.S., Padavala, V.S., Kolapalli, V.R.M., A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present and future perspectives of green nano technological combinations (2020). Asian Journal of Pharmaceutical Sciences, 15 (1), pp. 42-59.

Rudramurthy, G.R., Swamy, M.K., Sinniah, U.R., Ghasemzadeh, A., Nanoparticles: Alternatives against drug-resistant pathogenic microbes (2016) Molecules, 21 (7), art. no. 836, DOI: 10.3390/molecules21070836.

Sang, H., Lee, H.B. Molecular mechanisms of succinate dehydrogenase inhibitor resistance in phytopathogenic Fungi (2020) Research in Plant Disease, 26 (1), pp. 1-7.

Sardella, D., Gatt, R., Valdramidis, V.P Physiological effects and mode of action of ZnO nanoparticles against postharvest fungal contaminants (2017). Food Research International, 101, pp. 274-279. DOI: 10.1016/j.foodres.2017.08.019.

Sierotzki, H., &Scalliet, G. (2013). A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. Phytopathology, 103, 880–887.

Slavin, Y.N., Asnis, J., Häfeli, U.O., Bach, H. Metal nanoparticles: Understanding the mechanisms behind antibacterial activity (2017) Journal of Nanobiotechnology, 15 (1), art. no. 65, DOI: 10.1186/s12951-017-0308-z.

Strandberg, J. O. (1992). Alternaria species that attack vegetable crops: Biology and options for disease management. In J. Chelkowski & A. Visconti (Eds.), Alternaria Biology, Plant Diseases, and Metabolites, Topics in Secondary Metabolism, 3 (pp. 175–208). Amsterdam: Elsevier Science Publishers B. V.

Sun, Q., Li, J., Le, T., Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives (2018). Journal of Agricultural and Food Chemistry, 66 (43), 11209–11220.

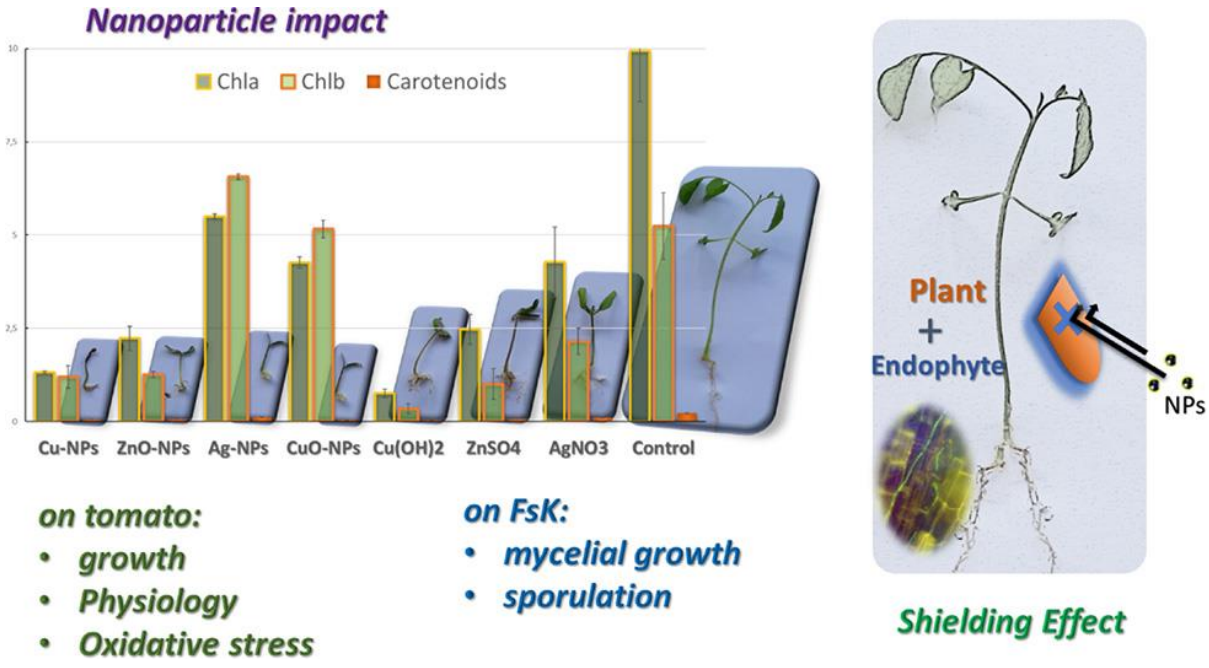
Sun, L., Liao, K., Li, Y., Zhao, L., Liang, S., Guo, D., Hu, J., Wang, D., Synergy between polyvinylpyrrolidone-coated silver nanoparticles and azole antifungal against drug-resistant *Candida albicans* (2016). Journal of Nanoscience and Nanotechnology, 16 (3), pp. 2325-2335.

Veloukas, T., Markoglou, A. N., & Karaoglanidis, G. S. (2013). Differential effect of SdhB gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea*. Plant Disease, 97, 118–122.

Xiao, C. L., & Boal, R. J. (2009). Preharvest application of a boscalid and pyraclostrobin mixture to control postharvest graymold and blue mold in apples. Plant Disease, 93, 185–189.

Xue, J., Luo, Z., Li, P., Ding, Y., Cui, Y., Wu, Q., A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles (2014). Scientific Reports, 4, art. no. 5408, DOI: 10.1038/srep05408.

7 Metal nanoparticles: Phytotoxicity on tomato and effect on symbiosis with the *Fusarium solani* FsK strain



Malandrakis, A.A., Kavroulakis, N., Avramidou, M., Papadopoulou, K.K., Tsaniklidis, G., Chrysikopoulos, C.V. Metal nanoparticles: Phytotoxicity on tomato and effect on symbiosis with the *Fusarium solani* FsK strain (2021) Science of the Total Environment, 787, art. no. 147606, DOI: 10.1016/j.scitotenv.2021.147606.

7. Metal nanoparticles: Phytotoxicity on tomato and effect on symbiosis with the *Fusarium solani* FsK strain

Abstract

The effect of copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc oxide (ZnO-NPs) nanoparticles (NPs) on plant growth, physiological properties of tomato plants and their symbiotic relationships with the endophytic *Fusarium solani* FsK strain was investigated. Fungitoxicity tests revealed that the FsK strain was significantly more sensitive to Cu-NPs and ZnO-NPs than CuO-NPs and Ag-NPs both in terms of mycelial growth and spore germination. All NPs were more toxic to FsK compared to their bulk counterparts except for $AgNO_3$, which was 8 to 9-fold more toxic than AgNPs. Apart from $AgNO_3$, NPs and bulk counterparts did not affect the number of germinated tomato seeds even in higher concentrations, while root length was significantly reduced in a dose dependent way in most cases. Dry weight of tomato plants was also significantly reduced upon treatment with NPs and counterparts with most pronounced effects in the cases of $AgNO_3$, Cu-NPs, ZnO-NPs, and $ZnSO_4$. Root and shoot length of grown tomato plants was also affected by treatments while differences between NPs and bulk counterparts varied. A marked oxidative stress response was recorded in all cases of NPs/bulk counterparts as indicated by increased MDA and H_2O_2 levels of treated plants. Treated plants had significantly reduced chlorophyl-a and carotenoid levels compared to the untreated control. NPs and counterparts did not affect FsK colonization of roots indicating a possible shielding effect of tomato plants once the endophyte was established inside the roots. Vice versa, a possible alleviation of CuO-NPs, ZnO-NPs, and $ZnSO_4$ toxicity was observed in the presence of FsK inside tomato roots in terms of plant dry weight. The results suggest that phytotoxicity of NPs in tomato treated plants should be considered before application and while both FsK and tomato are sensitive to NPs, their reciprocal benefits may extent to resistance towards these toxic agents.

7.1. Introduction

Heralded by Richard Feynman's infamous "There's plenty of room in the bottom", nanotechnology initiated a new era in science with numerous applications and virtually infinite possibilities (Feynman 1960). Research focusing on nanotechnology applications in agriculture is rapidly gaining ground primarily driven by the promising potential of nanoparticles (NPs) for optimized efficacy of inputs and reduction of pesticide/xenobiotic footprint in the environment (Kah et al., 2018; Baker et al., 2017). Controlled release, enhanced bioavailability, target-specific delivery and improved residual action are some of the advantages of nanoparticles used as alternative pesticides or nutrient carriers (Kah et al, 2018; Pandey et al., 2018). Furthermore, their combined use with synthetic pesticides has demonstrated synergism in a number of cases achieving enhanced effectiveness against plant pests with lower doses (Malandrakis et al., 2019; 2020a,b). Under this scope, NPs are proposed as suitable candidates to be used as novel, environmentally compatible pesticide alternatives (Baker et al., 2017; Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). However, certain environmental concerns such as fate in ecosystems and effects on non-target organisms including humans should be addressed before their wider commercial release (Noori et al., 2020; Baker et al., 2017).

Plants, being an essential part of all ecosystems, are expected to interact directly or indirectly with nanoparticles, that could potentially inflict toxicity, accumulate via uptake or disturb interactions of plants with beneficial/symbiotic organisms (Ma et al., 2010; Courtois et al., 2019; Lewis et al., 2019). Phytotoxicity can result from physical or chemical interaction of NPs with root or other plant tissues via a number of physiological and biochemical mechanisms including membrane interactions, ion release, production of reactive oxygen species (ROS), inactivation of enzymes or DNA disruption (Karami Mehrian et al., 2016; Ma et al., 2010; Noori et al., 2020). Standard indicators of phytotoxicity include seed germination, root elongation, plant biomass, and chlorophyl content (Ma et al., 2010; Larue et al., 2014; Karami Mehrian et al., 2016; Noori et al., 2020; Ristroph et al., 2017). A number of studies evaluating phytotoxicity of metal NPs have been conducted reporting adverse effects on various aspects of plant growth and physiology in numerous plant species (Li et al., 2015a; de la Rosa, 2021). Reports are often conflicting although a consensus is obvious: toxicity threshold of NPs towards plants is species dependent and each case should be evaluated separately.

Tomato is a vegetable crop with great popularity and economic importance worldwide with significant nutritional value (Akanbi-Gada et al., 2019; Karami Mehrian et al., 2016). Previous studies evaluating phytotoxicity of tomato caused by silver, zinc oxide, titanium oxide, copper, and ferric NPs are available although the comparative toxicity with their bulk counterparts is limited (Chen et al., 2020; Karami Mehrian et al., 2016; Akanbi-Gada et al., 2019; Noori et al., 2020; Sun et al., 2020). Limited are also the studies about the indirect impact of NPs on the plant performance via their interaction with the rhizospheric/endophytic soil microorganisms. The importance of a potential disruption of plant-beneficial microbe interactions by NPs is evident in cases of N_2 fixing, phosphate solubilizing bacteria, arbuscular mycorrhizae and plant growth promoting microorganisms (Wang et al., 2016). FsK is a nonpathogenic *Fusarium solani* strain which colonizes roots and induces plant response mechanisms against both pathogens and pests, mediated by the ethylene signaling pathway (Kavroulakis et al., 2007; Garantonakis et al., 2018; Kavroulakis et al., 2018). The sensitivity of FsK against a variety of fungicides, commonly used in agricultural practice was tested and the compatibility of this strain in integrated disease management programs was proposed (Malandrakis et al., 2018). A key question besides phytotoxicity risks posed by NPs, is whether they can affect the survival and colonization of FsK and its symbiotic interactions with the tomato plant.

Under this light, the scope of this study was to evaluate: (a) the effect of silver, copper, copper oxide, and zinc oxide NPs and their bulk counterparts on growth (seed germination, root elongation and dry weight) and physiology (oxidative stress and photosynthetic pigment content) of tomato plants, (b) their fungitoxic activity against the tomato-endophyte FsK, and (c) the possible impact of NPs/counterparts on root colonization and symbiotic interactions between FsK and tomato plants.

7.2. Materials and Methods

7.2.1 Nanoparticles, reagents and fungicides

Silver [Ag-NPs] (<100nm particle size), zinc oxide [ZnO-NPs] (particle size <50 nm), copper [Cu-NPs] (particle size 25 nm), copper oxide [CuO-NPs] (particle size <50 nm) nanoparticles (NPs) as well as zinc sulphate [$ZnSO_4$] and silver nitrate [$AgNO_3$] used in this study were purchased from Sigma Aldrich, MO, USA. A copper hydroxide containing a commercial fungicide (Copperblau-N 50 WP) used as a bulk counterpart of copper NPs was purchased from NITROFARM (Greece). Stock solutions-suspensions of commercial fungicide, reagents and nanoparticles used in fungitoxicity and phytotoxicity bioassays were prepared using distilled-sterilized water. Appropriate quantities of stock solutions were added aseptically to sterilized growth medium prior to inoculation or seed placement. To prevent their aggregation, nanoparticle suspensions were subjected to sonication for 30 min with Transonic 420 (Elma, Germany) before use. Zeta potentials and hydrodynamic diameter measurements for the nanoparticles (see table 7.S1) were measured with a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA) in triplicate.

Table 7.S1 Zeta potential and diameter size of nanoparticles (50 mg/mL) used in the study.

	Zeta potential (mV) (mean ± SD[a])	**Size (d.nm) (mean ± SD)**
Ag-NPs	-29.6 ± 0.5	82.8 ± 12.6
Cu-NPs	29.6 ± 0.2	45.2 ± 25.9
CuO-NPs	-9.4 ± 0.3	78.7 ± 5.3
ZnO-NPs	13.8 ± 0.4	68.6 ± 10.2

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

7.2.2 Fungal isolate and culture conditions

The fungitoxic effect of silver, copper and zinc containing NPs and their bulk/ionic counterparts against the biocontrol agent FsK, a previously characterized, non-pathogenic *F. solani* tomato fungal endophyte, was evaluated (Kavroulakis et al., 2007). The fungal isolate was grown on sterilized potato-dextrose-agar medium (PDA) and maintained in growth chambers in the dark at 25^{o}C or at 4 ^{o}C for long term storage.

7.2.3 Fungitoxicity tests

7.2.3.1 *Effect of NPs on FsK mycelial growth*

The fungitoxic effect of NPs and their bulk/ionic counterparts on the fungal strain FsK was assessed *in vitro* utilizing poison agar bioassays. The inhibitory effect of antifungal agents was determined by measuring colony radial growth on PDA medium containing appropriate concentrations of NPs or their counterparts. In order to obtain fungitoxicity-curves, concentrations of 0, 5, 10, 50, 100, 250, 500 and 1000 μg mL^{-1} Cu-NPs, CuO-NPs, Ag-NPs, ZnO-NPs, $Cu(OH)_2$, $AgNO_3$, and $ZnSO_4$ were added in sterilized PDA medium, which was poured in Petri dishes and left to solidify. After the growth medium cooled, inoculum consisting of a 5-mm mycelial plug cut from the edge of 5-day old FsK colonies grown on PDA was placed in the center of the plate with the mycelium facing down in direct contact with the medium. Plates were transferred for incubation in growth chambers at 25 °C in the dark for 7 days. Following inoculation and incubation procedures described above, mean colony diameters were then measured and percent inhibition was calculated using the formula: % inhibition = 100- (mean colony diameter of treated/mean colony diameter of untreated control)×100. In order to compare sensitivities of FsK to metal NPs and bulk counterparts, EC_{50} values (effective concentration causing 50% inhibition of mycelial growth) were calculated. Three replicate plates were used per concentration while all tests were repeated twice.

1.3.5.1 Effect of NPs on FsK spore germination

The potential of NPs and their bulk counterparts to inhibit FsK spore germination was assessed *in vitro* on PDA. Conidial suspensions of the fungal strain were obtained by inoculating 250 mL glass flasks containing Potato Dextrose Broth (PDB) with four 5-mm mycelial plugs, cut from the edge of rapidly growing colonies. Following incubation for 4 days in the dark at 25 ^{o}C in growth chambers under continuous shaking at 200 rpm, conidia were then harvested by filtration using a cheese cloth and the concentration of spores was determined using a haemocytometer. The spore concentration was adjusted to 100 conidia/100 μL by serial dilutions in sterilized-distilled water and 100 conidia were spread on the surface of petri dishes containing PDA amended or not with the appropriate nanoparticle / counterpart concentrations. Concentrations of 0, 1, 5, 10, 25, 50, 100, 250, 500 and 1000 μg/mL of each metal NP or bulk counterpart were used to obtain fungitoxicity-curves. Three replicate dishes of each compound concentration were incubated for 2 days in the dark at 25 ^{o}C. The number of forming colonies was counted and the percent inhibition of colony formation was calculated by the formula: % inhibition = 100-(mean number of colonies of treated/mean number of colonies of untreated control)×100. EC_{50} values based on relative percent inhibition were calculated for each compound. The experiment was conducted twice.

7.2.4. NPs phytotoxicity tests

7.2.4.1 Germination assays

Tomato seeds (*Solanum lycopersicon*, cv. ACE 55) used in germination assays were surface disinfected in a 2.5% NaOCl water solution, rinsed trice with distilled, sterilized water and then air dried. Germination was assessed in 15-mL Falcon centrifuge tubes filled with 10 mL water agar (WA) medium amended with concentrations of 0, 10, 100 and 1000 μg/mL of copper, silver and zinc oxide NPs as well as their bulk/ionic counterparts $Cu(OH)_2$, $ZnSO_4$ and $AgNO_3$ under aseptic conditions. In each tube, one seed was placed on the surface of the treated or untreated WA using a sterilized forceps and exercising sufficient pressure to ensure contact with the medium. Tubes were covered with aluminum foil and incubated for 6 days in a growth chamber at 25 ^oC with a 16h:8h day: night photoperiod. Twenty tubes per treatment were used and the germination experiment was repeated twice. At the end of the experiment, the number of germinated seeds and root length (length of the longest root) in each treatment was recorded and germination percentage (GP%) was calculated according to the formula:

GP% = mean number of germinated seeds of treatment/mean number of germinated seeds of the control×100 (1)

7.2.4.2 Impact of NPs on plant growth

The impact of NPs and their bulk/ionic counterparts on tomato plant growth was evaluated in terms of root and shoot length and dry weight of treated tomato seedlings grown on artificial growth medium. Sterilized Hornum-Agar (HA) medium (40 g/L NH_4NO_3, 30 g/L KNO_3, 30 g/L $MgSO_4.7H_2O$, 10 g/L $NaH_2PO_4.H_2O$, 2 g/L Fe-EDTA (9% Fe), 120 mg/L $MnSO_4.H_2O$, 120 mg/L H_3BO_3, 40 mg/L $CuSO_4.5H_2O$, 40 mg/L $ZnSO_4.7H_2O$ and 8 mg/L $Na_2MoO_4.2H_2O$ and 0.8% agar diluted 1:100 in tap water and pH adjusted to 6.8.) was selected to provide essential nutrients for tomato plants and achieve a uniform distribution of NPs during the plants growth. After autoclaving, 500 mL of HA medium amended with appropriate concentrations of metal nanoparticles and reagents was poured in aluminum containers 20x15x10 cm [length x width x height] and left to solidify. Concentrations of 5, 10, 50, 100, 500 and 1000 μg/mL Cu-NPs, CuO-NPs, Ag-NPs, ZnO-NPs, $Cu(OH)_2$, $AgNO_3$ and $ZnSO_4$ were used in the bioassays. Control treatments consisted of unamended HA medium. In each container, ten 4-day old pre-germinated tomato seeds were equally distributed on the surface of the solidified medium. Containers were covered with transparent lids and incubated in a growth chamber at 25 ^oC with a 16h:8h day:night photoperiod and 70% RH for 2 weeks. Root and shoot length as well as total dry weight were recorded after that period. Two containers per treatment were used and the experiment was repeated twice.

7.2.4.3 Physiological analysis

Physiological response of tomato seedlings grown on HA medium treated with selected concentrations of each NP or bulk counterpart was analyzed. Tomato plants were grown as described previously in aluminum containers containing HA medium amended with NPs and reagents at concentrations determined by the growth inhibition experiments causing 50% inhibition of growth in terms of dry weight. Specifically, concentrations of 300 μg/mL Cu-NPs, 1000 μg/mL CuO-NPs, 800 μg/mL $Cu(OH)_2$, 1000 μg/mL Ag-NPs, 200 μg/mL $AgNO_3$, 250 μg/mL ZnO-NPs and 300 μg/mL $ZnSO_4$ were used. After 3 weeks incubation in a growth chamber at 25 ^oC with a 16h:8h day:night photoperiod and 70% RH, plant tissues were harvested and analyzed in physiological experiments. Two containers per treatment were used and the experiment was repeated twice.

7.2.4.3.1 Extraction for lipid peroxidation and H_2O_2 assays

In order to evaluate the oxidative stress response of tomato plants caused by metal NPs and their bulk counterparts, hydrogen peroxide (H_2O_2) levels were determined. Specifically, 150 mg of plant material (FW) was reduced in fine powder with liquid nitrogen and homogenized in 4 ml 0.1% trichloroacetic acid (TCA) at 4°C by vigorous vortexing. After centrifugation at 6,500 rpm for 15 min at 4°C, the supernatant was used for the determination of both lipid peroxidation levels and H_2O_2 concentration (Sotiras et al., 2019).

