Tropical Plant Pathology (2026) 51:19
https://doi.org/10.1007/s40858-026-00794-9

ORIGINAL ARTICLE

Copper resistance and hormetic-like response in *Xanthomonas euvesicatoria* pv. *perforans*

Erivaldo Laurentino da Silva[1] · Maurício Rossato[1] · Alice Maria Quezado-Duval[2] · Marisa Alvares da Silva Velloso Ferreira[1]

Received: 23 July 2025 / Accepted: 11 February 2026

Abstract
Bacterial spot (BS) is a worldwide important tomato disease caused by *Xanthomonas* species. *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*) is the most prevalent causal agent of the disease in Central Brazil. Copper resistant *Xep* isolates have been detected, which might have been driven by the intensive use of copper-based products to manage the disease. The *copLAB* gene cluster is one of the genetic determinants of copper resistance in *Xanthomonas*. In this study, a collection of 45 Brazilian *Xep* isolates was characterized for the presence of the *copA* gene and their *in vitro* sensitivity to copper. The *copA* gene was detected by PCR in 28.8% of the isolates which showed higher minimum inhibitory concentration (MIC) values than the *copA*- isolates. Two isolates (EH 2016-08 and EH 2017-69) *copA*+ required preconditioning to express the resistant phenotype, and the same procedure resulted in an increase in copper MIC of EH 2020-12 (*copA*-). Growth stimulation at copper concentrations below the MIC was observed for R (EH 2017-27) and S (EH 2020-12) isolates in a hormetic-like effect, a dose response phenomenon characterized by low-dose stimulation and high-dose inhibition. This effect seemed to be independent of *copA* presence. Increase in biofilm production was observed for R and S isolates by subinhibitory doses of copper, but there was no stimulatory effect on virulence of R or S isolates on tomato plants. To our knowledge this is the first report of a hormetic-like effect in copper-sensitive and resistant isolates of *Xep*.

Keywords Subinhibitory-dose · Tomato · Stimulation · Bacterial spot · *Solanum lycopersicum*

Introduction

Bacterial spot (BS) is a disease of economic relevance that affects tomato (*Solanum lycopersicum*) and pepper/sweet pepper (*Capsicum* spp.). It is caused by three species/four variants, currently classified as *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*), *X. euvesicatoria* pv. *euvesicatoria* (*Xee*), *X. vesicatoria* (*Xv*), and *X. hortorum* pv. *gardneri* (*Xhg*) (Jones et al. 2004; Barak et al. 2016; Constantin et al. 2016; Morinière et al. 2020; Osdaghi et al. 2021). These species are recommended for regulation as quarantine pests A2, present but not widely distributed in the European and Mediterranean region (EPPO 2023). In Brazil, the two most prevalent species on tomato crops are *Xhg* and *Xep*, with the latter occurring predominantly in Goiás and Distrito Federal in Central Brazil (Araújo et al. 2017). Since its discovery in Florida in 1991, *Xep* has spread rapidly throughout the world, probably favored by pathogen movement with seeds and planting materials (Timilsina et al. 2025). BS development is favored by temperatures ranging between 24–30°C and high humidity (Strayer-Scherer et al. 2019). The inoculum may be present in crop residues for short periods, weeds or introduced into pathogen-free areas through seeds and seedlings. Therefore, the use of healthy propagative material and preventive applications of copper-based products are important allies in disease management (Osdaghi et al. 2021).

Copper-based bactericides are widely used in BS management. Copper performs different functions within the cell by complexing proteins, interfering in metabolism, electron transport, protein configuration, oxidative stress response, transcriptional regulation, and cellular signaling (Sharma et al. 2019). If present in high concentrations, it

✉ Erivaldo Laurentino da Silva
erivaldolaurentino2@gmail.com

[1] Departamento de Fitopatologia, Universidade de Brasília, Brasília, Distrito Federal 70910-900, Brasil

[2] Laboratório de Fitopatologia, Embrapa Hortaliças, Gama, Distrito Federal 70275-970, Brasil

Published online: 31 March 2026

can lead to the production of reactive oxygen species, causing disruption of the plasma membrane, protein denaturation, and damage to genetic material (Chillappagari et al. 2010; Yue et al. 2023). Resistance to this chemical was first detected in *X. vesicatoria* by Marco and Stall (1983) and later in *Pseudomonas* and other *Xanthomonas* species as well (Griffin et al. 2017; Behlau et al. 2020). The plasmid-borne gene cluster *copLAB,* present in several *Xanthomonas* species around the world, is involved in copper resistance (Cu^R) and is horizontally transferred (Behlau et al. 2013, 2017a, b, 2020). While *copL* protein regulates the expression of *copA* and *copB*, these two latter genes encode copper-binding proteins which sequestrate copper ions thus protecting the bacterial cells from its toxic effects (Voloudakis et al. 2005; Marin et al. 2019). A second cluster of plasmid-borne genes has been identified in *Xanthomonas* and *Pseudomonas* strains*,* named *copABCD,* encoding a set of periplasmic, outer or inner membrane proteins. Besides resistance mechanisms, bacteria also rely on homeostasis to regulate the amount of copper within the cells. Copper homeostasis genes *cohL*, *cohA* and *cohB* are located on the chromosome, and are homologous to the copper resistance genes *copL*, *copA*, and *copB* present on plasmids (Behlau et al. 2011). Bibi et al (2023) characterized a non-plasmid borne copper resistance genomic island which was located within the chromosome of several *Xep* strains. Genes in the island were associated with mobility, phage-related genes and transposase, suggesting that these genes may present two modes of horizontal transfer. A global genomic analysis revealed that chromosomal Cu^R was more prevalent, while plasmid-borne resistance conferred greater copper tolerance (Kaur et al. 2024). Teixeira et al. (2008) showed that the *copA* gene has a determinant role in copper resistance of *X. citri* pv. *citri*, since the inactivation of this gene leads to copper sensitivity. In *X. campestris* pv. *campestris*, the *copA* gene encodes a copper oxidase protein located in the periplasm, being more determinant for copper resistance than *copL* and *copB* genes (Hsiao et al. 2011). However, Marin et al. (2019) pointed out that these genes could have been mistakenly characterized as copper resistance genes due to their homology to the homeostasis genes *cohLAB*.

Plant pathogenic bacteria can be exposed to antibiotics at subinhibitory doses either directly or indirectly. Exposure to a subinhibitory dose can occur due to inappropriate application techniques, intentional dose reduction, decreased application frequency, or when the substance is washed off from the leaves, resulting in reduced doses reaching the target pathogen (Silva et al. 2018). This exposure may lead to a phenomenon called hormesis, which is a biphasic dose response phenomenon in which low, subinhibitory doses of a stressor agent leads to a positive response (stimulation) in the organism, while high doses lead to inhibition (Calabrese And Baldwin 2002). The effect of subinhibitory doses of antibiotics has already been observed in different bacteria such as *Escherichia coli, Erwinia amylovora, Clostridium* and *Vibrio* species (Drummond et al. 2003; Deng et al. 2012; Migliore et al. 2013; Li et al. 2020; Revitt-Mills And Robinson 2020; Peng et al. 2021; Mo et al. 2023). This suggests that the hormesis effect may also occur in phytopathogenic bacteria exposed to antibiotics and/or chemicals used in plant disease management, such as copper-based bactericides. Additionally, resistant and sensitive isolates may also have distinct responses to non-damaging subinhibitory doses of copper.

Considering the impact of BS for tomato production in Brazil, and the occurrence of copper resistance in *Xep* in Brazil (Araújo et al. 2012b), the aim of this study was to characterize a collection of 45 *Xep* isolates from Brazilian tomato fields for the presence of the *copA* gene and their *in vitro* sensitivity to copper. The effect of subinhibitory doses of copper on bacterial growth and the induction of resistance in sensitive isolates was investigated. The effects of copper at low doses on biofilm production and virulence on tomato plants were evaluated for both resistant and sensitive *Xep* isolates.

Materials and methods

Bacterial isolates

Forty-five isolates of *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*) were obtained from symptomatic tomato plant samples collected at Brazilian States of Goiás (22), São Paulo (13), Minas Gerais (4), Ceará (4), Santa Catarina (1) e Paraná (1) from 2014 to 2020 and identified by multiplex PCR (Araújo et al. 2012a). Two isolates of *X. euvesicatoria* pv. *euvesicatoria* (*Xee*), from *Capsicum* sp., were used as copper-sensitive (89-P) and copper- resistant (96-P) controls. The resistant status of this strain was determined by a previous study conducted at Embrapa Hortaliças where 73 isolates from *Capsicum* were characterized for their copper sensitivity. Isolate 96-P was amongst 10% of the collection that grew at the highest copper sulfate concentration tested (200 µg/mL), while 89-P was sensitive at all concentrations (50,100 and 200 µg/mL. All strains used in this study belong to the Phytopathogenic Bacteria Collection of Embrapa Hortaliças, Brasília, Brazil.

Amplification of the *copA* gene by PCR

Genomic DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's recommendations, except that in the protein precipitation step, three rounds of centrifugation were employed instead of one time. The final DNA

concentration was determined by NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer and dilutions were performed to achieve a final concentration of 25 ng/µL for subsequent use. PCR was conducted with primers *copAF/copAR* to amplify an 870 bp region of the *copA* gene (1,872 bp), one of the genes present in the *copLAB* operon (Behlau et al. 2013). Reactions were composed by 10 µL of PCR master mix 2x (Promega), 0.2 pmol of each primer (*copAF/copAR*), 2.6 µL of ultrapure water, and 2 µL of DNA from each isolate (25 ng/µL). The amplification reactions took place in a thermocycler (Bio Rad T100TM Thermal Cycler) with the following program: initial denaturation for 5 min at 95 °C, followed by 29 cycles of 95°C/30 s, annealing at 64°C/30 s, extension at 72 °C/45 s, and a final extension at 72 °C/10 min. The amplicons were analyzed by electrophoresis at 120 V on a 1% agarose gel, with 1X TBE running buffer. GelRed® Nucleic Acid Gel Stain (Biotium®, USA) was used for gel staining, and amplicons were visualized under ultraviolet (UV) light on a transilluminator using the LPIX system (Loccus ®, Brazil).

Determination of the minimum inhibitory concentration (MIC) of copper on bacterial growth

The MIC of copper was determined according to Marin et al. (2019), adapted for 96-well microtiter plates. In sterile microtiter plates, 200 µL of molten casein-yeast extract medium (CYE) containing copper at the following concentrations: 0 (control), 150 (T1), 175 (T2), or 200 µg mL^{-1} of Cu^{2+} (T3) were added. Copper hydroxide (Supera®, Oxiquímica Agrociência, Brazil) was used as the copper source. The *Xep copA*$^-$ isolates were cultured in 523 medium for 48 h at 28 °C, while *copA*$^+$ isolates were cultured on solid CYE medium containing Cu^{2+} (20 µg mL^{-1}) for the same period, aiming to activate the genes involved in copper resistance (Basim et al. 2005). Using a UV-Vis Spectrophotometer (UV-1203, Shimadzu Corporation®), a bacterial suspension of each isolate at a concentration of ~10^8 CFU mL^{-1} in sterile distilled water (SDW) (OD600nm = 0.3) was prepared (Jones et al. 2000). An aliquot of 20 µL of each suspension was deposited onto the medium in each well, followed by incubation of the microplates for 4 days at 28°C. The isolate was classified as phenotypically sensitive to copper based on its ability to grow in any of the treatments (T1, T2, or T3), and as resistant (R) if its MIC was > T3. *Xee* 96-P and 89-P isolates were used as controls for resistance (*copA*$^+$) and sensitivity (*copA*$^-$), respectively. A second experiment was conducted on solid CYE medium in Petri dishes. Bacterial suspension was diluted in 5 mL of medium (molten state, 45 °C) to a final concentration of ~10^8 CFU mL^{-1}. The inoculated medium was poured into plates and 10 µL of a copper hydroxide solution was deposited at four perpendicular and equidistant droplets 4 cm apart. The tested concentrations were: 100, 150, 200, 250, 300 and 500 µg mL^{-1} for *copA*$^-$ isolates, and 150, 200, 250, 300, 500, 1000, and 1500 µg mL^{-1} for *copA*$^+$ isolates. SDW was used as control. The MIC was determined by the lowest concentration at which the presence of an inhibitory halo (region where the medium is completely translucent and without visible growth of bacterial colonies) was detected. For this experiment, isolates were considered resistant (R) when doses of 300 µg mL^{-1} did not inhibit growth, without the formation of an inhibitory halo, and therefore with an MIC greater than 300 µg mL^{-1}.

Effect of subinhibitory doses of copper on the growth of *Xep*

The effect of subinhibitory doses of copper on bacterial growth was assessed following Migliore et al. (2013), with adaptations for microplate assays. To investigate whether the effects of subdoses were specific at species or isolate levels and related to the presence of the *copA* gene or *in vitro* resistance to copper, two isolates each of *Xep* and *Xee* were selected: EH 2020-12 (*copA*-) and EH 2017-27 (*copA*+) for *Xep*, and 89-P (*copA*$^-$) and 96-P (*copA*$^+$) for *Xee*. In microtiter plates, 100 µL of liquid CYE medium were combined with 50 µL of bacterial suspension at 4x10^8 CFU mL^{-1} followed by the addition of 50 µL of copper hydroxide stock solution to each well resulting in a final concentration of ~10^8 CFU mL^{-1}. For *copA*$^-$ isolates, the final copper concentrations were: 0.0; 0.2 0.4; 0.8; 1.6; 3.12; 6.25; 12.5; 25; 50; 100 and 200 µg mL^{-1}, and for *copA*$^+$ isolates were: 0.0; 0.24; 0.48; 0.97; 1.95; 3.9; 7.8; 15.6; 31.25; 62.5; 125 and 250 µg mL^{-1}. Two additional controls were included: wells containing only medium, and medium + copper hydroxide at 200 and 250 µg mL^{-1}, for sensitive and resistant isolates, respectively. Bacterial growth (600nm) was quantified under agitation at 282 rpm, at 28 °C, using an Epoch™ Microplate Spectrophotometer (BioTek® Instruments, Inc., USA). Hourly absorbance readings for 48 hours were used to calculate the Area Under the Bacterial Growth Curve (AUBGC) using Microsoft Excel® 2023 to calculate the area by the formula Shaner and Finney (1977) from OD values:

$$AUBGC = \Sigma \left[\frac{\left(OD_i + OD_{i+1}\right)}{2} \right] \times \left(H_{i+1} - H_i\right)$$

In the formula: OD_i is the optical density at hour i from a number i of hours; OD_{i+1} is the optical density at hour $i+1$; H_i is the first hour, $H_{i+}1$ is the second hour.

The experiments, conducted in a completely randomized design (CRD) with eight wells (replicates) per combination, were repeated once. The data obtained were subjected to ANOVA and Dunnett's test at a 95% confidence level using R software (RStudio 2020).