7.2.4.3.2 Hydrogen peroxide assay

Hydrogen peroxide accumulation was measured spectrophotometrically as described by Tsaniklidis et al. (2020) with some modifications. The reaction mixture consisted of 0.25 mL plant extracts, 0.25 mL of 0.1 M potassium-phosphate buffer (pH 7.0), and 0.5 ml of 1 M KI. The reaction color was developed for 45 min in darkness and absorbance was measured at 390 nm. Hydrogen peroxide levels were calculated using a calibration curve prepared with eight known concentrations of H_2O_2. Transformation formula: y=102.5+0.0569x (mmol/g fw).

7.2.4.3.3 Thiobarbituric Acid Reactive Substances (TBARS)/Lipid peroxidation assay

Lipid peroxidation was measured as malondialdehyde (MDA) byproduct content determined by reaction with 0.5% 2-thiobarbituric acid in 20% TCA (w/v). For each assay, 1 mL of plant extracts, 2 mL of 20% (w/v) TCA and 2 mL of 0,5% (w/v) 2-thiobarbituricacid (TBA) were used. The mixture was heated at 95 ^oC for 30 min and afterwards was cooled in ice. The concentration of MDA was calculated from the difference of the absorbance at 532

and 600 nm using the Beer–Lambert's equation (extinction coefficient of MDA was 155 $mM^{-1} * cm^{-1}$) (Heath and Packer 1968).

7.2.4.3.4 Photosynthetic pigments

Pigment content of treated and control tomato plants was measured in leaves following the method described below. 150 mg of fresh leaf sample was added in an eppendorf tube containing 1.8 mL of cold acetone (80%) and vortexed vigorously. Tubes were then incubated in the dark for 1 h, while being vortexed every 15-mins. Subsequently, tubes were centrifuged at 6.500 rpm for 5 min at 4°C. Chlorophyll and carotenoid concentrations were determined spectrophotometrically at absorbance wavelengths of 470, 647, and 663 nm by using the equations described by Lichtenthaler and Buschmann (2001):

$$[\text{Chl a}] = 12.25A_{663} - 2.79A_{647} \quad (3)$$

$$[\text{Chl b}] = 21.5A_{647} - 5.1A_{663} \quad (4)$$

$$[\text{Car}] = \{1000A_{470} - 1.82\,[\text{Chl a}] - 85.02\,[\text{Chl b}]\}/198 \quad (5)$$

7.2.5 Effect of NPs on the association between tomato plants – FsK

In order to investigate the potential impact of NPs on the beneficial association between the endophytic FsK *F. solani* strain and tomato plants, pot experiments with soil substrate treated with selected concentrations of nano or bulk metals were conducted.

7.2.5.1 Plant material and inoculation

Tomato seedlings (*Solanum lycopersicon*, cv. ACE 55) originated from tomato seeds surface sterilized in 2.5% NaOCl and sown directly into pots. Each pot contained 400 cm^3 of peat amended with 0.8 g/L of a NPK fertilizer (20-20-20). Pots were covered with aluminum foil and transferred in a controlled-environment growth chamber at 20–25 ^oC with a 16 h photoperiod at 65% RH for a week until the emergence of young tomato seedlings.

FsK conidial suspensions used as inoculum in the *in-planta* experiments were acquired according to the following procedure: A 5-mm mycelial plug cut from the edge of a rapid growing FsK colony, was transferred in PDA containing Petri dishes and incubated for 6 days at 25 ^oC in the dark for conidiation. Conidia were harvested by scraping the colony, transferring the collected mycelium/conidial mass in distilled-sterilized water and sieving using a cheese cloth in order to remove mycelial fragments. The resulting suspension was then centrifuged at 4000 g and conidia were re-suspended in an appropriate volume of 0.85% NaCl to achieve the desired inoculum concentration using a haemocytometer. One week after sowing, FsK

inoculum was applied in the soil of tomato seedlings as water drench with 10^4 conidia per cm^3 of potting mix.

7.2.5.2 Application of NPs on FsK inoculated/ non-inoculated tomato plants

One week after tomato seedlings were inoculated with FsK, metal NPs and bulk/ionic counterparts were applied as water suspensions (100 mL total water volume per pot) in the soil by drenching in appropriate concentrations. In the control treatment an equal amount of distilled water was used. NP containing suspensions were sonicated for 30 min before drenching to deter particle aggregation. Concentrations of NPs and counterparts applied were selected making sure that they were sublethal to FsK and additionally based on their mean dry weight inhibitory concentration determined in tomato toxicity experiments described above. Specifically, concentrations of 300 μg/mL Cu-NPs, 1000 μg/mL CuO-NPs, 800 μg/mL $Cu(OH)_2$, 250 μg/mL ZnO-NPs, 300 μg/mL $ZnSO_4$, 1000 μg/mL Ag-NPs and 200 μg/mL $AgNO_3$ were used in the experiments. The experiment included two subsets: a set of NPs/bulk counterpart treated tomato plants inoculated with and an identical set not inoculated with FsK. Each treatment consisted of 4 pots containing 4 tomato plants. The whole experiment was repeated twice.

7.2.5.3 Tomato root tissue harvesting and DNA extraction

The effect of NPs and their counterparts on FsK colonization of tomato root tissues was examined by comparing control and metal-treated tomato plants. Whole roots collected from 16 plants per treatment were washed to remove soil and then dried in sterilized filter paper. Genomic DNA was extracted from root tissue samples using the "NuncleoSpin® Plant II genomic DNA extraction" kit (MACHEREYNAGEL GmbH & Co.KG, Duren, Germany) according to the manufacturer's
protocol.

7.2.5.4 Quantification of FsK colonization using qPCR

The effect of NPs on the symbiotic relationships of FsK with tomato plants was evaluated by quantifying the presence (colonization) of FsK inside treated and non-treated tomato roots using Real Time qPCR. *F. solani* ITS region-specific primers FFsITS (5'-TGGTCATTTAGAG GAAGTAA-3') and RFsITS (5'GGTATGTTCACAGGGTTGATG -3'), were used for the Real Time PCR assay.

Copy numbers of the ITS gene in total DNA samples extracted from root tissues of FsK-inoculated plants were evaluated using an external standard curve as previously described (Garantonakis et al., 2018). Data were means of two technical replicates for each of three biological replicates. Values were normalized to ng of total DNA isolated.

7.3. Statistical analysis

The NPs and counterparts EC_{50} values for the FsK strain were calculated by regressing the relative inhibition of mycelial growth against the Log_{10} compound concentrations. The same analysis was conducted for the determination of the IC_{50} values of metal compounds in the tomato plant toxicity experiments. Statistical differences between treatments in fungitoxicity and phytotoxicity experiments were evaluated by analysis of variance and the resulting means were separated according to Tukey's HSD test ($\alpha = 0.05$). Correlations of growth and physiological parameters of NPs/counterpart treated tomato plants were evaluated using Pearson correlation coefficients. The SPSS v20 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

7.4.Results

7.4.1 Fungitoxic activity of NPs in vitro

The fungitoxic activity of metal nanoparticles in comparison with their bulk/ionic counterparts in terms of mycelial growth and spore germination inhibition of the FsK *F. solani* strain was evaluated *in vitro.* A dose-dependent response of FsK to metallic compounds tested was observed both in radial growth and spore germination. The respective EC_{50} values for each treatment are shown in Figure 7.1. Among NPs tested, Cu-NPs and ZnO-NPs were the most toxic against FsK both in mycelial growth and spore germination inhibition experiments. EC_{50} values calculated for Cu-NPs and ZnO-NPs were 260.8 and 554.4 μg/mL in mycelial growth and 18.6, 6.9 μg/mL in the spore germination tests respectively (see Fig.7.1). FsK was significantly less sensitive to CuO-NPs and Ag-NPs with EC_{50} values close to 1000 μg/mL both in mycelial growth and spore germination assays. ZnO-NPs and Cu-NPs were 2 to 4 times more toxic to FsK compared to their counterparts $ZnSO_4$ and $Cu(OH)_2$ respectively in terms of mycelial growth while differences were dramatically more profound in the case of spore germination (141 and 46 times more toxic respectively-see Fig. 7.1). On the contrary, CuO-NPs were less or equally toxic with $Cu(OH)_2$ while $AgNO_3$ was approximately 8 times more toxic than Ag-NPs in all bioassays (see Fig.7.1).

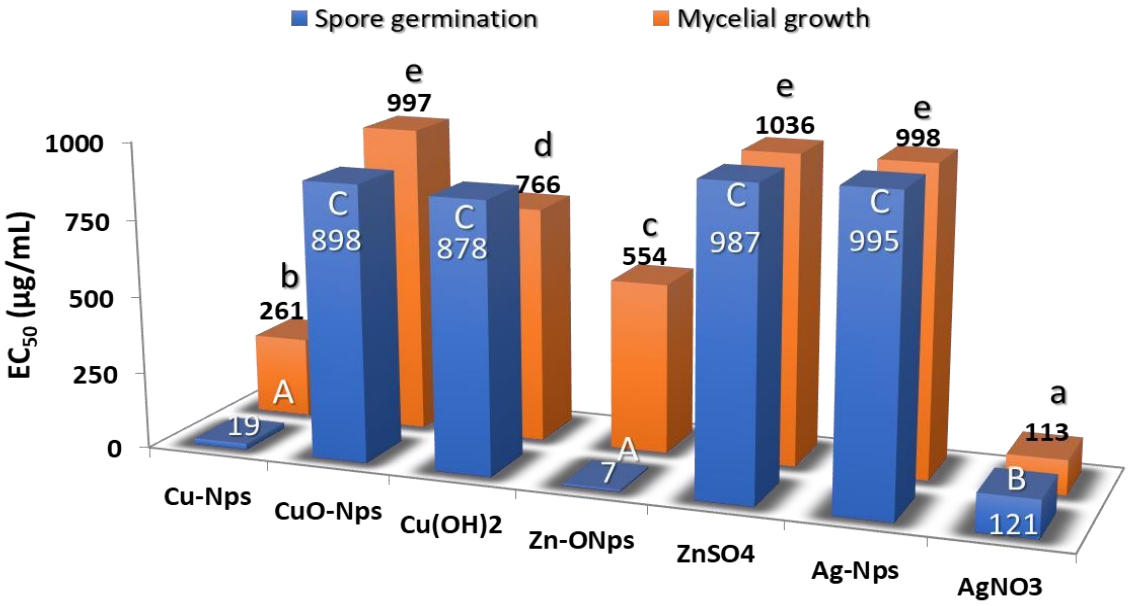


Figure 7.1. Mean sensitivity of FsK to NPs and their respective bulk/ionic counterparts in terms of mycelial growth and spore germination. Between treatments, bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

7.4.2 Phytotoxicity tests

7.4.2.1 Impact of NPs on tomato seed germination

The impact of metal NPs and their bulk counterparts on germination and root length of tomato seed was evaluated *in vitro.* Percent germination, root length and percent germination index rates of tomato seed treated with 10, 100 and 1000 µg/mL of metallic compounds compared to the untreated control seeds are presented in Table 7.1. Addition of NPs or their bulk/ionic counterparts did not affect the number of germinated seeds compared to the control even in the highest concentrations (1000 µg/mL). The only exception was observed in the case of $AgNO_3$ which exhibited a dose dependent decrease in the seed germination rate with a maximum inhibition of 43% at the highest concentration. In contrast, a significant, dose dependent impact on root elongation of germinating seeds was observed in all treatments (see Table 7.1). Addition of NPs or their bulk counterparts negatively affected root elongation of tomato seeds even at the lowest (10 µg/mL) concentration. Inhibition of root length at the 1000 µg/mL concentration ranged between 60 and 90 % compared to the control treatment (see Table 7.1). The less toxic compounds in terms of root elongation were Ag-NPs and CuO-NPs with an inhibition rate of approximately 60-65% while the remaining NPs and counterparts exhibited inhibition rates close to 85-90% at the highest tested concentration.

Table 7.1. Effect of NPs and their respective bulk/ionic counterparts on the germination of tomato seeds.

[a] Expressed as percent of the control treatment after 14 days incubation at 22 °C (n = 3).

	Germinated seeds (%) (mean[a] ± SD[b])			Root length (%) (mean ± SD)		
Treatment	10[c]	100	1000	10	100	1000
Ag-NPs	100.00 a	114.29 ± 10.40 b	100.00 b	48.55 ± 2.65 a	53.95 ± 4.50 bc	38.21 ± 4.54 b
$AgNO_3$	85.71 ± 0.42 a	71.43 ± 3.17 a	57.15 ± 4.69 a	65.64 ± 4.37 b	65.64 ± 2.46 c	13.49 ± 1.67 a
Cu-NPs	97.55 ± 1.05 a	100.00 b	80.75 ± 12.35 b	62.76 ± 2.05 b	21.80 ± 3.11 ab	15.27 ± 4.55 a
CuO-NPs	100.00 a	110.25 ± 11.5 b	85.71 ± 1.12 b	86.72 ± 3.69 bc	40.00 ± 5.14 b	35.63 ± 2.80 b
$Cu(OH)_2$	100.00 a	100.00 b	86.52 ± 5.23 b	75.43 ± 6.16 b	12.72 ± 2.61 a	14.53 ± 5.55 a
ZnO-NPs	71.43 ± 2.14 a	100.00 b	100.00 b	84.10 ± 3.45 bc	67.01 ± 2.28 c	14.29 ± 2.49 a
$ZnSO_4$	100.00 a	100.00 b	100.00 b	96.14 ± 3.17 c	76.97 ± 1.15 d	10.65 ± 2.00 a

[b] Standard deviation of the means (n = 10).

[c] Numbers indicate concentration of NPs/bulk counterparts in µg/mL of active ingredient.

7.4.2.2 Impact of NPs on tomato plant growth

The effect of metal nanoparticles tested as well as their bulk/ionic counterparts on root and shoot length of tomato plants grown in HA medium is shown in Figures 7.2 and 7.3. Among NPs tested, Cu-NPs were the most toxic followed by CuO-NPs, ZnONPs and Ag-NPs in terms of relative root length, with IC_{50} values of 17.88, 52.93, 197.72, and 693.20, respectively (See Fig. 7.2). In the case of shoot length, IC_{50} values of Cu-NPs, CuO-NPs, ZnO-NPs and Ag-NPs were 41.85, 75.69, 594.21 and 84.44 respectively. Comparison of the impact of NPs with their respective bulk/ionic counterparts on root and shoot development is shown in Figure 7.3. In all cases, statistically significant differences were found between metal nanoparticles and their counterparts as indicated by their respective IC_{50} values. Cu-NPs had a significantly higher adverse effect on root and shoot development compared to $Cu(OH)_2$ while in the ZnO-NPs and $ZnSO_4$ case, zinc nanoparticles were more toxic to root than shoot development where the reverse relationship was found (See Fig. 7.3). CuO-NPs and Ag-NPs were less toxic than their counterparts both in terms of root and shoot development.

Percent mean dry weight of tomato plants treated with 100, 500 and 1000 µg/mL of NPs and bulk/ionic counterparts is shown in Table 7.2. Reduction of tomato plant dry weight caused

by all treatments was dose-dependent in most cases. At the highest concentration, $AgNO_3$ had the most toxic effect causing a 89% reduction in dry weight compared with the control treatment, followed by Cu-NPs, ZnO-NPs and $ZnSO_4$. Ag-NPs, CuO-NPs and $Cu(OH)_2$ which exhibited the less toxic effect on tomato plants in terms of dry weight (See Table 7.2).

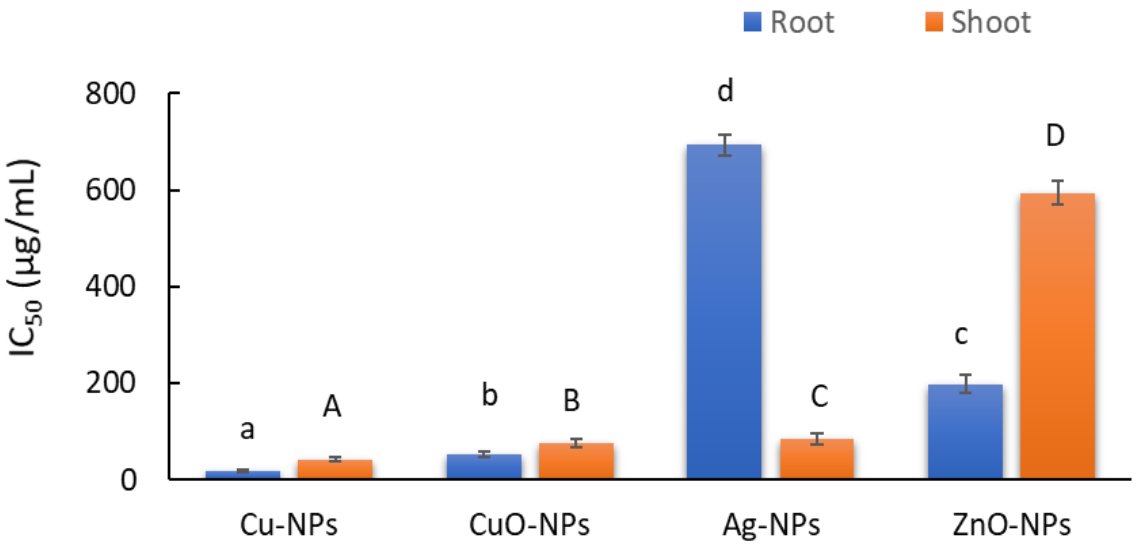


Figure 7.2. Comparison of mean toxicity of copper, silver and zinc containing nanoparticles on root and shoot length in tomato plants. Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

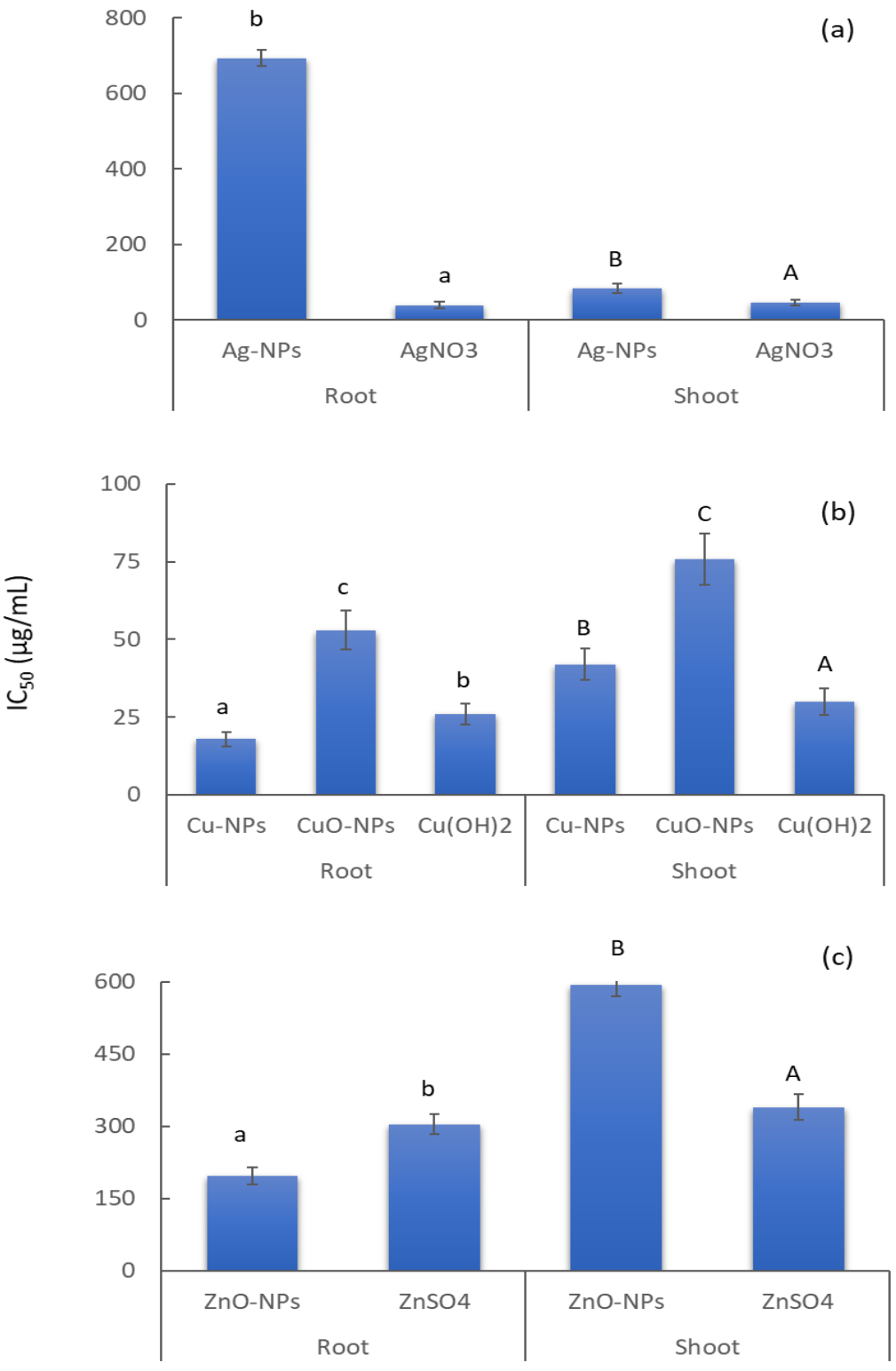


Figure 7.3. Effect of (a) Ag-NPs, (b) Cu-NPs and CuO-NPs, and (c) ZnO-NPs compared to their bulk/ionic counterparts $AgNO_3$, $Cu(OH)_2$ and $ZnSO_4$ respectively in terms of IC_{50} (concentration that causes 50% inhibition in length of root/shoot compared to the untreated control). Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

Table 7.2. Percent mean dry weight of tomato plants treated with NPs and their respective bulk/ionic counterparts.