Effects of copper preconditioning on bacterial growth and induction of resistance in *Xep*

(1) Two methodologies were applied in independent assays both using copper hydroxide as the copper source: (1) Copper solution droplets on solid CYE, and (2) Copper added to medium in microplate wells. For the first methodology two *Xep* isolates, EH 2017-69 and EH 2016-08, which were PCR positive for *copA* (*copA*$^+$), but did not exhibit the resistance phenotype *in vitro*, were cultured in CYE amended with 20 µg mL^{-1} of copper for 24 h to activate the resistance gene (Marin et al. 2019; Batista et al. 2021). To verify if the observed effect was isolate-specific, EH 2020-12 (*copA*$^-$) was also preconditioned. Subsequently, isolates were exposed to copper at 100, 150, 200, 250, and 300 µg mL^{-1} to assess, by the formation or absence of inhibitory halos, changes in MIC or whether preconditioning could lead to expression of resistance. A second assay was conducted in microplates to compare 48 h- growth curves between isolates EH 2020-12 (*copA*$^-$, sensitive) and EH 2017-27 (*copA*$^+$, resistant). The procedures and treatments were described in the "Effect of subinhibitory doses of copper on the growth of *Xep*" section, for S and R isolates. Two groups were formed, copper-preconditioned (P) and non-preconditioned (NP) isolates, spatially separated, each occupying half of the same microplate. Means of the hourly readings observed in four wells per combination of copper concentration/isolate were used to generate growth curves and calculate the AUBGCs. Each group was compared to its own control. The experiment was independently repeated once, and data were subjected to ANOVA and Dunnett's test at 95% confidence level.

Effect of subinhibitory copper hydroxide doses on *Xep* biofilm formation

The effect of subinhibitory doses on biofilm formation by copper-resistant (EH 2017-27, *copA*$^+$) and sensitive (EH 2020-12, *copA*$^-$) isolates was determined according to Li and Wang (2014). Procedures for inoculum preparation, medium composition, copper concentrations, controls, and number of replicates were described in the "Effect of subinhibitory doses of copper on the growth of *Xep*" section. After the bacterial inoculation into the microplate wells, they were maintained under static conditions at 28 °C for 48 h. Planktonic growth was quantified by OD (600 nm) measurements using a microplate reader Epoch. Biofilm formation was quantified after removing the bacterial growth using a micropipette and allowed to dry under laminar flow hood for 5 minutes. Wells were then washed twice with 260 µL of SDW for 5 minutes, followed by drying and addition of 260 µL of 0.1% crystal violet (CV). After 30 minutes, the excess CV was removed, and the wells were washed twice with SDW, gently pipetted to remove free CV, and dried in an inverted position under laminar flow hood for 2 hours. The elution of bound CV was achieved by adding 260 µL of 95% ethanol to each well. Microplates were kept at room temperature for 30 minutes, and absorbance was measured at 590 nm. The mean absorbance of 8 wells was compared to the control. Absorbance readings underwent ANOVA and Dunnett's test at a 95% confidence level using R software (RStudio 2020) and experiments repeated once.

Effect of subinhibitory copper hydroxide doses on bacterial spot severity in tomato plants

The experiment was conducted in a greenhouse at Embrapa Hortaliças, in a randomized block design with three treatments (commercial/labeled dose, subinhibitory dose and control) and four replications. The temperature registered during the period was between 20 and 30°C. Six-week-old tomato plantlets cv. Santa Cruz Kada Gigante (Feltrin Sementes®, Brazil) with four fully formed leaves were transplanted to 2.5 L plastic pots containing a mixture of clay, sand, rice straw, and commercial substrate in proportions of 3:1:1:1, supplemented with mineral fertilization according to the crop's recommendations. Copper hydroxide (HC) solutions were prepared in water at the commercial dose (Supera®–4mL L^{-1}), and at 25 µg mL^{-1} (subinhibitory dose with growth stimulatory effect). With a pre-compression sprayer (Guarany®, 1L) at a working pressure of approximately 3.0 bar (43.5 psi), each solution was applied to the adaxial surface of the leaflets until the runoff-point. After drying, the leaflets were inoculated with *Xep* (two isolates were used separately at each experiment: EH 2020-12 (S) and EH 2027-17 (R) at ~ 5 x 10^{8}UFC mL^{-1}) using a 200 mL-manual portable sprayer. Non-inoculated plants sprayed with water (not included in the statistical analysis), and *Xep*-inoculated plants were included as controls. The plants were subject to a misting intermittent system (10 mm/10 seconds/h) up to the disease severity evaluation date (five days after inoculation). It was carried out on a leaflet basis (four/leaf; two leaves/plant), by using a diagrammatic scale (Duan et al. 2015). Experiments were repeated once for both isolates and the obtained data were subjected to ANOVA and T-Test at 95% confidence level, and a combined analysis was performed using AgroEstat® 1.1.0.712 2014 (Barbosa And Maldonado Junior 2015).

Results

Amplification of the *copA* gene by PCR

The expected band of ~870 bp of the *copA* gene region was observed in 13 out of 45 *Xep* isolates, as well as in the *Xee* copper-resistant isolate (96-P), the positive control (Fig. S1).

Determination of the minimum inhibitory concentration (MIC) of copper for *Xep*

The MICs of copper determined for each isolate are shown in Table S1. Except for EH 2017-36 (MIC = 500 µg mL^{-1}), it was observed that all *Xep copA*$^-$ isolates (and *Xee*89-P) exhibited a sensitive phenotype with MICs equal to or less than 300 µg mL^{-1} and 150 µg mL^{-1}. On the other hand, the *copA*$^+$ isolates EH 2017-69 and EH 2016-08 showed a sensitive phenotype with MICs of 150 µg mL^{-1}, when copper droplets were applied to the medium. With the method of copper diffusion in medium, five (EH 2016-08, EH 2017-24, EH 2017-69, EH 2019-103 and EH 2019-108) out of the 13 *Xep copA*$^+$ isolates and the isolate *Xee* (96-P), did not exhibit a resistant phenotype (growth at 200 µg mL^{-1}) even after the preconditioning to activate R genes.

Effect of subinhibitory doses of copper on bacterial growth

All four *Xep* and *Xee* isolates (*copA*$^-$, S or *copA*$^+$, R) exhibited hormetic-like responses when growing in liquid medium amended with copper (Tables S2 and Table S3). This response was observed when bacterial growth in copper-containing medium was higher than that observed in the control, as expressed by the Area Under the Bacterial Growth Curve (AUBGC). In these concentration intervals, the AUBGCs showed statistically significant higher percentages of stimulation compared to the controls (Figs. 1 and 2). The experiment was repeated with similar results. While for EH 2020-12 (*Xep*, *copA*$^-$, S) the stimulatory range was 0.2–100 µg mL^{-1}, for 89-P (*Xee*, *copA*$^-$, S) this range was restricted from 0.2 to 25 µg mL^{-1}. For *Xep* EH 2020-12, the higher OD600 values generated distinct growth curves with maximum stimulation values achieved consistently at concentrations of 25 and 50 µg mL^{-1} among experiment repetitions (Fig. 3).

For EH 2017-27 (*Xep copA*$^+$, R) and 96-P (*Xee copA*$^+$, R) (Table S3), the stimulation ranges due to copper exposure at subinhibitory doses were different, varying from 0.24 - 62.5 µg mL^{-1} (EH 2017-27) and 0.24 - 31.25 µg mL^{-1} (96-P), with concentrations of 62.5 and 125 µg mL^{-1} corresponding, respectively, to non-significant stimulation and inhibition for 96-P. For isolate EH 2017-27, concentrations of 7.8 and 15.6 µg mL^{-1} resulted in growth stimulation of 31%, with AUGC of 38,110 and 38,172, respectively, while copper at 31.25 µg mL^{-1} resulted in stimulation up to 32% (AUGC = 38.613) compared to the control (AUBGC = 29.201) (Fig. 4). Meanwhile, for 96-P, the highest stimulation was 30% at 0.24; 1.95 and 31.25 µg mL^{-1} compared to the control.