	% Dry weight (mean ± SD[a])		
Treatment	100[b]	500	1000
Cu-NPs	97.25 ± 3.24 c[c]	45.12 ± 4.39 ab	27.84 ± 7.70 b
CuO-NPs	105.13 ± 0.85 c	76.31 ± 7.02 c	52.55 ± 4.25 c
$Cu(OH)_2$	96.19 ± 6.11 c	85.74 ± 3.90 c	45.07 ± 3.89 c
ZnO-NPs	85.20 ± 5.25 b	55.81 ± 2.88 b	29.69 ± 5.10 b
$ZnSO_4$	78.52 ± 3.88 ab	45.90 ± 9.16 ab	24.83 ± 3.55 b
Ag-NPs	95.70 ± 9.32 c	75.55 ± 4.19 c	55.23 ± 6.30 c
$AgNO_3$	65.79± 5.00 a	35.97 ± 5.05 a	11.00 ± 2.12 a

[a] Standard deviation of the means (n = 4).
[b] Numbers indicate concentration of NPs/bulk counterparts in µg/mL of active ingredient
[c] Within columns, means followed by the same letter do not differ significantly according to Tukey's HSD test (α = 0.05).

7.4.2.3 Effect of NPs on physiological parameters

Effect of NPs and non-nanoparticle counterparts on lipid peroxidation, an indicator of plant membrane integrity, measured as MDA byproduct was evaluated in comparison with non-treated tomato plants. All treatments except for CuO-NPs and $ZnSO_4$ exhibited higher MDA levels than the control treatment (see Fig. 7.4a). The highest MDA value was recorded in the case of Cu-NPs (1.39 µmol/g FW) which did not differ statistically from the respective value (1.17 µmol/g FW) of $Cu(OH)_2$. This was also the case for Ag-NPs and $AgNO_3$ (0.94 and 1.07 µmol/g FW), while ZnONPs exhibited significantly higher (1.01 µmol/g FW) MDA levels than $ZnSO_4$ (0.87 µmol/g FW) (see Fig. 4a).

Hydrogen peroxide accumulation as a response to oxidative stress potentially caused by NPs and counterpart treatments was determined in tomato plant tissues. Both NPs and counterparts exhibited higher levels of H_2O_2 accumulation than the untreated control (see Fig. 7.4b). Among NPs, higher levels of H_2O_2 (0.52 µmol/g FW) were recorded in Cu-NPs-treated tomato plants followed by ZnO-NPs (0.44 µmol/g FW), Ag-NPs (0.35 µmol/g FW) and CuO-NPs (0. 17 µmol/g FW) respectively. CuNPs and ZnO-NPs treatments resulted in a statistically higher H_2O_2 response than their counterparts $Cu(OH)_2$ and $ZnSO_4$. No statistical difference was

found in hydrogen peroxide levels between silver NPs and $AgNO_3$. In contrast, $Cu(OH)_2$ treatment resulted in a higher H_2O_2 response than CuO-NPs (see Fig. 7.4b).

The photosynthetic response potential of tomato plants to NPs and counterpart treatments was evaluated by measuring Chl-a, Chl-b, and carotenoid concentrations. Chl-a content was significantly reduced compared to control in all treatments indicating that both nano and bulk metals exerted a high stress potential that could negatively affect growth and development of tomato plants (see Fig. 4c). Nano-size did not seem to affect Chl-a content in the case of copper and zinc containing treatments while a significant decrease on Chl-a content was observed in Cu(OH)2 and AgNO3 compared to CuO-NPs and Ag-NPs treatments respectively. A similar significant ($P < 0.01$) decrease in Chl-b levels was recorded in the case of Cu-NPs (1.2 mg/g FW), ZnO-NPs (1.25 mg/g FW), $Cu(OH)_2$ (0.34 mg/g FW), ZnSO4 (1.00 mg/g FW) and AgNO3 (2.15 mg/g FW) treatments compared to the control (5.24 mg/g FW). In contrast treatment of tomato plants with CuO-NPs and Ag-NPs (5.16 and 6.56mg/g FW respectively) resulted in7. no significant decrease in Chl-b content compared to the control. All treatments had an adverse effect on the carotenoid content of tomato plants. Plants exhibited a 50 ($AgNO_3$) to 83% (CuO-NPs) reduction in the carotenoid levels when exposed to metal NPs and their bulk/ionic counterparts compared to the untreated control (see Fig. 7.4c).

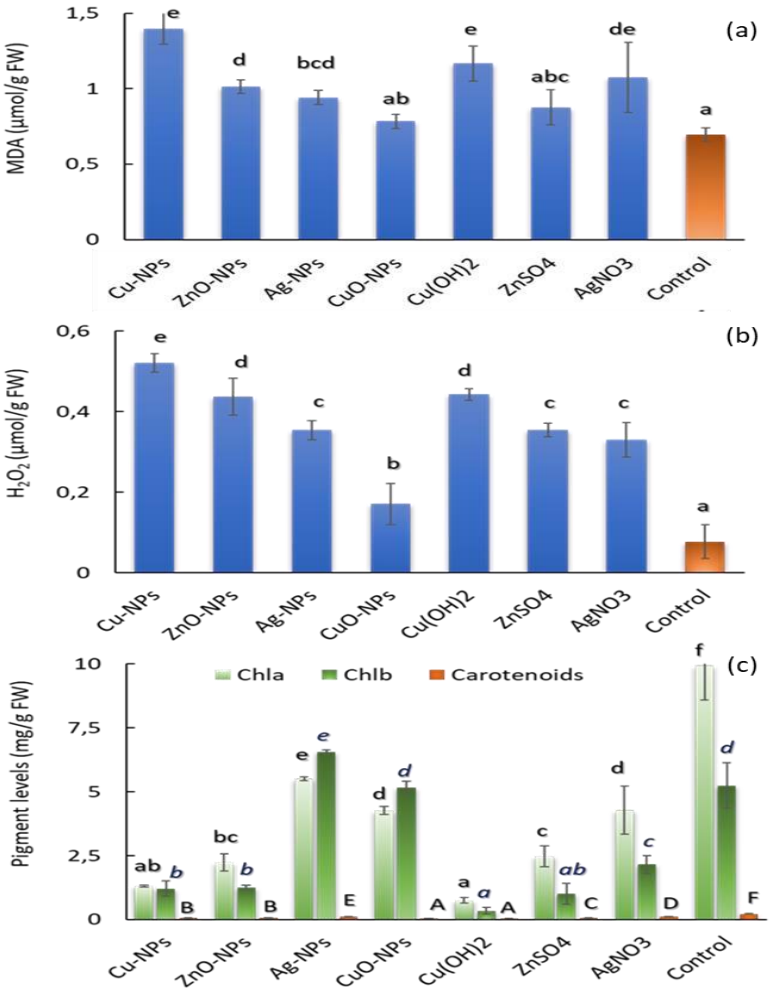


Figure 7.4. Effect of metal NPs and their bulk/ionic counterparts on (a) MDA, (b) H_2O_2 and (c) photosynthetic pigments levels of treated tomato plants. Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05). Between treatments in the pigment chart (c), capital letters indicate differences in carotenoid levels, small black letters in Chlorophyll-a (Chla) levels and blue italic letters in Chlorophyll-b (Chlb) levels.

7.4.3 Effect of NPs on the symbiotic relationship between tomato plants – FsK

The ability of endophytes to colonize host plants is essential for the establishment of a successful symbiotic relationship. Sub-lethal doses of NPs and their bulk/ionic counterparts applied as root drenches were used to evaluate potential adverse effects of treatments on tomato root colonization of FsK. Quantitative real time PCR was used for the quantification of root colonization and the results were expressed in terms of copy numbers per ng of FsK DNA. Although considerable variability was observed between treatments, no statistically significant difference was found between the tomato roots treated with NPs/counterparts and the untreated control (see Fig. 7.5). No differences in colonization of FsK were found between NPs and their bulk/ionic counterparts with the exception of $AgNO_3$, which resulted in significantly less FsK DNA levels present in tomato roots than Ag-NPs (see Fig. 7.5). This probably indicates a shielding effect exerted by tomato root tissue on the FsK strain once the endophyte is established inside them. The lack of systemic action of metallic compounds could be the reason for the absence of significant differences in tomato root colonization compared with the untreated control.

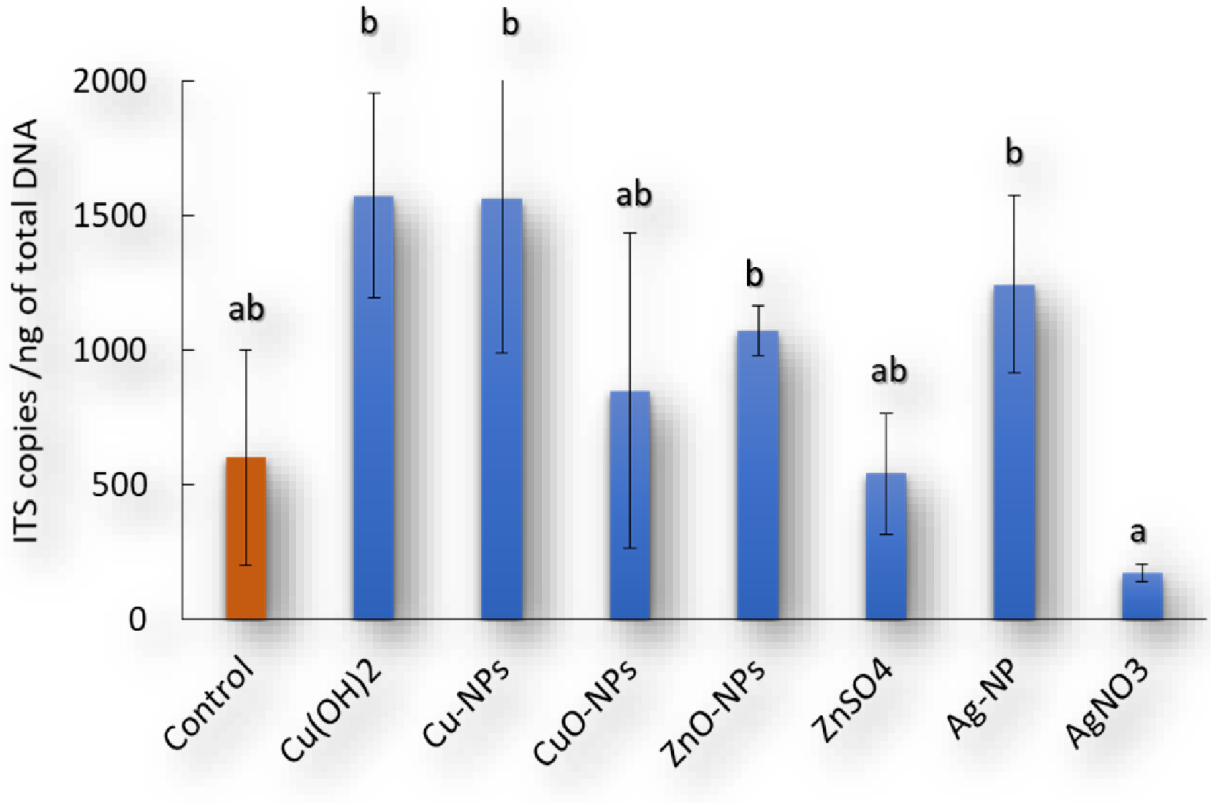


Figure 7.5. Effect of nanoparticles application on fungal (FsK) colonization within root tissues. Quantification of fungal colonization within root tissues by qPCR using primers specific for ITS gene (primary axis). Bars marked by the same letter do not differ significantly according to Tukey's HSD test ($\alpha = 0.05$).

Table 7. 3 Effect of metal NPs and counterparts on Fresh, Dry weight and shoot length of tomato seedlings in the presence or absence of the endophytic FsK *F. solani* strain.

Treatment	Fresh weight[a] (mean ± SD[c])		Dry weight[a] (mean ± SD)		Shoot length[b] (mean ± SD)	
	-FSK	+FSK	-FSK	+FSK	-FSK	+FSK
control	16.81 ± 3.24 a	16.18 ± 2.01 b	1.46 ± 0.42 d	1.20 ± 0.21 bc	54.5 ± 10.62 a	61.25 ± 8.06 b
Cu-NPs	17.10 ± 2.53 a	16.29 ± 2.43 b	1.19 ± 0.27 bcd	0.90 ± 0.25 abc	62.38 ± 11.28 a	65.75 ± 12.05 b
CuO-NPs	14.13 ± 3.05 a	14.05 ± 2.33 b	0.65 ± 0.32 ab	0.75 ± 0.19 ab	65.5 ± 2.65 a	63.00 ± 9.56 b
$Cu(OH)_2$	16.98 ± 0.92 a	16.33 ± 1.47 b	1.27 ± 0.13 cd	0.93 ± 0.03 abc	59.25 ± 6.9 a	65.00 ± 5.10 b
ZnO-NPs	15.39 ± 5.85 a	15.34 ± 3.4 b	0.77 ± 0.32 abc	1.09 ± 0.29 bc	61.63 ± 17.01 a	58.13 ± 8.08 b
$ZnSO_4$	17.55 ± 3.31 a	15.40 ± 1.02 b	0.81 ± 0.03 abc	1.17 ± 0.13 bc	70.25 ± 7.63 a	57.75 ± 6.5 b
Ag-NPs	14.10 ± 1.47 a	14.83 ± 1.62 b	0.95 ± 0.12 abcd	1.32 ± 0.3 c	55.00 ± 9.28 a	50.50 ± 1.22 ab
$AgNO_3$	10.45 ± 2.73 a	7.18 ± 3.52 a	0.49 ± 0.18 a	0.5 ± 0.34 a	53.13 ± 3.22 a	35.50 ± 11.82 a

[a] Mean Fresh and Dry weight measured in grams.
[b] Shoot length measured in mm.
[c] Standard deviation of the means (n=3).
[d] Within columns, means followed by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

In an attempt to investigate the potential of FsK to alleviate phytotoxicity caused by NPs and their bulk/ionic counterparts, growth parameters such as fresh, dry weight and shoot length of treated tomato plants were measured in the presence or absence of FsK. In non-inoculated tomato plants, no significant differences were found between treatments with xenobiotics and the control in terms of fresh weight and shoot length (see Table 7.3). On the contrary, the dry weight was significantly affected by treatment of tomato plants with NPs and counterparts in the absence of FsK. Specifically, CuO-NPs, ZnO-NPs, $ZnSO_4$ and $AgNO_3$ significantly reduced the dry weight of tomato plants compared with the untreated control (See Table 7.3). When plants were inoculated with FsK, significant differences in fresh weight and shoot length were only recorded between NPs/counterpart treatments and the control only in the case of $AgNO_3$, which exhibited a dramatic reduction (43 and 57% respectively). In

contrast, FsK-inoculated plants did not suffer any significant dry weight loss compared with the control in all NPs/counterparts cases except for $AgNO_3$. The later exhibited a significant decrease (41%) in dry weight compared with the control (see Table 7.3).

7.5. Discussion

In this study, the potential of copper, silver and zinc oxide NPs to cause phytotoxicity or interfere with the assosiation between tomato plants and their endophytic partner *Fusarium solani* FsK strain was investigated via plant growth, physiological analysis and colonization bioassays. Tomato seeds sown on growth medium containing all tested NPs germinated successfully and in rates similar or even higher than the control treatment even at the highest 1000 μg/mL concentration. This was also the case for the NPs bulk/ionic counterparts except for $AgNO_3$, which inhibited seed germination in a dose-dependent manner. In contrast, tomato-seedling root length was significantly reduced in a dose dependent way in most cases of NPs and bulk counterparts. Ag-NPs and CuO-NPs had the less negative effects on root elongation and were significantly less toxic than their counterparts $AgNO_3$ and $Cu(OH)_2$ respectively. The remaining NPs were equally toxic with their bulk counterparts in terms of root elongation. Khaldari et al. (2021) observed negative effects of green synthesized CuO-NPs on tomato root elongation even at the lowest concentration, while seed germination was only affected at the highest over a number of concentrations ranging from 4 to 4000 μg/mL. Chen et al. (2020) reported an increase on seed germination of tomato grown in iron, zinc and copper NPs-amended Murashige–Skoog (MS) nutrient medium compared with the control, although in lower concentrations. Similar reports on the effect of Ag-NPs on tomato plants indicate that silver NPs may not be a limiting factor for seed germination in concentrations up to 5000 μg/mL but significantly reduce root elongation in doses as low as 25 μg/mL (Cox et al., 2016; Song et al., 2013; Karami Mehrian et al., 2016).

Growth experiments of tomato plants grown on HA medium amended with metal NPs and their counterparts revealed a significant toxic effect of treatments on biomass, root and shoot length. Specifically, dry weight of plants was significantly reduced upon treatment with NPs and counterparts in concentrations over 500 μg/mL with most pronounced effects in the case of $AgNO_3$, Cu-NPs, ZnO-NPs and $ZnSO_4$. Cu-NPs resulted in a stronger reduction in tomato dry weight than $Cu(OH)_2$ while $AgNO_3$ were 5 times more toxic than Ag-NPs in terms of dry weight. Root and shoot length of grown tomato plants was also affected by treatments with NPs and their bulk counterparts. Cu-NPs incorporation to the growth medium resulted the highest root and shoot length inhibition among NPs tested, followed by CuO-NPs and ZnO-NPs. Shoot length was less negatively affected by NPs than their respective bulk counterparts in all cases. This was also the case for CuO-NPs and Ag-NPs as far as root length is concerned. On the contrary, Cu-NPs and ZnO-NPs resulted a more pronounced reduction in root length than $Cu(OH)_2$ and $AgNO_3$ respectively.

Tomato plant biomass significantly decreased (59-78%) upon exposure to CuO-NPs while NPs treatment also significantly reduced root (68-75%) and shoot (42-47%) length as compared to untreated controls (Pagano et al., 2016). Phytotoxicity of silver NPs in tomato plants was demonstrated by reduced wet weight, root length, lower chlorophyll contents, higher superoxide dismutase activity and less fruit productivity (Song et al., 2013; Noori et al., 2020). A significant reduction in root and shoot length of *S. lycopersicon* was also observed upon treatment with ZnONPs as well as its bulk counterpart form (Ahmed et al., 2019). In the present study, NP treatments had a more pronounced toxic effect in root rather than in shoot length as indicated by the respective IC_{50} values. The only exception was in the case of AgNPs where relative shoot length inhibition was greater than the one observed in roots. A probable higher translocation factor of Ag-NPs towards the shoot could be responsible for this enhanced toxicity. This could be attributed to the PVP coating of Ag-NPs which results in a negative charge of this NP in contrast to Cu-NPs and ZnONPs which were positively charged. Koelmel et al. (2013) has reported a surface charge- dependent bioaccumulation of Au-NPs in various rice organs with negatively charged NPs being more toxic and mostly accumulating in the above ground rice organs. Additionally, Noori et al. (2020) have reported a significantly higher translocation factor (TF) of AgNP-exposed tomato plants compared to $AgNO_3$ which results in the release of positively charged Ag^+ ions. On the contrary, positively charged ZnO-NPs were found to attach to negatively charged soybean and tomato roots resulting in a low translocation factor (Zn shoot to root concentration ratio) (Ristroph et al., 2017; Akanbi-Gada et al., 2019).

A number of physiological and biochemical mechanisms including membrane interactions, ion release, production of reactive oxygen species (ROS), inactivation of enzymes, disruption of the photosynthetic mechanism or damage to DNA are considered responsible for NPs phytotoxicity (Karami Mehrian et al., 2016; Ma et al., 2010; Noori et al., 2020). In an attempt to investigate the potential involvement of NPs and their bulk counterparts on lipid peroxidation and oxidative stress response of tomato, experiments determining MDA and hydrogen peroxide (H_2O_2) levels of treated and untreated *S. lycopersicon* plants were conducted. Overall, treatment of tomato plants with NPs/bulk counterparts resulted in a marked oxidative stress response both in terms of MDA and H_2O_2 levels which were significantly elevated in treated plants compared to the untreated control. The greatest oxidative response was observed in the case of Cu-NPs (2-8 fold increase in MDA and H_2O_2 levels compared to the untreated control respectively) followed by ZnO-NPs and Ag-NPs. This had an expected impact to the corresponding fresh weight (FW) of tomato plants as revealed by the negative correlation observed between H_2O_2 levels and FW. Comparison between NPs and their bulk/ionic counterparts showed different patterns of oxidative response. Oxidative stress caused by Cu-NPs and ZnO-NPs was greater than the one imposed by their counterparts $Cu(OH)_2$ and $ZnSO_4$ possibly indicating a higher toxicity of those metals related to nano properties. In contrast, treatment with Ag-NPs and CuO-NPs resulted in a lesser or equal oxidative response than that of their counterparts $AgNO_3$ and $Cu(OH)_2$ indicating that the ionic form of those metals are more reactive than the nano form. Similar studies on the oxidative response of AgNPs on tomato plants have reported a significant increase of MDA and H_2O_2 levels compared with untreated plants, which were significantly lower than that of plants

treated with $AgNO_3$ (Noori et al., 2020; Jiravova et al., 2016). High concentrations of CuO-NPs (>0.5 μM) have been reported to trigger oxidative bursts leading to elevated H_2O_2 levels, challenging oxidative plant-defense mechanisms and resulting in the disruption of membrane integrity/phytotoxicity in barley and rice plants (Shaw et al., 2014). A similar elevation of oxidative stress in terms of H_2O_2 production as response to ZnO-NPs treatment has been reported in tomato plants (Akanbi-Gada et al., 2019). Oxidative response to NPs and counterparts treatments in the present study were correlated with chlorophyl-a and carotenoid levels which were significantly reduced compared to the untreated control. Reduction of photosynthetic pigments was more pronounced in cases with high H_2O_2 and MDA confirming the suggestion that plant biomass and chlorophyll levels are more sensitive indicators of phytotoxicity than seed germination or root elongation (Ma et al., 2010). Photosynthetic pigment level alterations have been associated with metal nanoparticle treatments in tomato plants in various studies, including treatments with copper, zinc and zinc oxide NPs (Noori et al., 2020; Akanbi-Gada et al., 2019; Lopez-Lima et al., 2021).

A number of studies have investigated the potential of metal NPs to suppress both plant pathogens and beneficial/symbiotic microorganisms (Li et al., 2015b; Oktarina and Singleton, 2019; Malandrakis et al., 2019,2020a,b,2021). The importance of the potential toxic action of NPs towards beneficial soil microorganisms including bacteria and fungi derives from the direct implication of these organisms to soil and plant health and productivity, and the agroecosystem/environmental sustainability (Dimkpa 2014; Ameen et al., 2021). A better understanding of such ramifications requires studies involving combined plant- microbe interactions under the nanoparticle's influence (Ameen et al., 2021). FsK is a root colonizing *Fusarium solani* strain proven to induce tomato plant resistance mechanisms against pathogens and abiotic stress (Kavroulakis et al., 2007; Garantonakis et al, 2018; Kavroulakis et al., 2018). A key question besides tomato phytotoxicity and FsK fungitoxicity risks posed by NPs, is whether they can affect the survival and colonization of FsK and its symbiotic interactions with the tomato plant. *In vitro* fungitoxicity tests revealed that FsK was significantly more sensitive to Cu-NPs and ZnO-NPs than CuO-NPs and AgNPs both in terms of mycelial growth and spore germination. All NPs were more toxic to FsK compared to their bulk counterparts except for $AgNO_3$ which was 8 to 9fold more toxic than Ag-NPs. A similar fungitoxic profile was reported in another *F. solani* strain exposed *in vitro* to the above metal NPs and bulk counterparts (Malandrakis et al., 2019).

When applied in sublethal doses in a soil-based tomato-endophyte system, NPs and bulk counterparts did not exhibit any significant effect on FsK colonization of tomato roots. This could be due to the fact that the endophyte was already established inside tomato roots which acted as a barrier, before treatment with metal NPs/counterparts. On the other hand, tomato plants colonized by the FsK strain did not suffer any adverse effects in terms of dry mass from CuO-NPs, ZnO-NPs and $ZnSO_4$ treatments compared with the untreated control. In the absence of FsK, the above treatments of tomato plants resulted in a significant decrease in dry mass compared with the untreated control. This indicates a potential plant-endophyte mutual protection against NPs and heavy metals with obvious implications on plant health and production. A number of studies have reported toxicity of NPs on beneficial soil fungi

including biocontrol agents and other beneficial symbiotes such as arbuscular mycorrhizal fungi (AMF) and their impact on colonization and plant growth. Oktarina and Singleton (2019) have reported a significant fungitoxic effect of AgNPs in terms of colony diameter and spore production in the beneficial soil fungus *Trichoderma harzianum* in concentrations of 200, 600 and 1000 µg/mL) in growth media. A similar study reported a significant reduction in maize growth and colonization by the (AMF) fungus *Funneliformis mosseae* in the presence of 500 mg/ kg of ZnO-NPs and $ZnSO_4$ (Li et al., 2015b). On the other hand, AM fungi have demonstrated an alleviation potential against the negative effects of Ag-NPs and ZnONPs exposure in maize (*Zea mays* L.) and tomato plants (Cao et al., 2020; Noori et al., 2017; Wang et al., 2016).

7.6 Conclusion

Metal nanoparticles significantly negatively affected growth and physiology of tomato plants as indicated by dry mass, oxidative stress responses and photosynthetic pigment levels. Differences in plant and FsK responses between nano and bulk/ionic counterparts were observed indicating potential differences in the mode of toxic action of the two categories. NPs and counterparts applied in sublethal concentrations did not affect FsK colonization of tomato roots, while a possible alleviation of metal toxicity was observed in the presence of FsK in the case of CuO-NPs, ZnO-NPs0 and $ZnSO_4$. These results suggest that phytotoxicity of NPs in tomato treated plants should be considered before application and while both FsK and tomato are sensitive to NPs and counterparts, their symbiotic benefits can extent to mutual resistance towards these toxic agents.

7.7 References

Ahmed, B., Rizvi, A., Zaidi, A., Khan, M.S., Musarrat, J. Understanding the phytointeraction of heavy metal oxide bulk and nanoparticles: evaluation of seed germination, growth, bioaccumulation, and metallothionein production (2019) RSC Advances, 9 (8), pp. 4210-4225.

Akanbi-Gada, M.A., Ogunkunle, C.O., Vishwakarma, V., Viswanathan, K., Fatoba, P.O. Phytotoxicity of nano-zinc oxide to tomato plant (*Solanum lycopersicum* L.): Zn uptake, stress enzymes response and influence on non-enzymatic antioxidants in fruits (2019) Environmental Technology and Innovation, 14, art. no. 100325,DOI: 10.1016/j.eti.2019.100325.

Ameen, F., Alsamhary, K., Alabdullatif, J.A., ALNadhari, S. A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi (2021) Ecotoxicology and Environmental Safety, 213, art. no. 112027, DOI: 10.1016/j.ecoenv.2021.112027.

Baker, S., Volova, T., Prudnikova, S.V., Satish, S., Prasad M.N., N. Nanoagroparticles emerging trends and future prospect in modern agriculture system (2017) Environmental Toxicology and Pharmacology, 53, pp. 10-17.

Cao, J., Feng, Y., Lin, X., Wang, J. A beneficial role of arbuscular mycorrhizal fungi in influencing the effects of silver nanoparticles on plant-microbe systems in a soil matrix (2020) Environmental Science and Pollution Research, 27 (11), pp. 11782-11796.

Chen, Y., Lu, J., Liu, M., Li, H., Sun, Q., Nechitaylo, G.S., Bogoslovskaya, O.A., Olkhovskaya, I.P., Glushchenko, N.N. Tomato response to metal nanoparticles introduction into the nutrient medium (2020) IET Nanobiotechnology, 14 (5), pp. 382-388.

Courtois, P., Rorat, A., Lemiere, S., Guyoneaud, R., Attard, E., Levard, C., Vandenbulcke, F. Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage sludge: A review of effects on microorganisms, plants and animals (2019) Environmental Pollution, 253, pp. 578-598.

Cox, A., Venkatachalam, P., Sahi, S., Sharma, N. Silver and titanium dioxide nanoparticle toxicity in plants: A review of current research (2016) Plant Physiology and Biochemistry, 107, pp. 147-163.

de la Rosa, G., Vázquez-Núñez, E., Molina-Guerrero, C., Serafín-Muñoz, A.H., VeraReyes, I. Interactions of nanomaterials and plants at the cellular level: current knowledge and relevant gaps (2021) Nanotechnology for Environmental Engineering, 6 (1), art. no. 7, DOI: 10.1007/s41204-020-00100-1.

Dimkpa, C.O. Can nanotechnology deliver the promised benefits without negatively impacting soil microbial life? (2014) Journal of Basic Microbiology, 54 (9), pp. 889-904.

Feynman, R.P., There's Plenty of Room at the Bottom (1960) Engineering and Science magazine, 23, 22-26.

Garantonakis, N., Pappas, M.L., Varikou, K., Skiada, V., Broufas, G.D., Kavroulakis, N., Papadopoulou, K.K. Tomato inoculation with the endophytic strain *Fusarium solani* K results in reduced feeding damage by the zoophytophagous predator *Nesidiocoris tenuis* (2018) Frontiers in Ecology and Evolution, 6 (AUG), art. no. 126, DOI: 10.3389/fevo.2018.00126.

Heath R.L., Packer L.: Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189198, 1968.

Jiravova, J., Tomankova, K.B., Harvanova, M., Malina, L., Malohlava, J., Luhova, L., Panacek, A., Manisova, B., Kolarova, H. The effect of silver nanoparticles and silver ions on mammalian and plant cells *in vitro* (2016) Food and Chemical Toxicology, 96, pp. 50-61.

Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D.; A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues (2018) Nature Nanotechnology, 13 (8), 677–684.

Karami Mehrian, S., Heidari, R., Rahmani, F., Najafi, S. Effect of Chemical Synthesis Silver Nanoparticles on Germination Indices and Seedlings Growth in Seven Varieties of *Lycopersicon esculentum* Mill (tomato) Plants (2016) Journal of Cluster Science, 27 (1), pp. 327-340.

Kavroulakis, N., Doupis, G., Papadakis, I.E., Ehaliotis, C., Papadopoulou, K.K. Tolerance of tomato plants to water stress is improved by the root endophyte *Fusarium solani* FsK (2018) Rhizosphere, 6, pp. 77-85.

Kavroulakis, N., Ntougias, S., Zervakis, G.I., Ehaliotis, C., Haralampidis, K., Papadopoulou, K.K. Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic *Fusarium solani* strain (2007) Journal of Experimental Botany, 58 (14), pp. 3853-3864.

Khaldari, I., Naghavi, M.R., Motamedi, E. Synthesis of green and pure copper oxide nanoparticles using two plant resources: via solid-state route and their phytotoxicity assessment (2021) RSC Advances, 11 (6), pp. 3346-3353.

Koelmel, J., Leland, T., Wang, H., Amarasiriwardena, D., Xing, B. Investigation of gold nanoparticles uptake and their tissue level distribution in rice plants by laser ablation-inductively coupled-mass spectrometry (2013) Environmental Pollution, 174, pp. 222-228.

Larue, C., Castillo-Michel, H., Sobanska, S., Cécillon, L., Bureau, S., Barthès, V., Ouerdane, L., Carrière, M., Sarret, G. Foliar exposure of the crop *Lactuca sativa* to silver nanoparticles: Evidence for internalization and changes in Ag speciation (2014) Journal of Hazardous Materials, 264, pp. 98-106.

Lewis, R.W., Bertsch, P.M., McNear, D.H. Nanotoxicity of engineered nanomaterials (ENMs) to environmentally relevant beneficial soil bacteria–a critical review (2019) Nanotoxicology, 13 (3), pp. 392-428.

Li, K.-E., Chang, Z.-Y., Shen, C.-X., Yao, N. Toxicity of nanomaterials to plants (2015a) Nanotechnology and Plant Sciences: Nanoparticles and Their Impact on Plants, pp. 101-123.

Li, S., Liu, X.-Q., Wang, F.-Y., Miao, Y.-F. Effects of ZnO nanoparticles, ZnSO4 and arbuscular mycorrhizal fungus on the growth of maize (2015b) Huanjing Kexue/Environmental Science, 36 (12), pp. 4615-4622.

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy.– In: Wrolstad R.E., Acree T.E., An H. et al. (ed.): Current Protocols in Food Analytical Chemistry. Pp. F4.3.1F4.3.8. John Wiley & Sons Inc, New York.

Lopez-Lima, D., Mtz-Enriquez, A.I., Carrión, G., Basurto-Cereceda, S., Pariona, N. The bifunctional role of copper nanoparticles in tomato: Effective treatment for Fusarium wilt and plant growth promoter (2021) Scientia Horticulturae, 277, art. no. 109810, DOI: 10.1016/j.scienta.2020.109810.

Ma, X., Geiser-Lee, J., Deng, Y., Kolmakov, A. Interactions between engineered nanoparticles (ENPs) and plants: Phytotoxicity, uptake and accumulation (2010) Science of the Total Environment, 408 (16), pp. 3053-3061.

Malandrakis, A., Daskalaki, E.R., Skiada, V., Papadopoulou, K.K., Kavroulakis, N. A *Fusarium solani* endophyte vs fungicides: Compatibility in a *Fusarium oxysporum* f.sp. *radicis-lycopersici* – tomato pathosystem (2018) Fungal Biology, 122 (12), pp. 1215-1221.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens (2019). Science of the Total Environment, 670, pp. 292-299.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Synergy between Cu-NPs and fungicides against *Botrytis cinerea* (2020a) Science of the Total Environment, 703, art. no. 135557, DOI: 10.1016/j.scitotenv.2019.135557.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola* (2020b) Science of the Total Environment, 747, art. no. 141287, DOI: 10.1016/j.scitotenv.2020.141287.

Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V. Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates (2021) Pesticide Biochemistry and Physiology, art. no. 104796, DOI:10.1016/j.pestbp.2021.104796.

Noori, A., Ngo, A., Gutierrez, P., Theberge, S., White, J.C. Silver nanoparticle detection and accumulation in tomato (*Lycopersicon esculentum*) (2020) Journal of Nanoparticle Research, 22 (6), art. no. 131, DOI: 10.1007/s11051-02004866-y.

Noori, A., White, J.C., Newman, L.A. Mycorrhizal fungi influence on silver uptake and membrane protein gene expression following silver nanoparticle exposure (2017) Journal of Nanoparticle Research, 19 (2), art. no. 66, DOI: 10.1007/s11051-016-3650-4.

Oktarina, H., Singleton, I. Can nano-silver products endanger beneficial soil fungi? (2020) IOP Conference Series: Earth and Environmental Science, 425 (1), art. no. 012070, DOI: 10.1088/1755-1315/425/1/012070.

Pagano, L., Servin, A.D., De La Torre-Roche, R., Mukherjee, A., Majumdar, S., Hawthorne, J., Marmiroli, M., Maestri, E., Marra, R.E., Isch, S.M., Dhankher, O.P., White, J.C., Marmiroli, N. Molecular Response of Crop Plants to Engineered Nanomaterials (2016) Environmental Science and Technology, 50 (13), pp. 7198-7207.

Pandey, S., Giri, K., Kumar, R., Mishra, G., Raja Rishi, R.; Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks (2018) Proceedings of the National Academy of Sciences India Section B - Biological Sciences, 88 (4), pp. 1287-1308.

Ristroph, K.D., Astete, C.E., Bodoki, E., Sabliov, C.M. Zein Nanoparticles Uptake by Hydroponically Grown Soybean Plants (2017) Environmental Science and Technology, 51 (24), pp. 14065-14071.

Shaw, A.K., Ghosh, S., Kalaji, H.M., Bosa, K., Brestic, M., Zivcak, M., Hossain, Z. Nano-CuO stress induced modulation of antioxidative defense and photosynthetic performance of Syrian barley (*Hordeum vulgare* L.) (2014) Environmental and Experimental Botany, 102, pp. 37-47.

Song, U., Jun, H., Waldman, B., Roh, J., Kim, Y., Yi, J., Lee, E.J. Functional analyses of nanoparticle toxicity: A comparative study of the effects of TiO2 and Ag on tomatoes (*Lycopersicon esculentum*) (2013) Ecotoxicology and Environmental Safety, 93, pp. 60-67.

Sotiras M, Papadakis I, Landi M, Tsaniklidis G, Tsiantas P, Psychoyou M (2019) Allocation pattern, photosynthetic performance and sugar metabolism in hydroponically grown seedlings of loquat (*Eriobotrya japonica* Lindl.) subjected to salinity. Photosynthetica 57:258–267

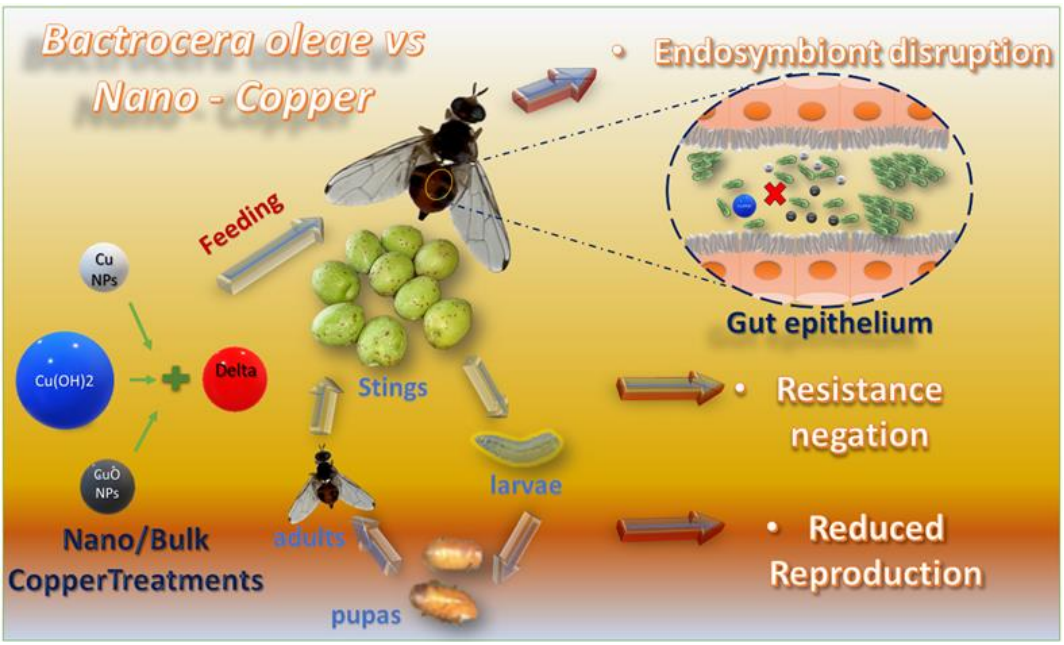
Sun, Q., Li, J., Le, T., Zinc Oxide Nanoparticle as a Novel Class of Antifungal Agents: Current Advances and Future Perspectives (2018). Journal of Agricultural and Food Chemistry, 66 (43), 11209–11220.

Sun, L., Wang, Y., Wang, R., Wang, R., Zhang, P., Ju, Q., Xu, J. Physiological, transcriptomic, and metabolomic analyses reveal zinc oxide nanoparticles modulate plant growth in tomato (2020) Environmental Science: Nano, 7 (11), pp. 3587-3604.

Tsaniklidis G, Pappi P, Tsafouros A, Charova SN, Nikoloudakis N, Roussos PA, Paschalidis KA, Delis C, (2020) Polyamine Homeostasis in Tomato Biotic/Abiotic Stress Cross-Tolerance. Gene 727: 144230

Wang, F., Liu, X., Shi, Z., Tong, R., Adams, C.A., Shi, X. Arbuscular mycorrhizae alleviate negative effects of zinc oxide nanoparticle and zinc accumulation in maize plants - A soil microcosm experiment (2016) Chemosphere, 147, pp. 88-97.

8 Copper nanoparticles interfere with insecticide sensitivity, fecundity and endosymbiont abundance in olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae)



Malandrakis, A.A., Varikou, K., Kavroulakis, N., Nikolakakis, A., Dervisi, I., Reppa, C., Papadakis, S., Holeva, M.C., Chrysikopoulos, C.V. Copper nanoparticles interfere with insecticide sensitivity, fecundity and endosymbiont abundance in olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) (2024) Pest Management Science, DOI: 10.1002/ps.8068.

8. Copper nanoparticles interfere with insecticide sensitivity, fecundity and endosymbiont abundance in olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae)

Abstract

The potential of copper nanoparticles (NPs) to be used as an alternative control strategy against olive fruit flies (*Bactrocera oleae*) with reduced sensitivity to the pyrethroid deltamethrin and the impact of both nanosized and bulk copper [$Cu(OH)_2$] on the insect's reproductive and endosymbiotic parameters were investigated.