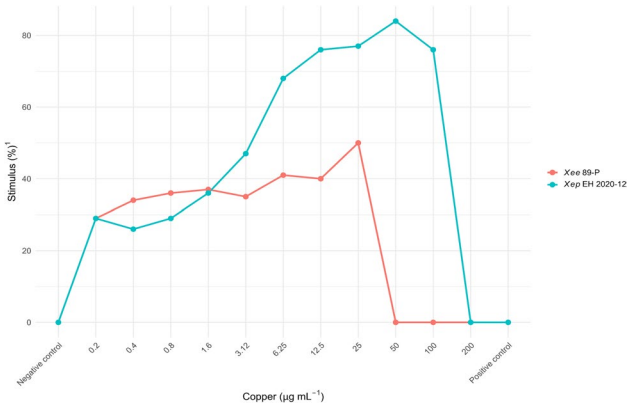

Fig. 1 Growth stimulation (%) in copper-sensitive and *copA*- *Xanthomonas euvesicatoria* pv. *perforans* (EH 2020-12) and *X euvesicatoria* pv. *euvesicatoria* (89-P) under different concentrations of added copper (source: copper hydroxide), determined by the values of the Area Under the Bacterial Growth Curve (AUBGC) compared to the growth without copper (positive control) after 48 hours of exposure. Positive control: bacterial growth on CYE medium, without added copper; negative control: CYE medium only

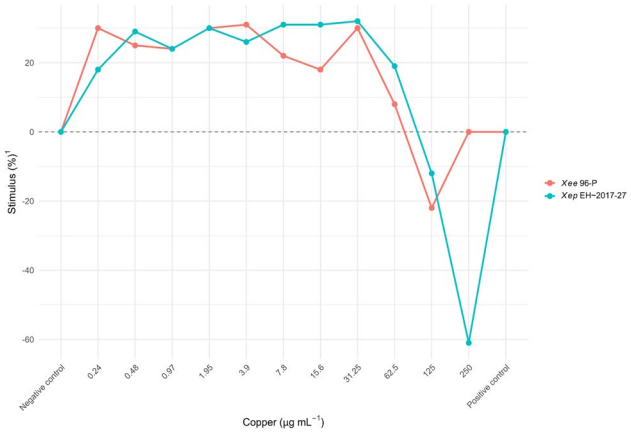

Fig. 2 Growth stimulation (%) in copper-resistant and *copA*+ *Xanthomonas euvesicatoria* pv. *perforans* (EH 2017-27) and *X. euvesicatoria* pv. *euvesicatoria* (96-P), under different concentrations of added copper (source: copper hydroxide), determined by the values of the Area Under the Bacterial Growth Curve (AUGC) compared to the growth without copper (positive control) after 48 hours of exposure. Negative values mean inhibition. Positive control: bacterial growth on CYE medium, without added copper; negative control: CYE medium only

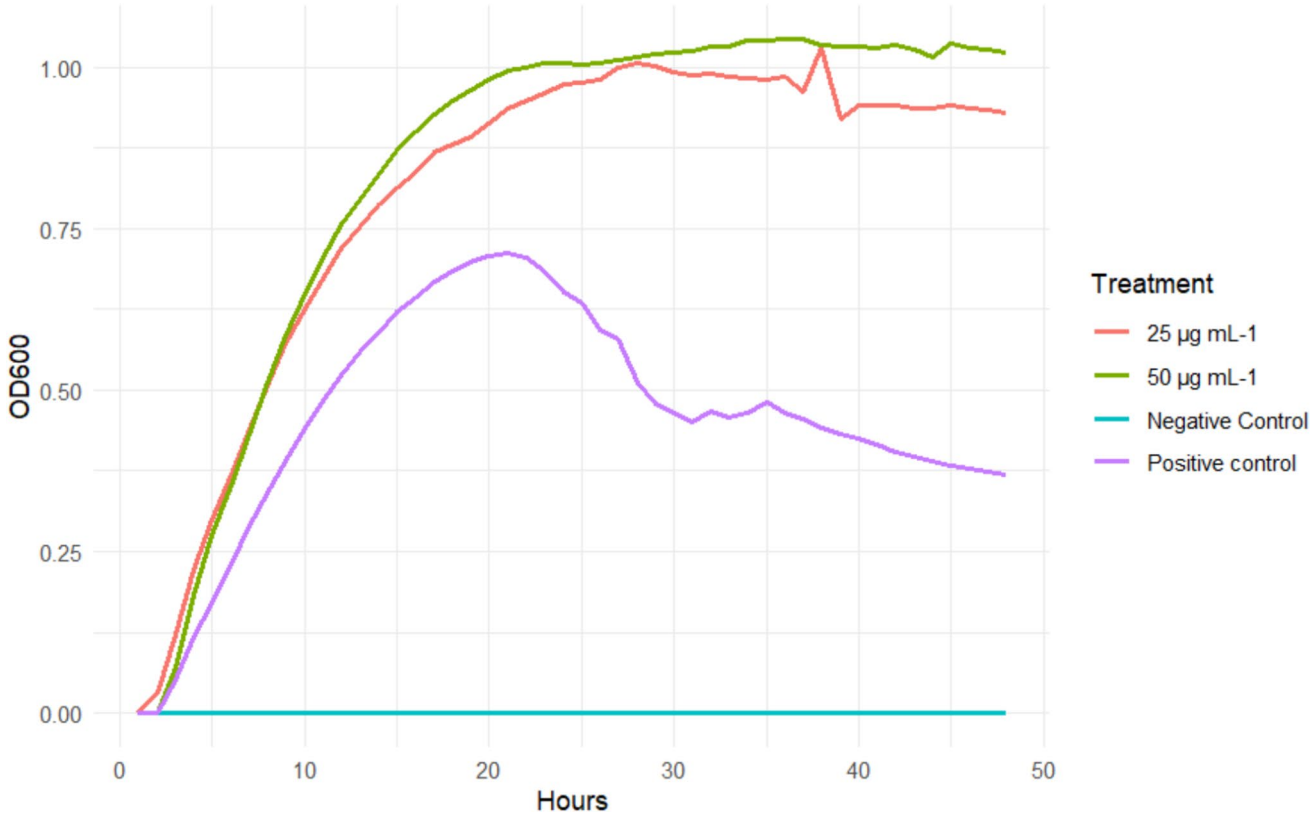

Fig. 3 *Xanthomonas euvesicatoria* pv. *perforans* isolate EH 2020-12 (S) 48 hour-growth curve in liquid CYE amended with copper at two subinhibitory concentrations: Positive control: bacterial growth without copper, Negative control: culture medium only

Effects of copper preconditioning on bacterial growth and induction of resistance in *Xep*

Copper droplet on solid medium For isolates EH 2016-08 and EH 2017-69, both *copA*$^+$ that did not express an R phenotype (Table S1), the preconditioning to copper at 20 µg mL^{-1} increased the resistance of the isolates. For EH 2016-08, an inhibitory halo was visible with 300 µg mL^{-1}, while for EH 2017-69, halo was not observed with this concentration inducing the isolate to express its resistance to copper. The preconditioning of isolate EH 2020-12 (*Xep, copA*$^-$) also increased resistance to copper raising its initial MIC from 200 to 300 µg mL^{-1} (Fig. 5). Concentrations lower than this value reduced bacterial density at the droplet deposition site, forming an opaque area (dark arrows) distinct from a perfect halo. On the other hand, inhibitory halos in the drop deposition site appeared translucid and without bacterial growth (white arrows) (Fig. 6).

Microplate assay Once the sensitive isolate EH 2020-12 responded to the preconditioning treatment by altering its tolerance to copper (Fig. 5) the isolate was grown for 48 hours and the growth curve obtained to better understand the influence of preconditioning with low doses. The different concentrations of copper applied led to reproducible results between experiments for both preconditioned (P) and non-preconditioned (NP) treatments of isolate EH 2020-12 (S). Overall, previously applied copper exhibited three effects on both P and NP isolates: inhibitory (200 µg mL^{-1}), growth reduction (100 µg mL^{-1}) and a hormetic-like (≤ 50 µg mL^{-1}), expressed by higher or lower AUBGC compared to the control of each isolate.