The application of nanosized and bulk copper applied by feeding resulted in significant levels of adult mortality, comparable to or surpassing those achieved with deltamethrin at recommended doses. Combinations of Cu-NPs or CuO-NPs with deltamethrin significantly enhanced the insecticide's efficacy against *B. oleae* adults. When combined with deltamethrin, Cu-NPs significantly reduced the mean total number of offspring compared with the control, and the number of stings, pupae, female and total number of offspring compared with the insecticide alone. Both bulk and nanosized copper negatively affected the abundance of the endosymbiotic bacterium *Candidatus Erwinia dacicola* which is crucial for the survival of *B. oleae* larvae.

Concluding, Cu-NPs can aid the control of *B. oleae* both by reducing larval survival and by enhancing deltamethrin performance in terms of toxicity and reduced fecundity, providing an effective anti-resistance tool and minimizing the environmental footprint of synthetic pesticides by reducing the required doses for the control of the pest.

8.1. Introduction

Chemical control of crop pests has revolutionized modern agriculture utilizing protective or systemic synthetic pesticides and still remains a cost efficient, key constituent of integrated pest management (IPM) ensuring food sustainability and safety.[1,2] However beneficial for agricultural output and disease transmission management, certain drawbacks of conventional insecticides, such as toxicity to non-target organisms and aquatic environmental pollution have resulted in the withdrawal of an unprecedentedly great number of insecticide active ingredients enforced by the implementation of strict EU regulations.[3] This fact in combination with the compromise of insecticide efficacy due to the emergence of resistant pests underline the necessity of alternative pest control measures capable of reducing yield losses and, at the same time, mitigating environmental risks.

The olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae), is the major pest of olive orchards in the Mediterranean basin and worldwide.[4] It's larvae feed on the olive fruit causing significant quantitative and qualitative losses in fruit and oil.[5,6] In Greece, annual economic losses due to *B. oleae* infestation are estimated to range between 30–35% of total production depending on the weather conditions.[7] The control of *B. oleae* in Greece is based on mass trapping in organic and the use of synthetic insecticides in conventional olive orchards.

Insecticides are applied either via cover (over the entire canopy of every tree) or as bait sprays where the insecticide mixed with a food attractant is applied on a small part of the canopy usually using 10-fold higher doses than the ones in cover sprays. The later method is recommended and implemented by the Greek Ministry of Rural Development and Food via the Directorates of Agriculture and Veterinary Regional Units in Greece.[8] Chemical insecticides used over the last decade against *B. oleae* include organophosphate insecticides (OPs) such as dimethoate or phosmet, pyrethroids such as deltamethrin and recently the macrocyclic lactone spinosad. Copper-containing pesticides are a very common and effective option for the control of the major olive fungal (*Cycloconium* spp., *Gloeosporium* spp. and *Pseudocercospora cladosporioides*) pathogens.[9] Besides its fungicidal action, copper was also reported to exhibit both an ovipositional deterrent effect on the olive fly and a symbionticidal action expressed as the inactivation of certain gut bacterial species essential for the survival of *B. olea* nymphs.[10-12] This is of great importance for the control of the pest and indirectly aids pest control by reducing *B. oleae* infestation rates. However, despite their effectiveness, chemical insecticides are susceptible to resistance development due to the extensive use -and often misuse- of site-specific active ingredients.

In Greece, the emergence of insecticide resistance in *B. oleae* was delayed due to the high insect mobility and low selection pressure of bait sprays.[13] Eventually, resistance of the insect to OPs has emerged due to target site modifications whereas reduced sensitivity to pyrethroids -mainly attributed to metabolic resistance- soon followed.[14,15] Moderate levels of pyrethroid resistance in Greece have been reported over a decade ago, although substantially higher levels have been observed recently in certain regions such as the island of Crete.[15] Typically, pyrethroid metabolic resistance has been associated with detoxification utilizing P450s, carboxylesterases or Glutathione S-transferases (GSTs).[16] Previous studies have reported *B. oleae* populations with reduced sensitivity to alpha cypermethrin, and although reports from farmers for reduced effectiveness of deltamethrin exist, to the best of the authors knowledge, no systematic studies on populations of *B. oleae* resistant to deltamethrin are available in Greece.

Nano pesticides, containing nanoparticles (NPs) as active ingredients, are promising and environmentally compatible insecticide alternatives owing to a number of unique properties they possess including high effectiveness, enhanced drug delivery, residual action and low resistance risk.[1,17,18] Silver and copper nanoparticles have been shown to exert acute toxic and reproduction-related as well as microbiome-alternating effects on another fruit fly, *Drosophila melanogaster* in ways that differ from those of their bulk counterparts.[19-21] Furthermore, recent studies have demonstrated that metal NPs, including copper-containing NPs, can be used alone or in combination with conventional pesticides to combat pesticide resistance and thus reduce needed doses to control pathogens and pests.[22] Up to date, no study is available on the effect of copper-containing NPs on *B. oleae* survival, reproduction and endobiont abundance or their potential to control olive fruit flies with reduced sensitivity to deltamethrin.

Under this light, the aim of this study was to: (a) evaluate the effectiveness of copper-containing NPs against *B. oleae*, (b) investigate the potential of bulk and NP copper to be used in combinations with deltamethrin against *B. oleae* in order to enhance insecticide effectiveness, and (c) investigate the effect of copper NPs alone or combined with deltamethrin on the reproductive behaviour, fecundity and abundance of the gut endosymbiotic bacterium *Candidatus Erwinia dacicola* of the insect.

8.2. Materials and Methods

8.2.1 Nanoparticles, reagents and insecticides

Copper [Cu-NPs] (particle size 25 nm), copper oxide [CuO-NPs] (particle size <50 nm) nanoparticles (NPs) and Ethylenediaminetetraacetic acid [EDTA] used in this study were purchased from Sigma Aldrich, MO, USA. The copper hydroxide containing commercial fungicide (Copperblau-N 50 WP), used as a bulk counterpart of copper NPs, was purchased from Nitrofarm (Greece), while the tested insecticide deltamethrin (Decis 2.5 EC) was kindly provided by Bayer CropScience AG.

Stock solutions-suspensions of the commercial fungicide, insecticide and nanoparticles used in acute toxicity bioassays were prepared using distilled-sterilized water. Nanoparticle suspensions were subjected to sonication for 30 min with a Transonic 420 (Elma, Germany) sonicator before use in order to deter aggregation. Zeta potential and hydrodynamic diameter measurements for the nanoparticles and insecticide (Table 8.1) were caried out with a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA) in triplicate.

8.2.2 Scanning Electron microscopy

Cu-NPs and CuO-NPs 1000 μg/mL suspensions were prepared and then combined with 100 deltamethrin μg/mL suspensions to acquire the respective samples for electron microscopy. Prepared samples were dried in a Critical Point Dryer (BAL-TEC 030) and observed in the Scanning Electron Microscope JSM-IT700HR (20 kV).

8.2.3 Sampling of infested olive fruit to acquire *B. oleae* individuals

Approximately 10 kg of infested olive fruits (cv *Koroneiki*) (with visible oviposition sites marks and tunnels) were collected at a weekly basis starting from early August from an unsprayed olive orchard in Nerokourou village (Crete, Kolymvari region, 2 ha, 35° 31′ 45, 67″ N–23° 46′ 39, 32″ E-71 m). Infested fruits were transferred to the laboratory and placed inside a wooden insect cage (dimensions 300 × 300 × 300 mm) under controlled conditions (25 ± 0.5 °C, 65 ± 5% R.H. and a 16L:8D photoperiod). Olive fruit were inspected daily and pupae were collected and transferred to new cages where water, and a common diet containing water, sugar, and protein (in a ratio of 5:4:1) were made available for newly-hatched adult flies.[23] At the end of September, newly emerged from pupae *B. oleae* adults were used for lab experiments.

8.2.4 Toxicity bioassays

To estimate the efficacy of copper nanoparticles, the commercial pyrethroid insecticide and their combinations against *B. oleae* adults, toxic compounds were supplied to insects by feeding. Specifically, treatments consisted of feeding suspensions containing the above mentioned sugar and protein diet amended with 1,200 μg/mL Cu-NPs, CuO-NPs or $Cu(OH)_2$, 125 μg/mL deltamethrin and their respective combinations. Overall, the experimental design consisted of: 8 (7+1 control) pesticide/NP treatments × 8 individual insects (4 ♀ and 4 ♂ adults) per replicate × 10 replicates.

Specifically, single olive fruits of uniform size collected from olive trees (*Olea europea* cv Amfissis) were transferred to small top-less PVC cylindrical containers (9 cm in diameter and 11 cm high, 0.4 mm thick) covered with fine muslin to ensure ventilation and prevent insects from escaping. Each fruit was mounted in a standard position at the bottom of the container using plasticine (Fig. 8.1). Adult olive fruit flies (four pairs of individuals, 10 days old) were released into each plastic container. Food and water were supplied in the form of droplets dripped on top of the muslin that covered the containers (Fig. 8.1a). Pesticides and copper nanoparticle treatments were applied by adding 1.5 mL from each solution in each cotton bud using a small-volume plastic disposable pipette. Flies in cages provided with a diet containing only food droplets without toxic agents were used as the control. Ten replications were tested per treatment. All the cages were kept in a growth chamber at 25 ± 0.5 °C, 65 ± 5% R.H. and a 16L:8D photoperiod. The efficacy (percentage of mortality) of the applied feeding solutions against the adult fly was evaluated by counting the number of dead *B. oleae* adults after 48, 120, 168 and 216 h (2,5,7 and 9 days) of application. Flies were considered as dead when no sign of movement (flying or walking) was observed.

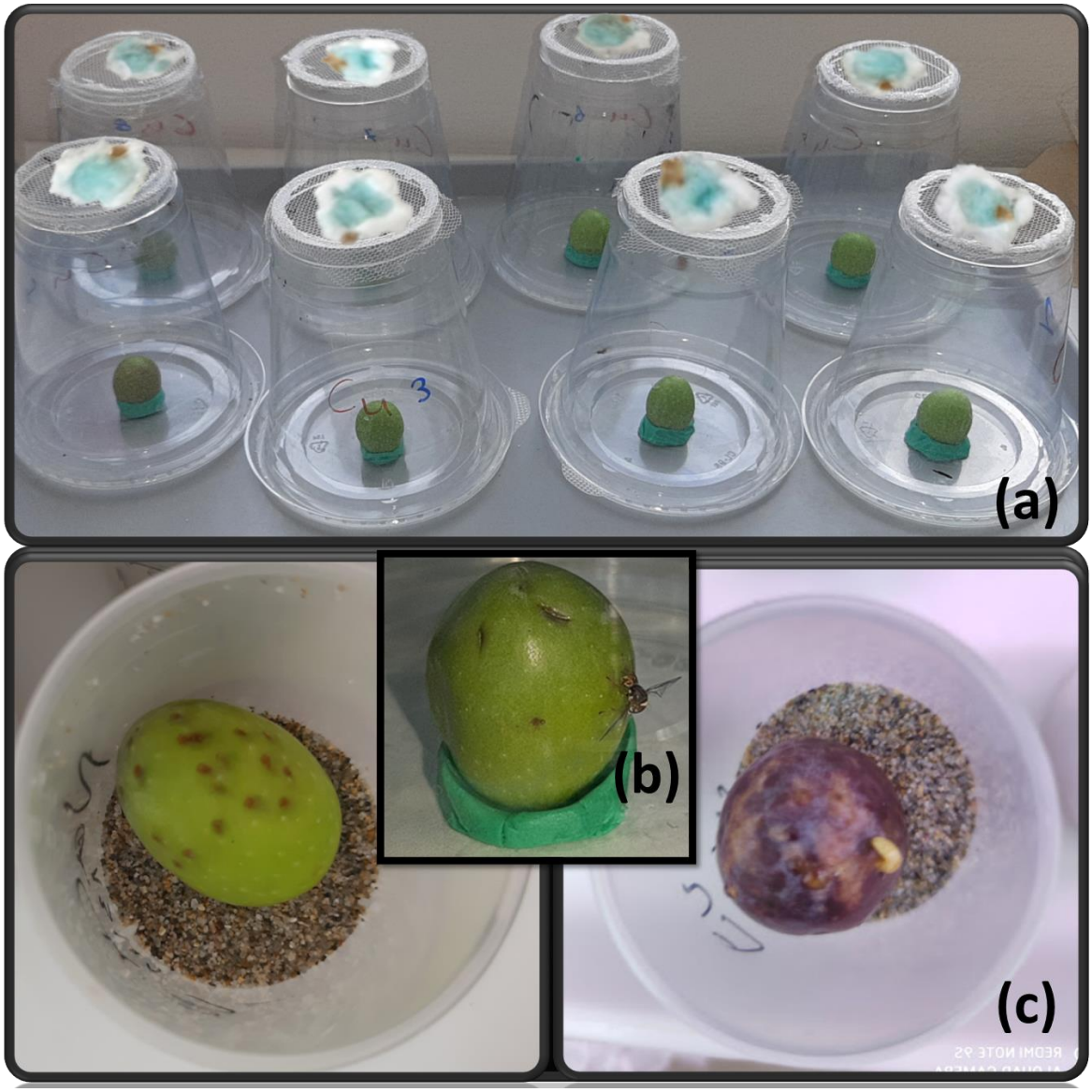


Figure 8.1. Bioassays on the effect of copper compounds, deltamethrin and combinations on *B*. *oleae* survival and fecundity parameters: (a) plastic containers with muslin on top and cotton buds used for feeding treatments; (b) olive fruit mounted at the bottom of containers using plasticin; (c) plastic containers with sand on the bottom used for larval emergence observations.

In order to investigate the potential involvement of copper ion release on the toxicity observed by copper nanoparticle treatments, additional bioassays according to the previous described experimental design were conducted using CuO-NPs (1,200 μg/mL) and Cu-NPs (1,200 μg/mL) and their combination with the strong chelating agent EDTA (500 μg/mL).

Flies were fed using treated cotton buds for 2 days before replacing them with ones containing only water and hydrolyzed protein which were subsequently refreshed every two days. Oviposition stings on the olive fruits were counted and then each fruit was replaced with a fresh one every two days. Total number of stings per female was evaluated by the formula

(number of stings/fruit/number of females surviving pesticide treatment). Infested olive fruits were transferred in small and well-ventilated plastic pots (25 mL of volume) with sand on the bottom for larval emergence and development (Fig. 1c). The vials were examined on a daily basis and the number of pupas produced was recorded for a total time period of 20 days. Subsequently, pupae were monitored for eclosion for another 30 days. Adults emerging from pupas were counted and their sex was determined by observation under a stereoscope.

8.2.5 Bacterial DNA extraction

To evaluate the impact of insecticidal treatments on the population of the endosymbiont bacterium '*Ca*. *E. dacicola*', DNA from *B. oleae* female adults treated with bulk and nanosized copper and their combinations with deltamethrin or water (control), was amplified. At least three biological replicates consisting of insects dissected into thorax-head and abdomen specimens were stored in 1.5 ml microcentrifuge tubes at -20^{o}C until their use for DNA extraction.

Total genomic DNA extraction was performed from thorax-head and abdomen specimens separately using the Qiagen DNeasy Food Mericon kit (Qiagen, Germany) following the manufacturer's instructions. DNA sample quality and quantity was determined using a Nano spectrophotometer (Implen) and DNA concentrations were adjusted to 0.167 and 0.116 ng/μl for thorax-head and abdomen specimens, respectively. Samples were subsequently stored at -20^{o}C until use in real-time PCR amplifications.

8.2.6 Relative quantification of bacterial populations using Real-time PCR

'*Ca. E. dacicola'* population levels in the thorax-head or abdomen parts of *B. oleae* adults treated with the insecticide deltamethrin, copper compounds and their combinations, in comparison with water treated controls were assessed using Real-time PCR. Primers ED1 and EdEnRev were used for the specific amplification of a 90bp '*Ca. E. dacicola*' 16S rRNA gene fragment in the DNA samples.[24,25] Ct values obtained from the real-time PCR amplification were normalized using the housekeeping *B. oleae β*-actin gene, which was amplified using the primers Act2F and Act2R.[26]

Real-time PCR reactions were performed on a QuantStudio 5 (Thermo Fisher Scientific) real-time PCR device. Each 13μl reaction consisted of 1x SYBR™ Select Master Mix (Thermo Fisher Scientific), 300μM of each primer and 3μl of DNA template. The thermal profile used included incubation at 95 ^{o}C for 10 min, followed by 40 cycles of: 95^{o}C for 15 sec and 60^{o}C for 1 min. The specificity of the amplified PCR product was verified by melting-curve analysis performed immediately after the amplification over a temperature range of 65–95°C in 0.5°C increments.[26] Three technical replicates per sample were used and their average Ct value was calculated.

Standard curves for each gene (16S rRNA of '*Ca. E. dacicola'*, *β*-actin) and insect body part were obtained using four 10-fold serial dilutions of control DNA from a thorax-head or abdomen sample. Efficiency (E) and slope of the standard curves were similar, allowing for the calculation of the relative abundance (R) using the following formula:

$$R=2^{-\Delta\Delta Ct},$$

where $\Delta\Delta Ct= \Delta Ct_{treated\ sample}$- mean $\Delta Ct_{control}$, and ΔCt is the difference between the reference (*β*-actin) and the target gene (16S rDNA of '*Ca. E. dacicola'*) calculated for all samples (treated and control), *i.e.* $\Delta Ct= Ct_{target\ gene}- Ct_{reference\ gene}$.[26]

8.3. Statistical analysis

Statistical differences between treatments in toxicity and phytotoxicity experiments were evaluated by analysis of variance and the resulting means were separated according to Tukey's HSD test ($\alpha = 0.05$). Correlations of mortality rates and oviposition and fecundity parameters of NPs/counterpart treated *B. oleae* were evaluated using Pearson correlation coefficients. Means of mortality rates caused by CuO-Nps, Cu-NPs and their combination with EDTA were compared using one-way t-Tests. The SPSS v20 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

8.4. Results

8.4.1 Characterization of copper nanoparticles and deltamethrin

The zeta potential and hydrodynamic diameter of copper (Cu-NPs), copper oxide (CuO-NPs) nanoparticles, deltamethrin and their combinations measured by the Zetasizer as well as their respective pH values are shown in Table 8.1. Combination with deltamethrin reduced the size of both copper-containing nanoparticles. This reduction was more profound in the case of Cu-NPs, reaching a value of more than 50% of the original size when combined with deltamethrin. The initially positive Cu-NPs charge (18.2 mV) changed to negative (-6.8 mV) in the presence of the also negative charged deltamethrin (-12.0 mV) (Table 8.1). This size reduction and charge inversion suggests a capping effect exerted by deltamethrin, which enveloped Cu-NPs causing a reduced aggregation aided by the repelling force of the negatively charged deltamethrin which ultimately led to the reduction of nanoparticle size (Fig. 8.2).

Table 8.1. Zeta potential, diameter size and pH values of copper containing NPs (100 mg/mL), deltamethrin (125 mg/mL) and mixtures. Delta: deltamethrin.

	pH	Zeta potential (mV) (mean ± SD[a])	Size (d.nm) (mean ± SD) [a]
CuO-NPs	6.0	-18.8 ± 0.9	80.6 ± 45.1
Cu-NPs	5.7	18.2 ± 1.2	215.5 ± 23.0
Delta	5.6	-12.0 ± 2.2	216.3 ± 13.9
Delta + CuO-NPs	6.5	-17.2 ± 1.9	68.0 ± 7.0
Delta + Cu-NPs	5.7	-6.8 ± 0.3	104.3 ± 17.0

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

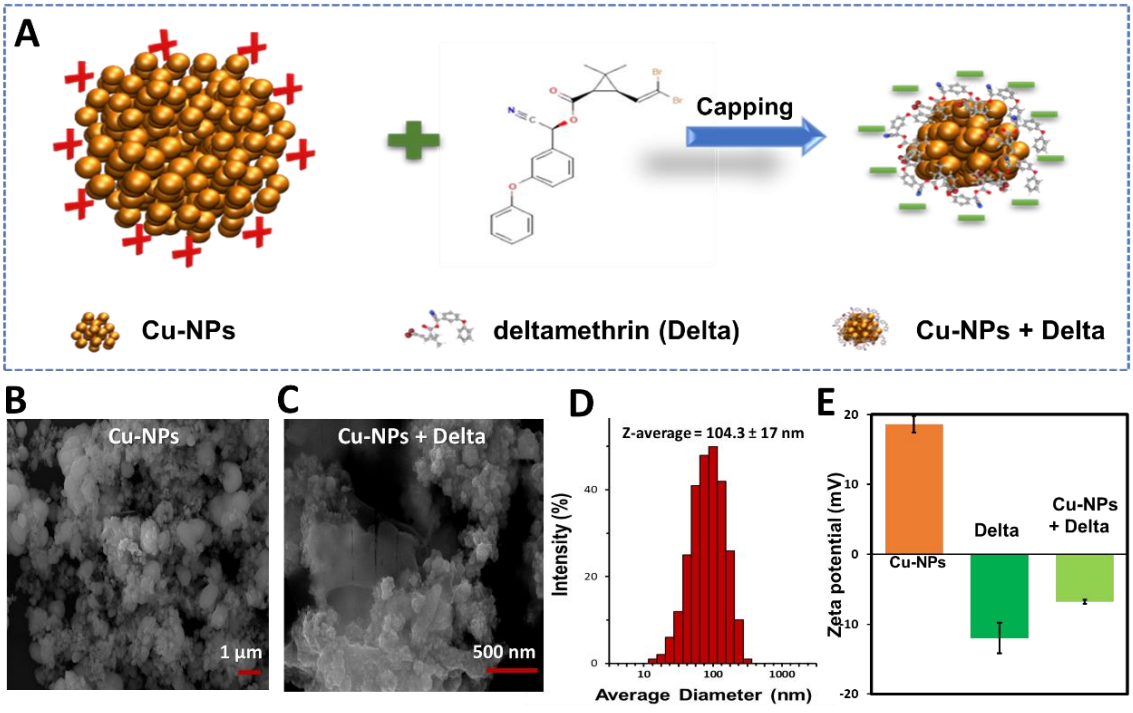


Figure 8.2: Characterization of copper nanoparticles and their mixture with deltamethrin (Delta). A) Capping effect and size reduction when Delta is combined with Cu-NPs. B) Representative SEM image of Cu-NPs. C) Representative SEM image of Cu-NPs+ Delta. D) Particle size distribution of Cu-NPs+ Delta. E) Zeta potential characterization of Cu-NPs, Delta and their combination.

8.4.2 Acute toxicity of copper compounds, deltamethrin and combinations

The *B. oleae* toxicity caused by Cu-NPs and CuO-NPs was compared to that of the insecticide deltamethrin and a commercial fungicide containing $Cu(OH)_2$. Mortality rates caused by feeds treated with copper compounds, the insecticide and their combinations are shown in Figures 3 and 4. When applied at the recommended dose for fungal diseases, copper hydroxide was the most toxic among treatments both at 2 and 9 days after treatment, with a mortality rate ranging from 74 to 93.75% (Fig. 3). Deltamethrin applied in the recommended for cover sprays dose caused lower mortality rates which ranged from 33 to 60.71% (Fig. 3). Cu-NPs exhibited similar to deltamethrin mortality rates ranging from 30 to 64% whereas CuO-NPs applied in the same dose was initially non-toxic to *B. oleae* but eventually exhibited a 39% mortality rate which was significantly higher than the one observed in the untreated control (14.77%) (Fig. 3). The above toxicity profile indicates that more copper ions are released by bulk counterparts than NP-copper as evident by the more profound toxic effect of $Cu(OH)_2$ when ingested by the insect.

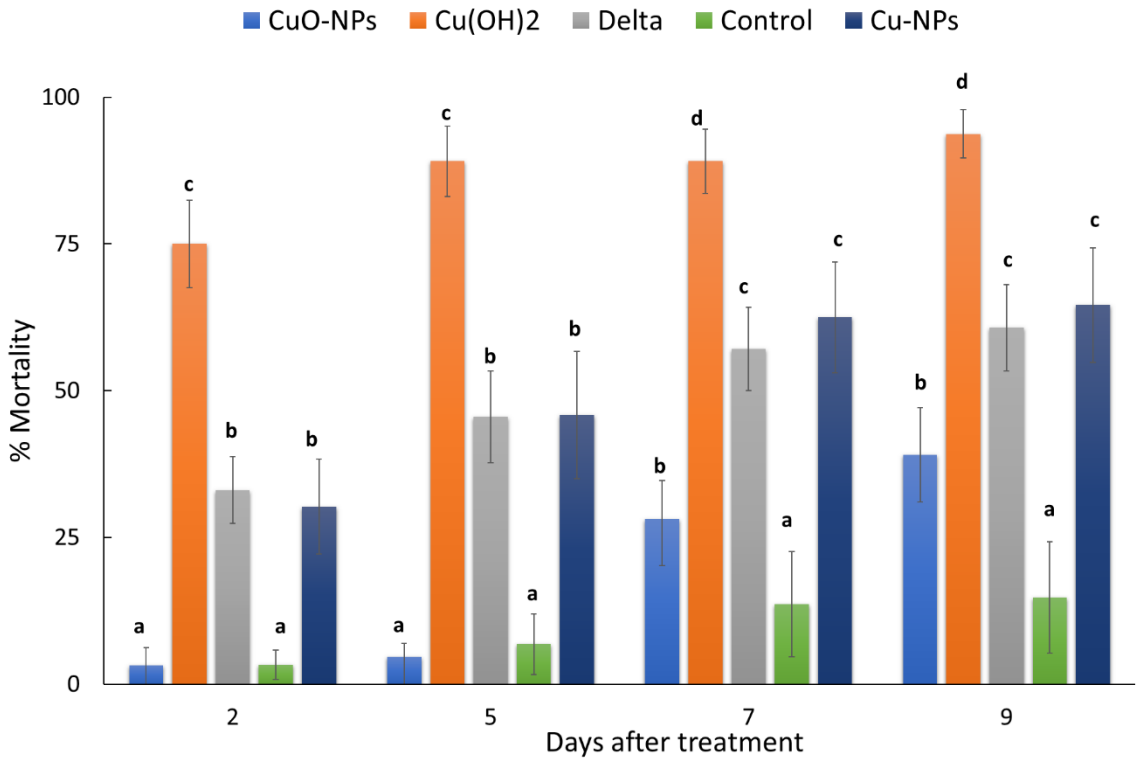


Figure 8.3. Mortality of *B. oleae* adults upon treatment with deltamethrin (125 μg/mL), copper nanoparticles (1200 μg/mL) and their bulk counterpart containing $Cu(OH)_2$ (1200 μg/mL). At the same day, bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

Combination of Cu-NPs with deltamethrin significantly increased mortality rates compared with the individual treatments (Fig. 8.4a). A similar, although delayed (7 or 9 days after treatment), effect was observed in the case of the combination of CuO-NPs with deltamethrin (Fig. 8.4b). On the contrary, the addition of $Cu(OH)_2$ to deltamethrin reduced the mortality caused by any of the two compounds (Fig. 8.4c).

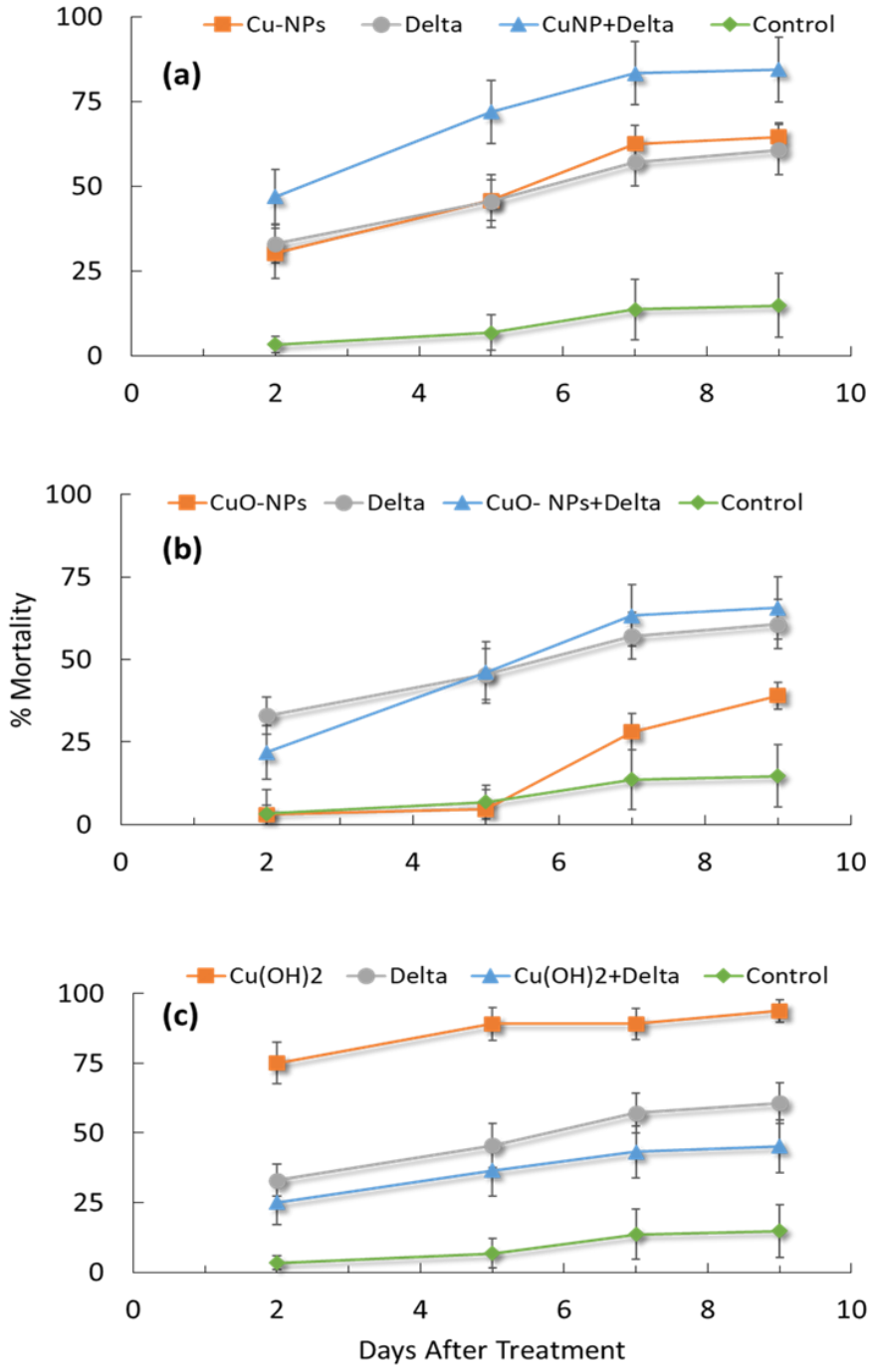


Figure 8.4. Effect of combination of (a) Cu-NPs (1200 µg/mL), (b) CuO-NPs (1200 µg/mL) and (c) $Cu(OH)_2$ (1200 µg/mL) with deltamethrin (Delta) (125 µg/mL) on adult mortality of *B. oleae* 2, 5, 7 and 9 days after treatment. Bars represent standard errors.

No significant difference on toxicity was observed when EDTA was added with either CuO-NPs or Cu-NPs indicating that other mechanisms besides copper ion release are involved on the toxicity excreted by copper nanoparticles on adult fruit flies (Fig. 8.5).

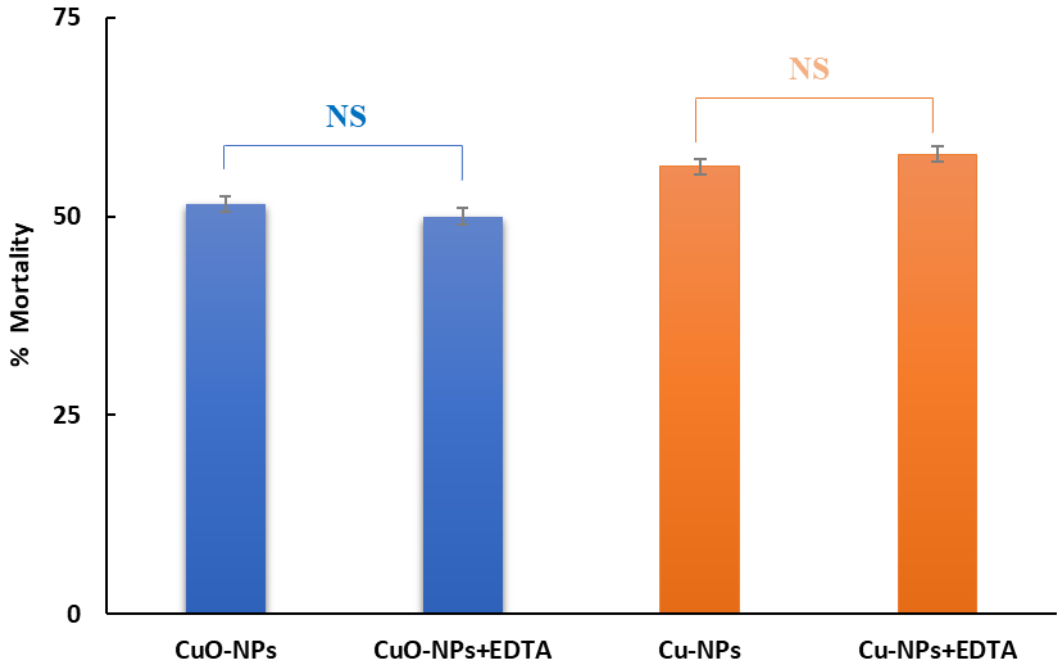


Figure 8.5. Effect of combination of EDTA (500 µg/mL) with CuO-NPs (1200 µg/mL) or Cu-NPs (1200 µg/mL) on adult mortality of *B. oleae* 2 days after treatment. NS indicates the absence of statistical differences between treatments based on t-Test analysis. Bars represent standard errors.

8.4.3 Effect on oviposition behavior and fecundity parameters of *B. oleae*

The overall effect of pesticides/NPs treatments and their combinations on oviposition parameters and offspring are shown in figure 6. Compared to the control, treatments did not affect the mean number of males, females and total offspring number. Cu-NPs, $Cu(OH)_2$ and their combinations with deltamethrin did not result in any differences in oviposition behavior of *B. oleae* female adults in terms of number of stings per olive fruit (Fig. 8.6). On the contrary, fruit flies treated with deltamethrin or CuO-NPs had a tendency to produce 2-fold more stings per fruit compared to the control treatment, although their combination seemed to neutralize that sting-inducing effect. Number of stings per olive fruit was positively correlated with the number of pupae and subsequent number of male or female offspring emerging from infested fruits per female as revealed by Pearson correlation analysis (Table 8.2). This indicates that stings resulted in fertile eggs that successfully lead to viable larvae capable to complete their life cycles. Mortality rates were negatively corelated with the

number of stings (r = -0.75, P=0.05) or the number of male offspring (r = -0.73, P=0.05) probably indicating a tendency of *B. oleae* to sting less for oviposition purposes and produce fewer male offspring under toxic conditions (Table 8.2). Sex ratio (♂/♀) was not significantly affected by treatments (0.5 – 0.69) except in the case of Cu-NPs which exhibited the maximum sex ratio (0.93).

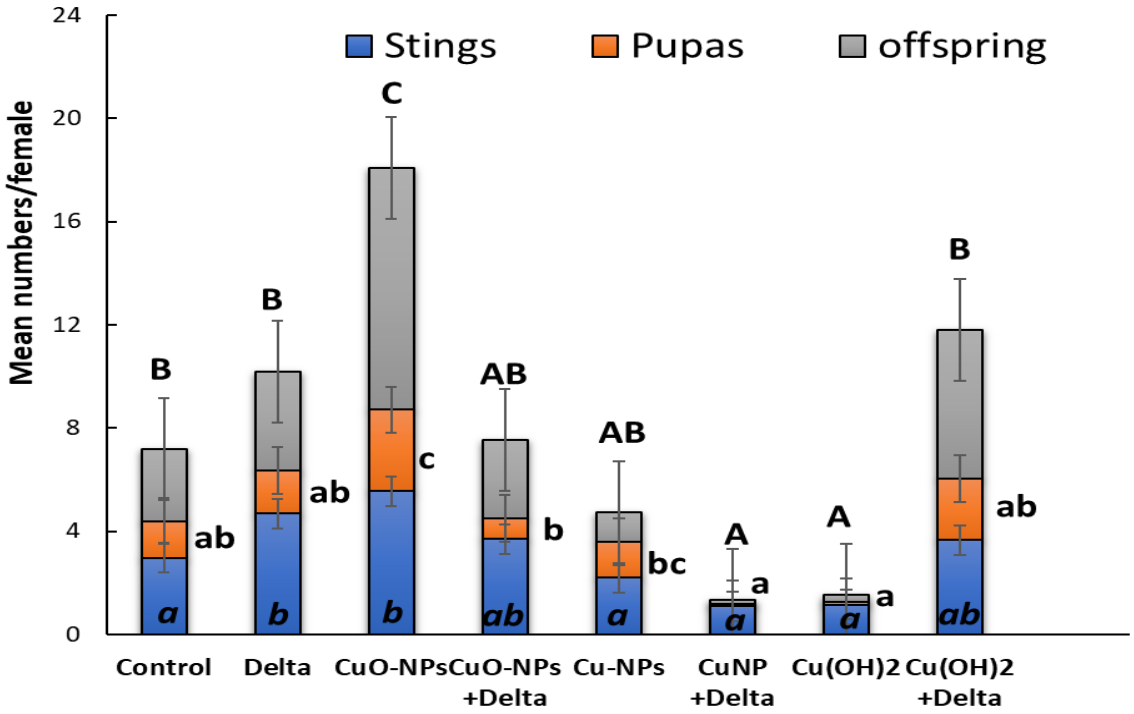


Figure 8.6. Effect of pesticides/NPs treatments on mean number of oviposition stings, pupas on olive fruit and offspring per female adult of *B. oleae,* 9 days post treatment. Delta: deltamethrin. Bars represent standard errors. Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

Table 8.2. Correlation between mortality rates caused by treatments and number of stings, pupae, male, female, and total adult offspring per female of *B. oleae.*

	% Mortality	Stings	Pupae	Male offspring	Female offspring	Total offspring
% Mortality	1.0 [a]	-0.75*	-0.63	-0.73*	-0.71	-0.60
Stings	-	1.0	0.94*	0.81*	0.82*	0.93*
Pupae	-	-	1.0	0.76*	0.77*	0.95*
Male offspring	-	-	-	1.0	0.98**	0.81*
Female offspring	-	-	-	-	1.0	0.85**
Total offspring	-	-	-	-	-	1.0

[a] Pearson correlation coefficient values.* Corresponds to a significance lever of P=0.05. ** Corresponds to a significance lever of P=0.01

When Cu-NPs were combined with deltamethrin they significantly reduced the number of stings, pupae, female and total number of offspring compared to deltamethrin alone (Fig. 8.7). In the case of CuO-NPs, their combination with deltamethrin reduced the mean number of pupas produced compared to the individual treatments but total number of offspring was not significantly different from that of deltamethrin alone (Fig. 8.3). On the contrary, the deltamethrin/$Cu(OH)_2$ mixture resulted in an increase in the mean number of pupas and offspring compared to the individual treatments (Fig. 8.6).

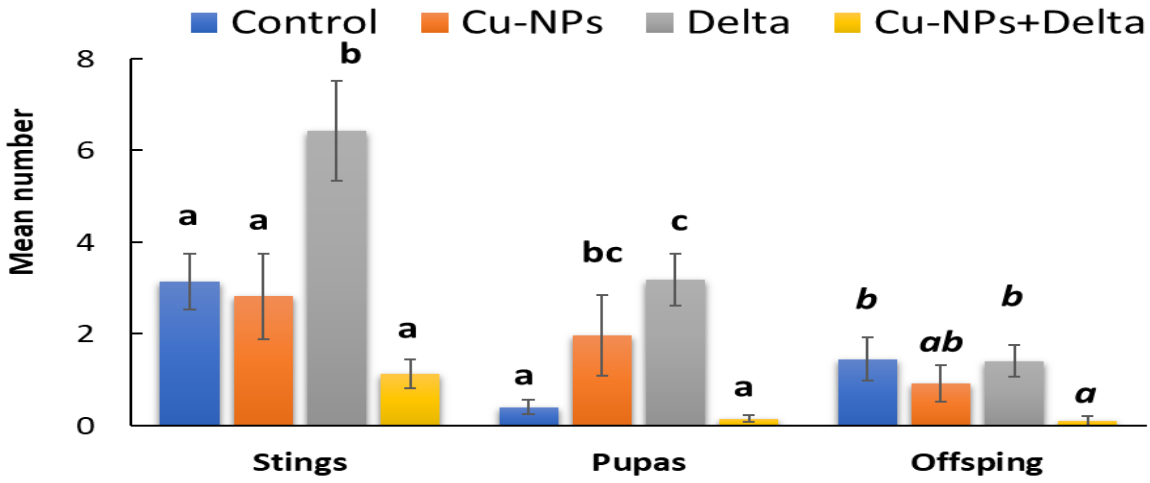


Figure 8.7 Effect of deltamethrin (Delta) and Cu-NPs combination on fecundity parameters of *B. oleae* compared with the individual and the control treatments, 9 days post treatment. Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05).

8.4.4 Relative quantification of the endosymbiotic bacterium '*Ca. E. dacicola*'

Quantitative real-time PCR analysis of the thorax-head samples from *B. oleae* individuals revealed that individual treatment with any of the copper compounds (bulk or nanosized) significantly reduced the relative abundance of *Ca. E. dacicola* in the thorax of the *B. oleae* compared with the control. This was also the case for the combination of $Cu(OH)_2$ with deltamethrin but not in the cases of deltamethrin and its combinations with CuO-NPs and Cu-NPs which exhibited similar levels of bacterial abundance compared with the control (Fig. 8.8).