Both P and NP treatments of isolate EH 2020-12 (S) showed a stimulatory interval of doses from 0.2 to 50 µg mL^{-1}, with the maximum value of AUBCG corresponding to a stimulus of 63% significantly compared to their controls (Table 1). Although the initial inoculum was the same for both isolates (OD_{600nm} = 0,3), the ability to grow in medium with copper was affected by the preconditioning, since the final AUBGCs observed were significantly higher for the P isolate than the NP and this pattern was consistent among the experiment's repetitions in the same conditions (Tables S4 and S5).

The experiment was repeated with the resistant isolate EH 2017-27 to verify the effect of the preconditioning on its growth curve under several subinhibitory copper doses. The P isolate showed a higher growth in liquid medium containing low doses of copper (expressed by the AUGC) than the

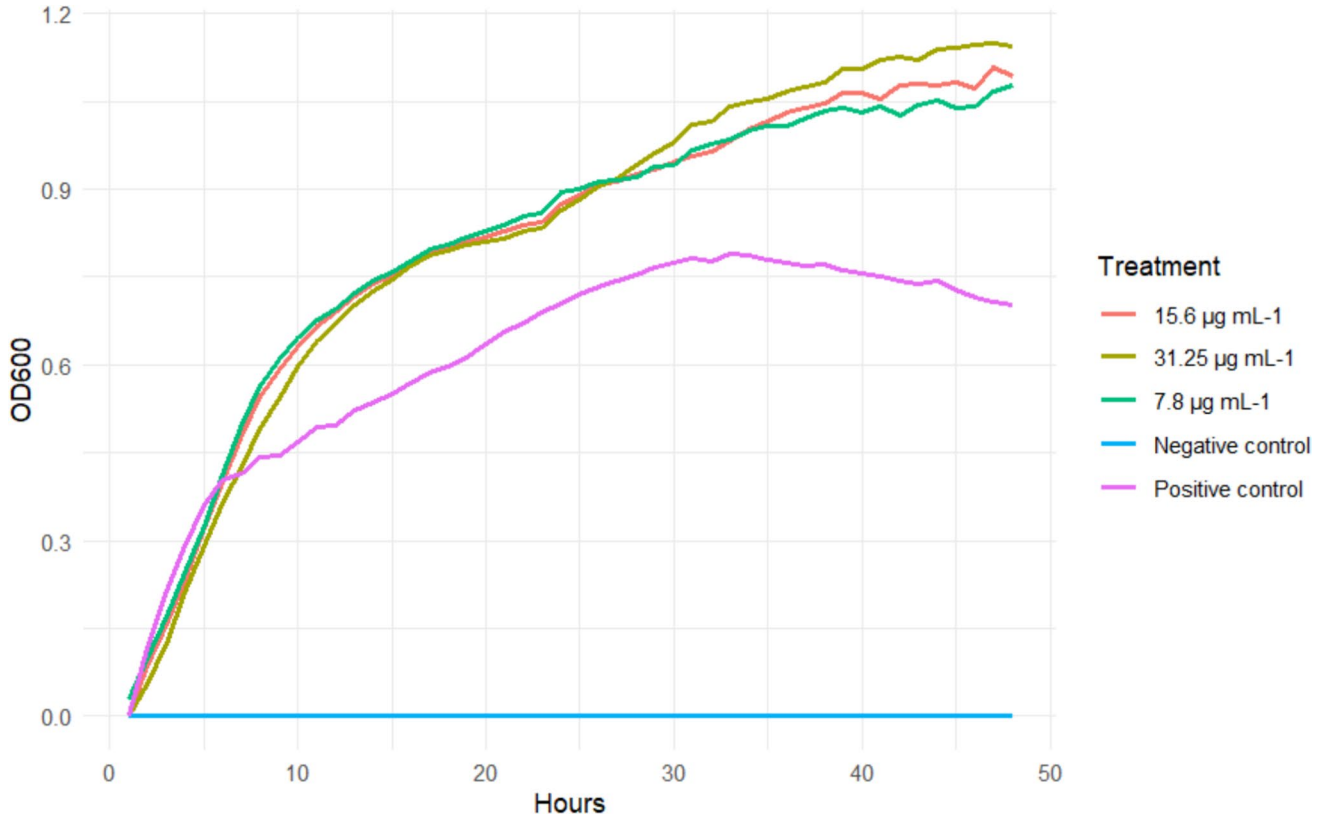

Fig. 4 *Xanthomonas euvesicatoria* pv. *perforans* isolate EH 2017-27 (R) 48h-growth curve in liquid CYE amended with subinhibitory copper of concentrations. Positive control: bacterial growth without copper; Negative control: culture medium only

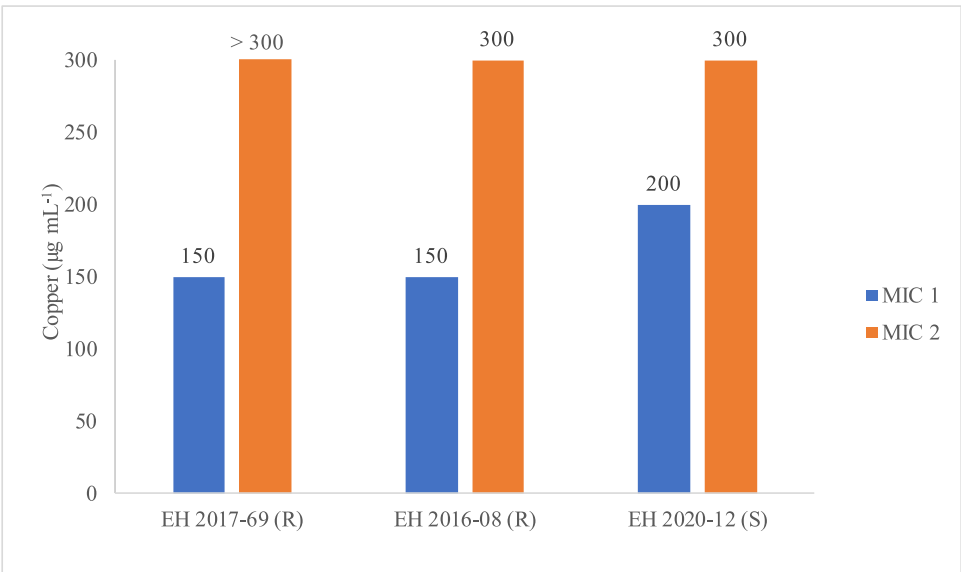

Fig. 5 Determination of the minimum inhibitory concentration of copper (source: copper hydroxide), before (MIC 1) and after preconditioning (MIC 2) with copper (20 µg mL^{-1}) in CYE medium for three isolates of *Xanthomonas euvesicatoria* pv. *perforans*. R= resistant, S= sensitive. For EH 2017-69 growth was observed at all concentrations tested after preconditioning up to 300 ug mL^{-1}, thus MIC 2 is higher than this concentration, being classified as phenotypically resistant in this study

NP isolate. The maximum AUBGC's value was found for copper at 3.9 and 1.95 µg mL^{-1} representing, respectively, 23 and 24% of stimulus in the growth compared to the control of each experiment (Table 2). On the other hand, for the NP treatment only the inhibitory doses (125 and 250 µg mL^{-1}) were statistically different from the control and the observed stimuli were not consistent among the experiment's repetitions (Tables S6 and S7).

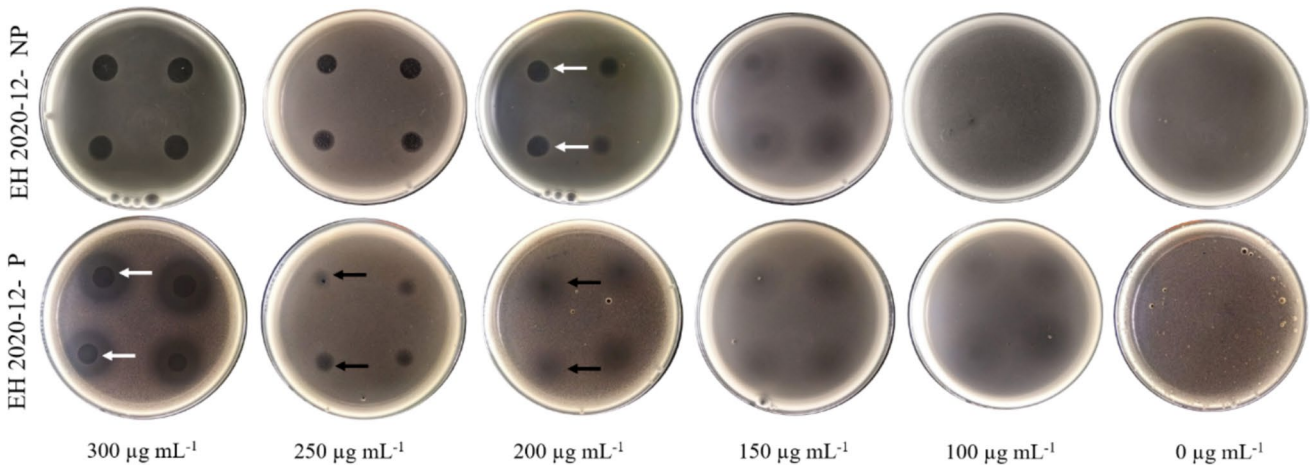

Fig. 6 Determination of the minimum inhibitory concentration by applying different concentrations of copper hydroxide (copper in metallic equivalent µg mL^{-1}) as drops onto CYE medium containing isolate EH 2020-12 (*Xanthomonas euvesicatoria* pv. *perforans copA*$^-$, S). P: isolate preconditioned with copper at 20 µg mL^{-1}/24h, NP: non-preconditioned isolate (control), White arrows: indicate translucent region with absence of bacterial growth (inhibitory halo), Dark arrows: indicate opaque region showing reduction of bacterial density at the copper deposition site, but without formation of a perfect inhibitory halo

Effect of subinhibitory copper hydroxide doses on *Xep* biofilm formation

Both copper-resistant EH 2017-27 (R) and sensitive EH 2020-12 (S) isolates showed stimulus on the biofilm production compared to the controls without added copper (Tables 3 and 4). For the R isolate growth stimulus was 2 and 12%, between the two experiments, at 25 µg mL^{-1} of copper. For the S isolate, higher biofilm growth compared to the control was observed with a wider range of copper concentrations, from 12.5 to 100 µg mL^{-1}.

Table 1 Areas Under the Bacterial Growth Curve (AUBGC) and growth stimulation (%) of the isolate EH 2020-12 (S) (*Xanthomonas euvesicatoria* pv. *perforans*) preconditioned with copper (20 µg mL^{-1}) and non-preconditioned followed by exposure to different concentrations of copper

Copper (µg mL^{-1})	Preconditioned		Non-preconditioned	
	AUBGC	Stimulus (%) [1]	AUBGC	Stimulus (%)
Negative Control	0,000*		0,000*	
200	0,000*		0,000*	
100	9,584*	−25	5,352*	−47
50	14,405	13	14,402*	43
25	19,831*	56	16,394*	63
12.5	20,671*	63	15,737*	56
6.25	19,876*	56	15,736*	56
3.12	19,250*	51	14,919*	48
1.6	19,378*	58	14,849*	47
0.8	18,246*	43	14,663*	45
0.4	18,151*	43	$15,426_a$	53
0.2	16,870*	33	15,096*	50
Positive Control	12,720	-	10,082	-

[1]Growth stimulation (%) compared to the positive control in the same column after 48h

*Different from the control in the same column by Dunnett's test at 95% confidence. Positive control: medium + bacteria; Negative control: culture medium only

Table 2 Areas Under the Bacterial Growth Curve (AUBGC) and growth stimulation (%) of the isolate EH 2017-27 (R) (*Xanthomonas euvesicatoria* pv. *perforans*) preconditioned with copper (20 µg mL^{-1}) and non-preconditioned, followed by exposure to different concentrations of copper

Copper (µg mL^{-1})	Preconditioned		Non-preconditioned	
	AUBGC	Stimulus (%) [1]	AUBGC	Stimulus (%)
Negative control	0,000*	-	0,000*	-
250	19,329*	−48	22,366*	−45
125	28,081*	−24	35,537*	−12
62.5	39,193	6	42,605	6
31.25	40,669	10	42,605	6
15.6	41,283*	12	42,879	6
7.8	42,843*	16	43,435	8
3.9	45,402*	23	43,694	8
1.95	45,650*	24	41,424	3
0.97	41,671*	13	44,183	10
0.48	36,583*	−1	44,034	9
0.24	35,384*	−4	39,263	−3
Positive control	36,825	-	40,328	-

[1]Growth stimulation (%) compared to the positive control in the same column after 48hrs. Negative values correspond to inhibition

*Different from the control in the same column by Dunnett's test at 95% confidence. Positive control: medium + bacteria; Negative control: culture medium only

Table 3 Influence of different doses of copper hydroxide on biofilm formation (BF) of *Xanthomonas euvesicatoria* pv. *perforans*, isolate EH 2017-27 (R) after 48 hours in two independent experiments

Copper (µg mL^{-1})	Experiment 1			Experiment 2		
	PG [1]	BF	BF stimulus (%) [2]	PG	BF	BF stimulus (%)
Negative control	0.000	0.000*	-	0.000	0.000*	-
250	0.639	1.632*	−29	0.557	1.339*	−45
100	0.564	1.286*	−44	0.577	1.304*	−47
50	0.540	1.814*	−22	0.665	2.219*	−9
25	0.643	2.361*	2	0.787	2.748*	12
12.5	0.672	2.273	−2	0.752	2.389	−2
6.25	0.697	2.230	−4	0.709	2.407	−2
3.12	0.698	2.369	2	0.716	2.177*	−11
1.6	0.715	2.277	−2	0.696	2.311	−6
0.8	0.724	2.201	−5	0.796	2.227	−9
0.4	0.7136	2.368	2	0.860	2.406	−2
0.2	0.733	2.242	−3	0.785	2.331	−5
Positive Control	0.730	2.314	-	0.851	2.450	-

[1]Planktonic Growth (PG) determined by optical density (OD) at 600nm after 48 h

[2]Stimulus on biofilm formation compared to the positive control in the same column. Negative values correspond to inhibition

*Different from the control in the same column by Dunnett's test at 95% confidence. Positive control: medium + bacteria; Negative control: culture medium only

Effect of subinhibitory copper hydroxide doses on bacterial spot severity in tomato plants

No significant differences ($p \geq 0.05$) were found between the blocks or between the treatments within each individual experiment, nor in the combined analysis of all experiments. For all repetitions of each experiment, no treatment was effective in reducing disease severity, and neither EH 2020-12 (S) nor EH 2017-27 (R) were stimulated by the hormetic-like dose sprayed on tomato leaves. The labeled dose applied led to a slight reduction of disease severity, but not significant by ANOVA test and T-Test ($p \geq 0.05$) (Table 5).