A similar trend of the effect of copper compound treatments on the abundance of the endosymbiont bacterium was observed in the abdomen of *B. oleae* individuals, although no statistically significant difference between any insecticidal agent and the control treatment was found (Fig. 8.8). The only exception was observed in the case of deltamethrin which resulted a significant decrease in the endosymbiont abundance in the abdomen compared with the untreated control. In any case, bulk and nanosized copper treatments had a more profound effect on the bacterial abundance in the thorax-head parts than in the abdomen section of the insects. This could be due to a possible increased concentration of the toxic compounds in the esophageal bulb of the insects and their limited translocation to the abdomen/gut section.

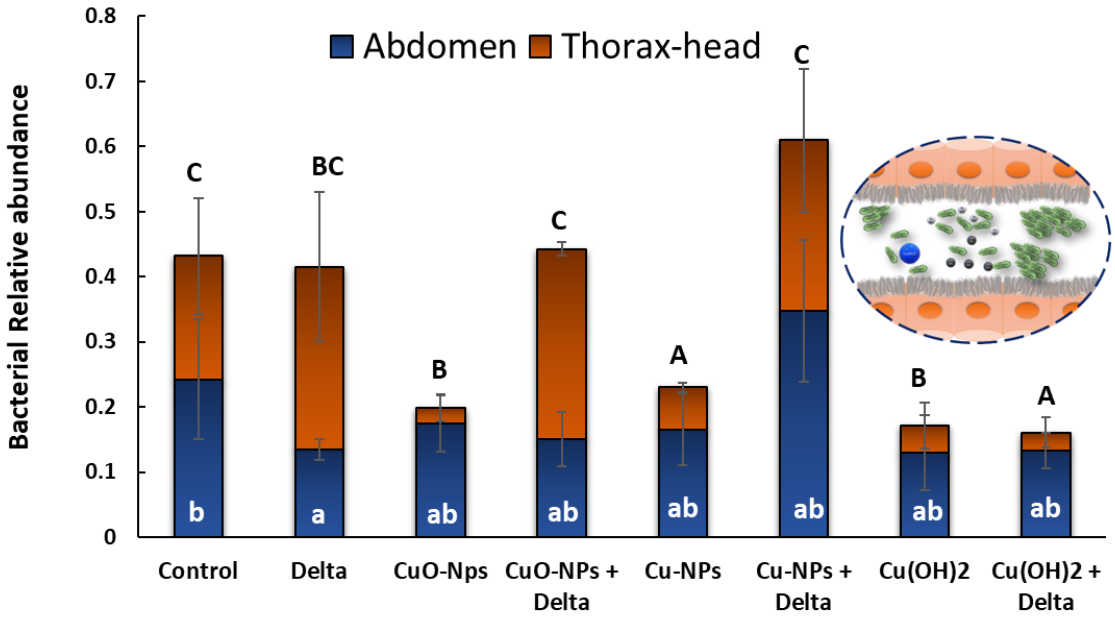


Figure 8.8 Relative abundance of '*Ca. E. dacicola*' in the thorax-head (orange bars) and abdomen (blue bars) parts of *B. oleae* adults treated with deltamethrin (Delta), bulk or nanosized copper and their combinations or water (control). Relative abundance was calculated based on the *β*-actin reference gene and expressed as the $R=2^{-\Delta\Delta Ct}$. Bars marked by the same letter do not differ significantly according to Tukey's HSD test (α=0.05). Bars represent standard errors. Oval schematic depicts fruit fly gut epithelium and bacterial interaction with copper compounds.

8.5. Discussion

The effects of copper NPs on the survival, oviposition behavior and fecundity parameters of *B. oleae* as well as the abundance of the endosymbiotic bacterium 'Ca. *E. dacicola*' alone or in combination with the insecticide deltamethrin were studied in laboratory bioassays.

Acute toxicity bioassays revealed a decreased sensitivity of the *B. oleae* surveyed population to the insecticide deltamethrin since the recommended dose for cover sprays caused 33 to 61% mortality rates, which are relatively low and are expected to lead to control failures. Practical resistance problems of *B. oleae* to pyrethroids have been reported in Greece only in the case of the extensively used alpha-cypermethrin. Alpha-cypermethrin applied in diagnostic doses has been reported to result in low mortality rates (22-40%), while the documented up-regulation of relevant detoxification genes suggested that a P450-mediated metabolic resistance mechanism could be responsible for the reduced sensitivity of *B. oleae* to this insecticide.[13] Deltamethrin was one of the pyrethroids that replaced alpha-cypermethrin in bait and cover sprays and has initially demonstrated a high control potential against *B. oleae*, although recent reports indicate a reduced effectiveness of this insecticide in the field.[7,27] To the best of the authors knowledge, the present study is the first report of a *B. oleae* field population with reduced sensitivity to deltamethrin in Greece.

Metal nanoparticles, especially those containing silver, zinc oxide, titanium oxide and copper, have exhibited a great potential as pesticide alternatives against arthropod pests of economic importance, including flies, moths, mosquitos, lice and ticks.[28,29] Green synthesized copper NPs showed strong larvicidal activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes, and the stored grain pests *Tenebrio molitor*, *Corcyra cephalonica*, *Sitophilus granarius* and *Rhyzopertha dominica*.[30-32] In the present study, copper NPs in doses equal to the recommended doses of copper hydroxide showed a differential toxicity to *B. oleae* adults. Specifically, Cu-NPs exhibited mortality rates similar to that of deltamethrin, while CuO-NPs had a delayed, lower toxic effect against the insect. This reduced toxic effect of CuO-NPs compared to Cu-NPs has been demonstrated in various plant pathogens.[33,34] On the contrary, their bulk counterpart copper hydroxide was more toxic than all treatments indicating differences in the mode of action between nano and bulk copper compounds. Stronger acute toxic effect of $Cu(OH)_2$ indicates that dissolved copper ions are more toxic than the nanoparticle forms of copper, in which slower ion release may account for the lower acute toxicity and the slower advancement of mortality of nano-copper compared to their bulk counterpart (especially in the case of CuO-NPs). Similarly, bulk/ionic forms of copper had a more profound toxic effect than CuO-NPs on the survival of the fruit fly *Drosophila melanogaster*.[20,21]

In an attempt to investigate the possible role of copper ion release on the observed toxicity of copper NPs against fruit fly adults, the strong chelating agent EDTA was combined with both CuO-NPs and Cu-NPs. EDTA, when tested against various plant pathogens or pests, has been shown to "deactivate" several metal nanoparticles by binding to metal ions

released.[34,35] This was not the case in the present study where the addition of EDTA did not cause any significant change in the toxicity of both CuO or Cu-NPs against *B. oleae*, probably indicating that mechanisms other than ion release could be responsible for the entomo-toxic effect copper NPs.

Interestingly, the combination of copper nanoparticles with the pyrethroid insecticide deltamethrin resulted an enhanced toxic effect against adults of *B. oleae* which was more profound in the case of Cu-NPs. This is of great importance and has obvious implications in resistance management of *B. oleae* populations with reduced sensitivity to pyrethroids already reported in Greece.[13] In a recent study, a CeO_2-based nanohybrid enhanced the activity of nitenpyram, sulfoxaflor, and clothianidin insecticides against both laboratory and field insecticide-resistant strains of *Nilaparvata lugens*.[36] The authors of the above study have proposed the nanohybrid's-induced reduction in host detoxification enzyme activities by downregulating ROS-dependent P450 gene expression, as the probable mechanism for overcoming insecticide resistance. This mechanism could be responsible for the observed enhanced toxicity of deltamethrin when combined with copper NPs against *B. oleae* adults with reduced sensitivity to deltamethrin, although further studies would be needed to validate such a hypothesis. Another study has reported an interference of PVP-coated Ag-NPs with detoxifying enzymes such as carboxylesterases (CarE), glucosidases (Glu) and glutathione S-transferases (GST) in the larval gut of *Spodoptera litura* F. and *Achaea janata* L causing an excessive oxidative stress.[37] In contrast, the addition of deltamethrin to $Cu(OH)_2$ alleviated the high toxicity caused by the fungicide to *B. oleae* causing lower mortality than each of the individual treatments. This could be due to the formation of a copper-deltamethrin conjugation, preventing both copper ions and the insecticide to reach their targets or inactivating each other.

Subsequently, the effect of copper nanoparticles, $Cu(OH)_2$, deltamethrin and their combinations on oviposition and reproduction parameters was assessed under laboratory conditions. Deltamethrin and CuO-NPs individual treatments induced the number of *B. oleae* oviposition attempts as indicated by the significantly higher number of stings per olive fruit recorded in comparison with the control treatment. This resulted in an elevated number of produced pupas per olive fruit by both treatments compared with the control. Furthermore, deltamethrin treatment induced the birth of female over male offspring. Similarly, Mauduit et al.[38] has reported a stimulation of reproductive traits of the beneficial insect European earwig (*Forficula auricularia* L.) caused by sublethal doses of deltamethrin. On the contrary, all the other *B. oleae* treatments (including the ones containing deltamethrin) did not differ significantly in terms of oviposition attempts and number of produced pupas compared with the control treatment. The total number of offspring per female was also unaffected by almost all treatments when compared with the control. The only exceptions were recorded in the case of $Cu(OH)_2$ and the Cu-NPs + deltamethrin combination which resulted in a significant reduction in the number of total offspring compared with the control. Apparently, copper hydroxide treatment resulted in less total number of offspring due to the high mortality rates caused by this fungicide. Nevertheless, $Cu(OH)_2$ has demonstrated oviposition inhibitory properties against *B. oleae* in a number of laboratory and field studies.[9,10,39,40] This was

attributed both to the direct and indirect oviposition-deterring properties of copper hydroxide due to the antibacterial effect of the compound against olive fruit bacteria producing oviposition-attractive compounds. [39,40]

Recent reports attribute the toxic effect of copper against *B. oleae* to the symbiosis disruption of the insect with the gut endosymbiotic bacterium *Candidatus Erwinia dacicola* which is essential for successful larval development in unripe olive fruits.[12,41] In the case of copper NPs, no report is available about a similar effect of any nanoparticle on the above olive fly endosymbiont although reports exist on Ag-NPs reducing diversity of midgut flora in treated larvae of *Drosophila melanogaster*.[19] In the present study, both bulk and nanosized copper reduced the abundance of the gut endosymbiotic bacterium, an effect which was more profound in the head-thorax part of *B. oleae*. This could be due to translocation limitations of copper compounds between the thorax and abdomen. It must be pointed out that mixtures containing deltamethrin and both Cu and CuO nanoparticles resulted in a reduced number of pupae and total number of offspring compared to the individual treatments alone. This effect was more profound in the case of Cu-NPs which were more toxic than CuO-NPs. However, this reproduction-affecting additive effect of nano copper compounds with deltamethrin was not observed is terms of toxicity to the endosymbiotic bacterium *Candidatus Erwinia dacicola.* On the contrary, nanocopper-deltamethrin combinations negated the inhibitory effect of copper NPs against the endosymbiont. This suggests that the observed reproduction properties-related reduction caused by the combination of copper NPs with deltamethrin is not associated with the abundance of the endosymbiotic bacterium *Candidatus Erwinia dacicola* in the gut of *B.oleae*. Furthermore, the fact that the combination of $Cu(OH)_2$ with deltamethrin had an antagonistic effect resulting in an increase both in the number of pupas and total number of offspring compared to the individual treatments indicates differences in the mode of genotoxic action between nano and bulk counterparts.

Copper NPs, when combined with deltamethrin, were reduced in size (Table 1, Figure 2) and thus could be more effective both in terms of acute and reproductive activity. Similar results, as far as toxicity is concerned, have been reported in the case of a boscalid + ZnO-NPs combination which resulted in a reduction in NPs size producing a synergistic effect between the two compounds against the plant pathogenic fungus *Alternaria alternata*.[35] However, this enhanced toxicity of NP-copper/ deltamethin combination was reversed in the case of the endosymbiotic bacterium *Candidatus Erwinia dacicola* which indicates that the enhanced toxicity towards *B. oleae* was due to an increased toxicity of deltamethrin, probably caused by the deactivation of detoxifying enzymes by nanocopper. In any case, the combination of deltamethrin with copper NPs can enhance its toxicity against *B. oleae*, a fact especially important in the case of olive fruit fly populations resistant to this insecticide and reduce insect populations even in sub-lethal doses.

While copper NPs show great potential against *B. oleae*, they may pose both known and unknown health and environmental risks. Their unique properties that enable them to have increased effectiveness compared to their bulk counterparts could also lead to toxic effects towards non-target organisms. Metal nanoparticles -especially smaller ones- have been shown

to possess cell membrane-penetration properties which could extend to plant or even human cells.[22] Furthermore, since their impact on biological systems is not yet fully understood, ecotoxicity tests commonly used for their bulk counterparts may not be sufficient to evaluate their toxicity. This also applies for their combinations with pesticides which may cause an additional toxicity effect to non-target organisms.[22] Thus, before copper and other metallic NPs can be applied in the field alone or in combination with agrochemicals in large-scale applications, dose optimization and several safety issues concerning their environmental fate and impact on non-target organisms should be addressed by ecotoxicological studies.

Concluding, copper containing nanoparticles were toxic to olive fruit fly *B. oleae* populations with reduced sensitivity to the pyrethroid insecticide deltamethrin, although less toxic than their bulk counterpart $Cu(OH)_2$. When combined with deltamethrin, NPs enhanced the insecticide's toxic effect against *B. oleae* especially in the case of Cu-NPs. This was also the case for the effect of the combinations in reproductive properties where nano-deltamethrin mixtures caused a reduction in the number of pupas and total number of offspring produced compared with the individual treatments. The opposite/antagonistic effect was observed in the case of the $Cu(OH)_2$ + deltamethrin combination indicating differences on the mode of action between NPs and their bulk counterpart. Furthermore, both bulk and nanosized copper negatively affected the abundance of the *B. oleae* endosymbiotic bacterium *Candidatus Erwinia dacicola* in olive fruit fly adults which could lead to reduced survival of larvae feeding on olive fruit. These results point out a promising potential for copper NPs to be used in combination with deltamethrin in IPM programs for the control of *B. oleae* population in bait-based sprays and as an anti-resistance management tool, reducing the environmental footprint of synthetic pesticides by requiring lower doses for the control of the pest.

8.6 References

1. Pandey S, Giri K, Kumar R, Mishra, G and Raja Rishi R, Nanopesticides: Opportunities in Crop Protection and Associated Environmental Risks. *Proc. Natl. Acad. Sci., India, Sect. B* **88**:1287-1308 (2018).
2. Malandrakis A, Daskalaki E.R, Skiada V, Papadopoulou KK and Kavroulakis N, A *Fusarium solani* endophyte vs fungicides: compatibility in a *Fusarium oxysporum* f.sp. *Radicis-Lycopersici* – tomato pathosystem. *Fungal Biol* **122**: 1215–1221 (2018).
3. Malandrakis AA, Kavroulakis N and Chrysikopoulos CV, Use of silver nanoparticles to counter fungicide-resistance in *Monilinia fructicola. Sci. Total Environ* DOI: 10.1016/j.scitotenv.2020.141287 (2020).
4. Varikou K, Garantonakis N and Malandrakis A, A novel bioassay for evaluating insecticide sensitivity: a case study of *Calocoris trivialis* Costa (Hemiptera: Miridae) in olives. *Ecotoxicology* **30**:441–447 (2021).
5. Manousis T and Moore NF, Control of *Dacus oleae* a major pest of olives. *Insect Sci. Appl.***8**:1–9 (1987).
6. Tamendjari A, Sahnoune M, Mettouchi S and Angerosa F, Effect of *Bactrocera oleae* infestation on the olive oil quality of three Algerian varieties: Chemlal, Azzeradj and Bouchouk. *Riv. Ital. Sostanze Gr.* **86**:103–111 (2009).
7. Varikou K, Garantonakis N, Birouraki A, Ioannou A and Kapogia E, Improvement of bait sprays for the control of *Bactrocera oleae* (Diptera: Tephritidae). *Crop Prot.* **81**:1–8 (2016).
8. Chaniotakis N, Protection of olive orchards inorganic farming: National Conference on Organic Farming, Kalamata, Greece, p. 135 (1994).
9. Gonçalves F and Torres L, Effect of copper oxychloride on the olive infestation by *Bactrocera oleae* in northeastern Portugal. *Acta Hortic*. **949**:333–340 (2012).
10. Prophetou-Athanasiadou DA, Tzanakakis ME, Myroyannis D and Sakas G, Deterrence of oviposition in *Dacus oleae* by copper hydroxide. *Entomol. Exp. Appl.* **61**:1–5 (1991).
11. Belcari A, Sacchetti P, Rosi MC and Del Pianta R, Control of the olive fly *(Bactrocera oleae*) through the use of copper products in central Italy. *IOBC/WPRS Bull.***28**:45–48 **(**2005).
12. Sinno M, Bézier A, Vinale F, Giron D, Laudonia S, Garonna AP and Pennacchio F, Symbiosis disruption in the olive fruit fly, *Bactrocera oleae* (Rossi), as a potential tool for sustainable control. *Pest Manage. Sci.* **76**:3199–3207 (2020).
13. Pavlidi N, Kampouraki A, Tseliou V, Wybouw N, Dermauw W, Roditakis E et al. Molecular characterization of pyrethroid resistance in the olive fruit fly *Bactrocera oleae*. *Pestic. Biochem. Phys.* **148**:1–7 (2018).
14. Margaritopoulos JT, Skavdis G, Kalogiannis N, Nikou D, Morou E, Skouras PJ, Tsitsipis JA and Vontas J. Efficacy of the pyrethroid alpha-cypermethrin against *Bactrocera oleae* populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag. Sci.* **64:**900–908 (2008).
15. Roditakis E, Grispou M, Staurakaki M, Roditakis N.E, Livadaras G, Vontas J., Nauen R, Godley N, Filippou E and Manousakis E, A novel approach to evaluate efficacy of baited insecticide applications for the olive fruit fly control. *IOBC/wprs WG Integrated Protection of Olive Crops* Kalamata, Greecepp. 65–66, (2015).
16. Vontas JG, Small GJ and Hemingway J, Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens. Biochem. J.* **357:**65–72 (2001).
17. Kah M, Kookana RS, Gogos A and Bucheli TD, A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. *Nat. Nanotechnol.* **13**:677–684 (2018).

18. Sun Q, Li J and Le T, Zinc oxide nanoparticles as a novel class of antifungal agents: current advances and future perspectives. *J. Agric. Food Chem.* **66:**11209–11220 (2018).
19. Han X, Geller B, Moniz K, Das P, Chippindale AK and Walker VK, Monitoring the developmental impact of copper and silver nanoparticle exposure in *Drosophila* and their microbiomes. *Sci. Total Environ.* **487**:822–829 (2014).
20. Alaraby M, Hernández A and Marcos R, New insights in the acute toxic/genotoxic effects of CuO Nanoparticles in the *in vivo* Drosophila model. *Nanotoxicolog* **10**:749–760 (2016).
21. Budiyanti DS, Moeller ME and Thit A, Influence of copper treatment on bioaccumulation, survival, behaviour, and fecundity in the fruit fly *Drosophila melanogaster*: toxicity of copper oxide nanoparticles differs from dissolved copper. *Environ. Toxicol. Pharmacol.* DOI: 10.1016/j.etap.2022.103852 (2022).
22. Malandrakis AA, Kavroulakis N and Chrysikopoulos CV, Metal nanoparticles against fungicide resistance: alternatives or partners? *Pest Manage. Sci.* **78**:3953–3956 (2022).
23. Tzanakakis ME, Tsitsipis JA and Steiner LF, Egg production of olive fruit fly fed solids or liquids containing protein hydrolysate. *J. Econ. Entomol.* **60**: 352–354 (1967).
24. Estes AM, Hearn DJ, Bronstein JL and Pierson EA, The olive fly endosymbiont, '*Candidatus* Erwinia dacicola', switches from an intracellular existence to an extracellular existence during host insect development. *Appl Environ Microbiol*.**75**:7097–7106 (2009).
25. Estes AM, Hearn DJ, Burrack HJ, Rempoulakis P and Pierson EA, Prevalence of '*Candidatus* Erwinia dacicola' in wild and laboratory olive fruit fly populations and across developmental stages. *Environ Entomol*. **41***:* 265–274 (2012).
26. Sinno M, Bézier A, Vinale F, Giron D, Laudonia S, Garonna AP and Pennacchio F, Symbiosis disruption in the olive fruit fly, *Bactrocera oleae* (Rossi), as a potential tool for sustainable control. *Pest Manage. Sci* **76**:3199-3207 (2020).
27. Akmoutsou P, Mademtzoglou D, Nakou I, Onoufriadis A, Papadopoulou X, Kounatidis I, Frantzios G, Papadakis G, Vasiliadis K, Papadopoulos NT and Mavragani-Tsipidou P, Evaluation of toxicity and genotoxic effects of spinosad and deltamethrin in *Drosophila melanogaster* and *Bactrocera oleae. Pest Manage. Sci* **67**:1534-1540 (2011).
28. Athanassiou CG, Kavallieratos NG, Benelli G, Losic D, Usha Rani P and Desneux N, Nanoparticles for pest control: current status and future perspectives. *J Pest Sci* DOI: 10.1007/s10340-017-0898-0 (2018).
29. Benelli G, Mode of action of nanoparticles against insects. *Environ Sci Pollut Res Int.* **25** :12329-12341(2018).
30. Vivekanandhan P, Swathy K, Thomas A, Kweka EJ, Rahman A, Pittarate S and Krutmuang P. Insecticidal efficacy of microbial-mediated synthesized copper nano-pesticide against insect pests and non-target organisms. *Int J Environ Res Public Health* DOI: 10.3390/ijerph181910536 (2021).
31. Badawy AA, Abdelfattah NAH, Salem S, Awad MF and Fouda A, Efficacy assessment of biosynthesized copper oxide nanoparticles (Cuo-NPs) on stored grain insects and their impacts on morphological and physiological traits of wheat (*Triticum aestivum* l.) plant. *Biology* **10:**art. no. 233, DOI: 10.3390/biology10030233 (2021).
32. Biradar W, Nadagouda S, Aralimarad P and Hiregoudar S, Entomotoxic effect of green nanoparticle an alternate strategy for stored grain pest management. *Int J Trop Insect Sci.* **41**:2829-2840 (2021).
33. Malandrakis AA, Kavroulakis N and Chrysikopoulos CV, Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens. *Sci Total Environ.***670:**292-299 (2019).