Table 4 Influence of different doses of copper hydroxide added on biofilm formation (BF) of *Xanthomonas euvesicatoria* pv. *perforans*, isolate EH 2020-12 (S) after 48 hours in two independent experiments

Copper (µg mL^{-1})	Experiment 1			Experiment 2		
	PG [1]	BF	BF stimulus (%)[2]	PG	BF	BF stimulus (%)
Negative control	0.000	0.000*	-	0.000	0.000*	-
200	0.000	0.000*	-	0.000	0.000*	-
100	0.186	2.044*	39	0.197	2.564*	67
50	0.207	2.597*	77	0.216	2.120*	38
25	0.203	2.346*	60	0.377	2.502*	63
12.5	0.280	1.793	22	0.325	1.769*	15
6.25	0.319	1.388	−5	0.376	1.140*	−30
3.12	0.443	1.554	6	0.625	1.366*	−11
1.6	0.671	1.317	−10	0.735	1.273*	−17
0.8	0.784	1.091	−26	0.762	0.841*	−45
0.4	0.822	1.379	−6	0.789	0.872*	−43
0.2	0.795	1.407	−4	0.784	0.923*	−40
Positive Control	0.793	1.466	-	0.796	1.538	-

[1]Planktonic Growth (PG) determined by optical density (OD) at 600nm after 48 h

[2]Stimulus on biofilm formation compared to the positive control in the same column. Negative values correspond to inhibition

*Different from the control in the same column by Dunnett's test at 95% confidence. Positive control: medium + bacteria; Negative control: culture medium only

Discussion

The preventive application of copper-based bactericides is one of the strategies commonly employed for bacterial spot management. However, challenges of its use in tomato fields may arise due to resistance development. The presence of copper-resistant isolates of *Xanthomonas* spp. (Abbasi et al. 2015; Griffin et al. 2017; Areas et al. 2018; Marin et al. 2019; Lai et al. 2021; Rana et al. 2023; Kaur et al. 2024), including *Xep* isolates, has already been detected in several countries including Brazil (Aguiar et at. 2000; Quezado-Duval et al. 2003; Araújo et al. 2012b). In the present study the *copA* gene was detected by PCR in 28.8% of the isolates, indicating the presence of the *copLAB* operon (Behlau et al. 2013). The primers used in this study were designed to specifically amplify the *copA* gene sequence, as demonstrated by Behlau et al. (2013). The 32 strains that lacked this gene (PCR negative) were mostly sensitive to copper, with the exception of one, EH 2017-36, which may have other genes involved (Voloudakis et al. 2005; Behlau et al. 2011) or even variations in the *copA* sequences that prevented amplification. As expected, MIC values determined by two methods were lower in *copA*$^-$ isolates and higher in *copA*$^+$ ones. Two *Xep* isolates (EH 2016-08 and EH 2017-69) required preconditioning for 24 hours in solid CYE amended with 20 µg mL^{-1} copper to express a resistant phenotype. However, using the same method of preconditioning, five *copA*$^+$ *Xep* isolates (EH 2016-08, EH 2017-24, EH 2017-69, EH 2019-103 and EH 2019-108) and *Xee* (96-P), were phenotypically sensitive (MIC = 150 µg mL^{-1}). Since neither the presence nor the absence of other genes involved in copper resistance was investigated, only *copA* was analyzed in this study, it was not possible to determine why the previous exposure to low doses of copper was not able to activate the resistance phenotype in five isolates, even though this is a commonly applied procedure for its activation (Basim et al. 2005; Marin et al. 2019; Batista et al. 2021). Also, it is important to mention that the procedure was effective in increasing resistance in the other two *copA*+ isolates. Therefore, this finding highlights the need for further studies involving genes other than *copA*. Additionally, the methodologies applied (liquid media in microtiter plates x solid medium) can differ substantially in terms of the level of exposure to which the bacteria are subjected when in contact with copper, since in a liquid medium under agitation the copper suspension may be better distributed and diffusion may differ from solid medium. So, despite contrasting MIC results, employing two distinct methodologies can contribute to a better understanding of the effect of a previous subinhibitory dose.

In this study, the chosen copper source was copper hydroxide, which is less soluble than other more commonly used sources such as copper sulfate. However, the use of solid media amended with copper hydroxide may also differentiate resistant from sensitive isolates of bacteria, as shown in studies with *Xanthomonas* (Khanal et al. 2020; Longhi et al. 2022) as well as with *Pseudomonas syringae* pv *phaseolicola*. (Zhang et al. 2017). As for *Xep*, the study of Khanal et al. (2020) showed that resistant strains grew at the same concentration range when exposed to both copper sources in solid media. Growth of the sensitive strain occurred only at the lowest concentration tested of both copper sources. Additionally, Longhi et al (2022) demonstrated that copper hydroxide and copper sulfate were able to inhibit *X. vasicola* pv. *vasculorum in vitro*, having similar MIC and differences in sensitivity to copper compounds were observed among strains. In the present study, the reduced growth or inhibition observed at higher copper concentrations, visualized with the bacterial growth curve in liquid CYE and the absence of growth on solid CYE, demonstrated that copper was available in the media. Moreover, copper hydroxide is the most commonly used copper-based product in the tomato producing areas in Distrito Federal and also widely employed for bacterial diseases management (Graham et al. 2016; Behlau et al 2017a, b).

Growth stimulation at copper concentrations below the MIC was observed for both *Xep* and *Xee* isolates. According to the response obtained by different isolates, this effect induced by subinhibitory doses of copper seems to be general and not isolate or pathovar-specific. Furthermore, its occurrence is not related to the presence of the *copA* gene or sensitivity to the chemical in *Xep* and *Xee*. The growth curves showed inhibition at higher doses (equal to or greater than the MIC), while lower doses exhibited a consistently stimulatory effect across repetitions, which suggests the occurrence of an hormetic response (Calabrese And Baldwin

Table 5 Influence of copper hydroxide on the severity of tomato bacterial spot caused by copper- sensitive (EH 2020-12) and resistant (EH 2017-27) isolates of *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*) five days after inoculation

Treatment	Severity (%)[3]	
	EH 2017-27 (R)	EH 2020-12 (S)
Subinhibitory dose (25 µg mL^{-1}) [1]	2.56 ^{ns}	1.67 ^{ns}
Commercial dose (1,400 µg mL^{-1}) [2]	1.80^{ns}	1.15^{ns}
Xep inoculated plants	2.72 ^{ns}	1.92^{ns}

[1]Copper in metal equivalent

[2]Commercial dose (Supera®)

[3]Values represent the mean obtained from 256 leaflets (= 4 leaflets/leaf x 2 leaves/plant x 4 plants x 4 blocks) divided into two experiments in a combined analysis. Means followed by the same letter in the column do not significantly (ns) differ ($p \geq 0.05$) at T-Test at 95% of confidence interval