34. Malandrakis AA, Kavroulakis N and Chrysikopoulos CV, Copper nanoparticles against benzimidazole-resistant *Monilinia fructicola* field isolates *Pestic Biochem Physiol.* DOI: 10.1016/j.pestbp.2021.104796 (2021).
35. Malandrakis AA, Kavroulakis N and Chrysikopoulos CV, Zinc nanoparticles: Mode of action and efficacy against boscalid-resistant *Alternaria alternata* isolates. *Sci Total Environ.* DOI: 10.1016/j.scitotenv.2022.154638 (2022).
36. Zeng Q, Yu C, Chang X, Wan Y, Ba Y, Li C, Lv H, Guo Z, Cai T, Ren Z, Qin Y, Zhang Y, Ma K, Li J, He S and Wan H, CeO_2 nanohybrid as a synergist for insecticide resistance management. *Chem Eng J* DOI: 10.1016/j.cej.2022.137074 (2022).
37. Yasur J and Usha Rani P, Lepidopteran insect susceptibility to silver nanoparticles and measurement of changes in their growth, development and physiology. *Chemosphere* **124**:92-102 (2015).
38. Mauduit E, Lécureuil C and Meunier J, Sublethal exposure to deltamethrin stimulates reproduction and has limited effects on post-hatching maternal care in the European earwig. *Environ Sci Pollut Res Int.* **28**: 39501-39512 (2021).
39. Checchia I, Perin C, Mori N and Mazzon L, Oviposition deterrent activity of fungicides and low-risk substances for the integrated management of the olive fruit fly *Bactrocera oleae* (Diptera, Tephritidae). *Insects* DOI: 10.3390/insects13040363 (2022).
40. Mojdehi MRA, Keyhanian AA and Rafiei B, Application of oviposition deterrent compounds for the control of olive fruit fly, *Bactrocera oleae* Rossi. (Dip. Tephritidae) control. *Int J Trop Insect Sci.* **42**:63-70 (2022).
41. Bigiotti G. Pastorelli R, Belcari A and Sacchetti P, Symbiosis interruption in the olive fly: Effect of copper and propolis on *Candidatus* Erwinia *dacicola. J Appl Entomol.* **143**:357-364 (2019).

9 General discussion and concluding remarks

9.1. General discussion

Modern agriculture faces significant challenges in achieving sustainable food production while minimizing the depletion of natural resources and environmental pollution. Recent advancements in technology, particularly in nanotechnology, present promising solutions to these issues. Research over the past decade highlights the potential of nanotechnology in various agricultural applications, such as nano-fertilizers, which enhance nutrient delivery through targeted release, thereby reducing waste and environmental impact. Additionally, nanotechnology-assisted plant protection products can improve crop yields while lessening the ecological footprint of traditional agrochemicals. The integration of nanosensors in precision farming allows for real-time monitoring of crop health, soil conditions, and environmental factors, leading to more informed agricultural practices. Furthermore, nanotechnology can aid in developing crops that are more resilient to extreme weather conditions caused by climate change. Overall, the application of nanotechnology not only boosts productivity but also promotes sustainability by optimizing resource use and reducing environmental harm, positioning it as a crucial element in the future of sustainable agriculture.

A major challenge chemical control faces is the loss of available conventional active ingredients due to strict environmental safety regulations which, combined with the loss of fungicide efficacy due to resistance development, constitute major problems of contemporary crop protection. Metal containing nanoparticles appear to have all the credentials to be the next-generation, eco-compatible fungicide alternatives and a valuable anti-resistance management tool. Could the introduction of metal NPs as nano-fungicides be the answer to both reducing environmental footprint of xenobiotics and dealing with fungicide resistance? The potential of metal nanoparticles to be utilized as nano-fungicides both as alternative to conventional fungicides or/and as partners in combating fungicide resistance is discussed in terms of effectiveness, potential antimicrobial mechanisms as well as synergy profiles with conventional fungicides. However, their "golden" potential to be used both as alternatives and partners of conventional fungicides to combat resistance and reduce environmental pollution, is challenged by undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity, constituting risks that should be considered before their commercial introduction as nano-pesticides at a large scale.

Under this light the main objectives of the present thesis were a) to study the effectiveness of metallic nanoparticles (MNPs) against phytopathogenic fungi both *in vitro* and *in vivo*, b) to investigate the mechanism of fungitoxic action of MNPs at the biochemical level, c) to explore the potential use of MNPs alone or in combination with conventional plant protection products to combat resistant phytopathogenic strains, as well as to reduce recommended doses and the environmental footprint of pesticides, d) to examine the potential use of MNPs against populations of the olive fruit fly, resistant to insecticides, and their impact

on the endosymbiotic microorganisms of the fly's gut flora, and e) to investigate the toxicological effects of MNPs on the growth and physiological parameters of tomato plants, as well as on the symbiotic organisms of their root system.

Metallic nanoparticles as fungicide alternatives

The effectiveness of copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc (ZnO-NPs) containing nanoparticles (NPs) was assessed *in vitro* against seven fungal species, known to cause foliar and soil-borne diseases. Mycelial growth assays revealed that Cu-NPs with mean inhibition rates, EC_{50}, ranging between 162 and 310 μg/mL were the most effective among the NPs tested in inhibiting fungal growth, followed by ZnO-NPs with EC_{50} ranging between 235 and 848 μg/mL. All fungal species were practically insensitive to CuO-NPs and Ag-NPs except for *B. cinerea*, which was equally sensitive to Ag-NPs and Cu-NPs (EC_{50} =307 μg/mL). Cu-NPs were more fungitoxic in terms of mycelial growth, to almost all species tested, than a reference protective fungicide containing $Cu(OH)_2$. Fungitoxicity experiments with the NPs tested and bulk size reagents containing the respective metals revealed that ZnO-NPs were more toxic to all fungal species tested than $ZnSO_4$, whereas Cu-NPs were more fungitoxic than $CuSO_4$ in all cases, except for *B. cinerea*, *A. alternata* and *M. fructicola*. The existence of a positive correlation between Cu-NPs and CuO-NPs toxicity and, at the same time, the absence of any correlation between NPs tested and their respective bulk metal counterparts indicated potential differences in the mode of action between bulk and nanosized antifungal ingredients. Although there was considerable variation between fungal species, all NPs were generally 10 to 100 fold more fungitoxic to spores than hyphae and in the majority of cases more effective than $Cu(OH)_2$, as revealed by colony formation bioassays. NPs significantly suppressed grey mold symptoms on plum fruit, especially Ag-NPs, which completely inhibited disease development. Consequently, tested NPs have the potential to be used as protective antifungal agents. Following this initial screening, selected MNPs were tested against fungicide-resistant fungal pathogens.

MNPs combating fungicide resistance

Combating drug-resistance is a daunting task, especially due to the shortage of available drug alternatives with multisite modes of action. In this study, the potential of MNPs to be used against fungicide-resistant fungal pathogens, their potential synergy with conventional fungicides as well as the underlying mechanism were investigated using four pathogen-NP cases. In the first case, the ability of copper nanoparticles (Cu-NPs) to suppress sensitive or resistant to fungicides *Botrytis cinerea* isolates, alone or in combination with conventional fungicides, was tested *in vitro* and *in vivo.* Sensitivity screening *in vitro* revealed two fungicide resistance phenotypes, resulting from target site mutations. DNA sequencing revealed three *B. cinerea* isolates highly resistant to benzimidazoles (BEN-R), thiophanare methyl (TM), and carbendazim, bearing the E198A resistance mutation in the *β*-tubulin gene, and four isolates highly resistant to the QoI pyraclostrobin (PYR-R) with a G143A mutation in the *cytb* gene. Cu-NPs were equally effective against sensitive and resistant isolates. An additive/ synergistic

effect was observed between Cu-NPs and TM in the case of BEN-S isolates both *in vitro* and when applied in apple fruit. A positive correlation was observed between TM and TM+Cu-NPs treatments, suggesting that an increased TM availability in the target site could be related with the observed additive/synergistic action. No correlation between $Cu(OH)_2$ and Cu-NPs sensitivity was found, indicating that different mechanisms govern the fungitoxic activity between nano and bulk counterparts. A synergistic profile was observed between Cu-NPs and fluazinam (FM) - an oxidative phosphorylation inhibitor - in all isolates regardless of resistance phenotype, suggesting that ATP metabolism could be involved in the mode of action of Cu-NPs. Furthermore, the observed cross sensitivity and antagonistic action between Cu-NPs and NaCl also provided evidence for copper ions contribution to the fungitoxic action of Cu-NPs. The results suggested that Cu-NPs in combination with conventional fungicides can provide the means for an environmentally safe, sustainable resistance management strategy by reducing fungicide use and combating resistance against *B. cinerea*.

Similar results were observed in the case of Cu-NPs, which could suppress mycelial growth in both sensitive (BEN-S) and resistant (BEN-R) *M. fructicola* isolates harbouring the E198A benzimidazole resistance mutation, more effectively than copper oxide NPs (CuO-NPs) and $Cu(OH)_2$. A significant synergy of Cu-NPs with 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 indicates 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 used against *M. fructicola*.

To test whether the effectiveness of MNPs could be affected by the metallic basis of NPs, Ag-NPs were tested against the above *M. fructicola* isolates in terms of effectiveness and fungicide resistance management both *in vitro* and *in vivo.* 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. Contrary to the profile observed in the case of Cu-NPs, the above 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 Ag nanoparticles and their ionic counterparts. Similarly to Cu-NPs, 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 resistance and at the same time reduce the environmental impact of synthetic fungicides by reducing doses needed for the control of the pathogen.

Finally, the antifungal potential of ZnO-NPs against *Alternaria alternata* isolates with reduced sensitivity to the succinate dehydrogenase inhibitor (SDHI) boscalid, resulting from target site modifications, was evaluated *in vitro* and *in vivo.* ZnO-NPs could effectively inhibit mycelial growth in a dose-dependent way in both boscalid (BOSC) sensitive (BOSC-S) and resistant (BOSC-R) isolates. The fungitoxic effect of ZnO-NPs against the pathogen was significantly enhanced when combined with boscalid compared to the individual treatments in all phenotype cases (BOSC-S/R) both *in vitro* and *in vivo*. Fungitoxic effect of ZnO-NPs could be –at least partly– attributed to zinc ion release as indicated by the positive correlation between sensitivities to the nanoparticles and their ionic counterpart $ZnSO_4$ and the alleviation of the ZnO-NPs fungitoxic action in the presence of the strong chelating agent EDTA. The superior effectiveness of ZnO-NPs against *A. alternata* compared to $ZnSO_4$ could be due to nanoparticle properties interfering with cellular ion homeostasis mechanisms. The observed additive action of the oxidative phosphorylation-uncoupler fluazinam (FM) against all phenotypes indicates a possible role of ATP-dependent ion efflux mechanism in the mode of action of ZnO-NPs. A potential role of ROS production in the fungitoxic action of ZnO-NPs was evident by the additive/synergistic action of Salicylhydroxamate (SHAM), which blocks the alternative oxidase antioxidant action. Mixture of ZnO-NPs and boscalid resulting in a "capping" effect for the nanoparticles significantly reducing their mean size probably accounted for the synergistic effect of the mixture against both sensitive and resistant *A. alternata* isolates. Summarizing, results indicated that ZnO-NPs can be effectively used against *A. alternata* while combination with boscalid optimizes their performance and provides an effective tool for combating SDHI-resistance and reducing the environmental fingerprint of synthetic fungicides by reducing recommended doses for the control of the pathogen.

All the above cases indicate that metal NPs can combat fungicide resistance especially when used in combination with the same fungicide that the pathogen has developed resistance to. This extraordinary resistance-negating effect can be attributed to a number of mechanisms such as increased bioavailability of the fungicide caused by a carrier effect of the NP, chemo sensitization of the fungal cell caused by the NPs or an indirect increased toxicity of the NPs due to a "capping" effect of the fungicide which results in smaller, and thus more toxic, NPs. In any case, besides managing fungicide-resistance, the combination of MNPs with fungicides was shown to drastically reduce the recommended fungicide doses which can contribute to the mitigation of the problem of environmental contamination caused by the excessive use of agrochemicals.

MNPs against insecticide-resistant *Bactrocera oleae* populations

Besides their antimicrobial properties, MNPs such as those containing silver, zinc oxide, titanium oxide and copper, have exhibited a great potential as pesticide alternatives against arthropod pests of economic importance, including flies, moths, mosquitos, lice and ticks. Furthermore, silver and copper nanoparticles have been shown to exert acute toxic effects on various and reproduction-related as well as microbiome-alternating effects on the *Drosophila melanogaster* fruit fly in ways that differ from those of their bulk counterparts. However, to date, no study is available on the effect of copper-containing NPs on *Bactrocera oleae,* which is the most economically important olive pest worldwide and faces significant control challenges due to the development of insecticide resistance. Under this light, the potential of copper nanoparticles (NPs) to be used as an alternative control strategy against olive fruit flies (*B. oleae*) with reduced sensitivity to the pyrethroid deltamethrin and the impact of both nanosized and bulk copper [$Cu(OH)_2$] on the insect's reproductive and endosymbiotic parameters were investigated. The application of nanosized and bulk copper applied by feeding resulted in significant levels of adult mortality, comparable to or surpassing those achieved with deltamethrin at recommended doses. Combinations of Cu-NPs or CuO-NPs with deltamethrin significantly enhanced the insecticide's efficacy against *B. oleae* adults. When combined with deltamethrin, Cu-NPs significantly reduced the mean total number of offspring compared with the control, and the number of stings, pupae, female and total number of offspring compared with the insecticide alone. Both bulk and nanosized copper negatively affected the abundance of the endosymbiotic bacterium *Candidatus Erwinia dacicola* which is crucial for the survival of *B. oleae* larvae. These results demonstrate that Cu-NPs can aid the control of *B. oleae* both by reducing larval survival and by enhancing deltamethrin performance in terms of toxicity and reduced fecundity, providing an effective anti-resistance tool and minimizing the environmental footprint of synthetic pesticides by reducing the required doses for the control of the pest.

Toxicological effects of MNPs on tomato and its root-symbiotic organisms

Plants, being an essential part of all ecosystems, are expected to interact directly or indirectly with nanoparticles, that could potentially inflict toxicity, accumulate via uptake or disturb interactions of plants with beneficial/symbiotic organisms. A number of studies evaluating phytotoxicity of metal NPs have been conducted reporting adverse effects on various aspects of plant growth and physiology in numerous plant species although reports are often conflicting highlighting that toxicity threshold of NPs towards plants is species dependent and each case should be evaluated separately.

Tomato is a vegetable crop with great popularity and economic importance worldwide, and although studies evaluating phytotoxicity of tomato caused by silver, zinc oxide, titanium oxide, copper, and ferric NPs are available, their toxicity compared with their bulk counterparts, as well as their interaction with tomato's endophytic symbiotic microorganisms is limited. Therefore, this study investigated the effect of copper (Cu-NPs, CuO-NPs), silver

(Ag-NPs) and zinc oxide (ZnO-NPs) nanoparticles (NPs) on plant growth, physiological properties of tomato plants and their symbiotic relationships with the endophytic *Fusarium solani* FsK strain. Fungitoxicity tests revealed that the FsK strain was significantly more sensitive to Cu-NPs and ZnO-NPs than CuO-NPs and Ag-NPs both in terms of mycelial growth and spore germination. All NPs were more toxic to FsK compared to their bulk counterparts except for $AgNO_3$, which was 8 to 9-fold more toxic than AgNPs. Apart from $AgNO_3$, NPs and bulk counterparts did not affect the number of germinated tomato seeds even in higher concentrations, while root length was significantly reduced in a dose dependent way in most cases. Dry weight of tomato plants was also significantly reduced upon treatment with NPs and counterparts with most pronounced effects in the cases of $AgNO_3$, Cu-NPs, ZnO-NPs, and $ZnSO_4$. Root and shoot length of grown tomato plants was also affected by treatments while differences between NPs and bulk counterparts varied. A marked oxidative stress response was recorded in all cases of NPs/bulk counterparts as indicated by increased MDA and H_2O_2 levels of treated plants. Treated plants had significantly reduced chlorophyl-a and carotenoid levels compared to the untreated control. NPs and counterparts did not affect FsK colonization of roots indicating a possible shielding effect of tomato plants once the endophyte was established inside the roots. Vice versa, a possible alleviation of CuO-NPs, ZnO-NPs, and $ZnSO_4$ toxicity was observed in the presence of FsK inside tomato roots in terms of plant dry weight. The results suggest that phytotoxicity of NPs in tomato treated plants should be considered before application and while both FsK and tomato are sensitive to NPs, their reciprocal benefits may extent to resistance towards these toxic agents.

9.2. Concluding remarks and future directions

Certain limitations of pesticides such as high environmental footprints, side effects to non-target organisms, increasing costs on RnD and registration of new active ingredients and the loss of their effectiveness due to resistance development, have rendered the development of eco-compatible alternatives a necessity. Nanotechnology could provide an answer to this challenge by introducing nanoparticles (NPs) acting nano-pesticides or as carriers for relevant active ingredients. Metal NPs used in this study were demonstrated to possess a great potential as eco-compatible, alternative pesticides with unique properties and effectiveness. Results also highlighted their potential for addressing the crucial agricultural issue of pesticide resistance. Specifically, the combination of MNPs with pesticides led to an increase in the effectiveness of the latter against both sensitive and resistant strains, enabling effective management of the resistance phenomenon while simultaneously allowing for reduced dosages and consequently minimizing the environmental footprint of these drugs. Driven by the significance of this finding, we were motivated and filed a patent titled "Use of metallic nanoparticles in plant protection formulations" (E-filing case number: 2418-0004776301). Future efforts should focus on the production of MNPs via green synthetic routes, which are more eco-friendly and can be fine-tuned to produce NPs with specific desired properties such as smaller-size, increased effectiveness and targeted drug delivery.

The effect of MNPs on the agroecosystem were investigated in terms of their phytotoxicity on tomato plants and a beneficial endosymbiotic fugus (FSK) that protects the plant from infections and various stresses. Results study revealed a differential response to various NPs (Cu-NPs, CuO-NPs, Ag-NPs and ZnO-NPs) and bulk counterparts, which involved reduced growth, chlorophyll-a and carotenoid contents and increased oxidative stress. These results suggested that phytotoxicity of NPs in tomato treated plants should be considered before application. Interestingly, FSK provided a protection against the phytotoxic effect of some NPs which could be a promising tomato protective agent against metallic NPs.

However promising as they are for crop protection, MNPs may pose both known and unknown health and environmental risks. Their very same unique properties that enable them to have increased fungitoxic action compared to their bulk counterparts could have undesirable/toxic effects towards non-target organisms. Their ability to penetrate fungal cell membranes -especially in the case of smaller NPs- could extend to plant or even human cells and cause toxic responses. Ecotoxicity tests typically used for their bulk counterparts may prove insufficient to evaluate NP toxicity since their effect on biological system is not yet completely understood. Phytotoxicity, human and environmental safety issues should be systematically investigated before their commercial release for use as nano-pesticides. The same applies for their combinations with fungicides which may result in a special, combined toxicity to non-target organisms. Concluding, since their effect on biological systems is not yet completely understood, MNPs should be further studied for undesirable effects towards non-target organisms such as phytotoxicity, toxicity to humans and environmental ecotoxicity before their commercial introduction as nano-pesticides at a large scale.