2002; Garzón And Flores 2013; Silva et al. 2018; Agathokleous and Calabrese 2019). For some non-phytopathogenic bacterial species the stimulatory effect of subdoses of different antibiotics has been reported (Migliore et al. 2013; Cui et al. 2018; Sun et al. 2023). To explain this effect, two mechanisms have been proposed, one is direct stimulation or induced adaptation, by a previous exposure to a lower dose of a stressor agent. A second mechanism might be overcompensation. In this case, a disruption of homeostasis occurs when an initial stress induces a chain reaction at the molecular level that can be expressed phenotypically, such as increased growth (Stebbing 1982; Davies et al. 1995; Calabrese 2008). However, these mechanisms are still poorly understood in plant bacteriology. In this study, the preconditioning of isolates R and S with subdoses of copper had different effects depending on the methodology used. Resistant isolates preconditioned in solid medium for 24 hours expressed resistance either fully (no inhibition at the highest copper concentration) or partially (isolate EH 2016-08, with copper MIC increasing from 150 to 300 µg mL^{-1}). This procedure of previous exposure to a lower dose of copper (20 µg mL^{-1}) is a standard method employed for activating resistance genes in *Xanthomonas* spp., sometimes referred to as 'pre-sensitization' or 'prior sensitization'. It aims to select resistant isolates present in a population through the phenotypic expression of genetic resistance (presence of *cop* genes) (Basim et al. 2005; Marin et al. 2019; Batista et al. 2021). It is possible that other genes in the *cop* operon may be involved in the resistance response after preconditioning, however, their role was not investigated in this study (Behlau et al. 2013; Marin et al. 2019; Behlau et al. 2020). On the other hand, the increase in copper MIC of the sensitive isolate EH 2020-12 which lacks the *copA* gene could be explained by hormesis-like effect, since the isolate does not contain the resistance gene and was subjected to the same treatment. Whether containing resistance genes to the stressor agent or not, pre-conditioning with subinhibitory doses can increase its tolerance to the same agent (Calabrese 2016). In the literature, examples involving fungicides from different chemical groups can be found. For example, subdoses of strobilurin reduced the sensitivity of the fungus *Cryptococcus gattii* to the fungicide itself and induced cross-resistance to azole fungicides (Bastos et al. 2019). In *Botrytis cinerea*, resistance induction to different groups of fungicides occurred after pre-conditioning with subinhibitory doses of pyrrolnitrin (Ajouz et al. 2010). The phenomenon has been studied not only for its effects on pathogens but also for its potential beneficial effects on biological control agents (Dharma et al. 2024). For antibiotics, this would also be the case of resistance induction in several bacteria after treatment with low concentrations (Revitt-Mills And Robinson 2020). In this study, the copper concentration used to precondition the sensitive isolate was in the hormetic range. The higher growth and stimulation observed in the preconditioned isolate compared to the non-preconditioned one suggests a greater capacity for adaptation to the new environment. Additionally, it has been demonstrated that a positive regulation of the chromosomal cluster *cohLAB*, explains copper tolerance in *Xanthomonas citri* subsp. *citri* strains that lack *copLAB* or *copABCD* (Marin et al. 2019). This gene is a copper homeostasis operon (Boyer et al. 2024), and may be involved in the increased resistance after preconditioning in *Xep* in a similar way. However, this was not investigated in this study and may be subject for new approaches that explore hormesis and hormetic-like effects from a genetic perspective.

In this study, an increase in biofilm production was also observed for *Xep* isolates, resistant (EH 2017-27) and sensitive (EH 2020-12), when exposed to some of the subinhibitory concentrations of copper. Stimulatory effects of low doses of antibiotics were observed in different studies involving bacteria, leading to increases in biofilm production (Hoffman et al. 2005; Linares et al. 2006; Ranieri et al. 2018). Resistant and sensitive isolates tend to form stronger and weaker biofilms, respectively (Qi et al. 2016), an increase in biofilm production can provide a competitive advantage to bacteria, as biofilm is an important virulence factor in *Xanthomonas* species (An et al. 2020).

Based on our results, no stimulatory effect on the virulence of either the resistant or sensitive *Xep* isolates was observed, and even the commercial copper dose (labeled dose) was ineffective in reducing severity under greenhouse conditions. Although the subinhibitory dose of copper used in the experiment stimulated growth, it did not affect virulence significantly. Virulence in *Xanthomonas* is controlled by several factors including ability to form biofilm, produce exopolysaccharides, lipopolysaccharides, adhesins, and extracellular degrading enzymes, secretion systems, effectors, and an efficient regulatory network that coordinates all these factors (An et al. 2020). It is known that, in a hormetic scenario, growth, virulence and stressor agent doses are not necessarily related in a linear or straightforward manner. The same dose can simultaneously stimulate both the pathogen growth and virulence (Di et al. 2016b) but this was not observed in the present study. On the other hand, a dose that inhibits growth might have a stimulatory effect on virulence, as reported in studies involving the pathogen *Sclerotinia sclerotiorum* (Di et al. 2016a; Lu et al. 2018). One reason for this is that when pesticide is applied to plants, the agent molecule can be affected by weather factors (temperature, water, humidity) (Azevedo And Chasin 2003), the pressure of the spraying equipment, and droplet size. These factors can alter the final dose on the leaf, resulting in a lower dose (Silva et al. 2018; Garzón And Flores 2013). Exposure to subinhibitory concentrations of different compounds

can lead to various effects in bacteria, such as population growth (Migliore et al. 2013), production of cell-protective compounds that reduce reactive oxygen species (Mo et al. 2023), synthesis of secondary metabolites (Peng et al. 2021), increases in mutation rate (Revitt-Mills And Robinson 2020), conjugation (Li et al. 2020), and increased cross-resistance tolerance levels between antibiotics (Martins et al. 2020).

To our knowledge this is the first report of a hormetic-like effect in copper-sensitive and resistant isolates of *Xep*. This effect led to stimulation of growth and biofilm production induced by copper at subinhibitory doses. However, the stimulatory subdose could not interfere with bacterial virulence in tomato plants. The mechanisms involved in stimulating *Xep* growth when exposed to low doses of copper can be explored in future studies aiming to understand how hormesis can affect the bacterial spot pathosystem and how subinhibitory doses can influence copper resistance dynamics and disease management in the field.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40858-026-00794-9.

Acknowledgments The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing a doctorate scholarship to ELS and the technical support at Embrapa Hortaliças.

Author contributions ELS: Conceptualization, experimental procedures, data analysis, writing the original draft, review and editing. MASVF: Funding acquisition and project administration, supervision, writing, review and editing. AMQD: Funding acquisition, supervision, resources, writing, review and editing. MR: Resources, writing, review and editing. All authors read and approved the submitted version.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). This research was supported by the Fundação de Apoio à Pesquisa do Distrito Federal -FAPDF. Grant # FAPDF 00193-00001496/2021-15.

Data availability The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Abbasi PA, Khabbaz SE, Weselowski B, Zhang L (2015) Occurrence of copper-resistant strains and a shift in *Xanthomonas* spp. causing tomato bacterial spot in Ontario. Can J Microbiol 61:753–61

Agathokleous E, Calabrese EJ (2019) Hormesis can enhance agricultural sustainability in a changing world. Glob Food Secur 20:150–155

Aguiar LA, Kimura O, Castilho AMC, Castilho KSC, Ribeiro RLD, Akiba F, Carmo MGF (2000) Copper resistance in Brazilian isolates of *Xanthomonas campestris* pv. *vesicatoria* of pepper and tomato. Agron 34:78–82

Ajouz S, Decognet V, Nicot PC, Bardin M (2010) Microsatellite stability in the plant pathogen *Botrytis cinerea* after exposure to different selective pressures. Fungal Biol 114:949–954

Al-Momani H, Aolymat I, Ibrahim L, Albalawi H, Al BD, Albiss BA, Almasri M, Alghweiri S (2024) Low-dose zinc oxide nanoparticles trigger the growth and biofilm formation of *Pseudomonas aeruginosa*: a hormetic response. BMC Microbiol 24:290

An SQ, Potnis N, Dow M, Vorhölter FJ, He YQ, Becker A, Teper D, Li Y, Wang N, Bleris L, Tang JL (2020) Mechanistic insights into host adaptation, virulence and epidemiology of the phytopathogen *Xanthomonas*. FEMS Microbiol Rev 44:1–32

Araújo ER, Costa JR, Ferreira MASV, Quezado-Duval AM (2012) Simultaneous detection and identification of the *Xanthomonas* species complex associated with tomato bacterial spot using species-specific primers and multiplex PCR. J Appl Microbiol 113:1479–1490

Araújo ER, Ferreira MASV, Quezado-Duval AM, Café-Filho AC (2012) Sensitivity of Xanthomonads causing tomato bacterial spot to copper and streptomycin and *in vivo* infra-specific competitive ability in *Xanthomonas perforans* resistant and sensitive to copper. Plant Pathol J 94:79–87

Araújo ER, Costa JR, Ferreira MASV, Quezado-Duval AM (2017) Widespread distribution of *Xanthomonas perforans* and limited presence of *X. gardneri* in Brazil. Plant Pathol 66:159–168

Areas MS, Gonçalves RM, Soman JM, Souza Filho RC, Gioria R, Silva Junior TAFD, Maringoni AC (2018) Resistance of *Xanthomonas euvesicatoria* strains from Brazilian pepper to copper and zinc sulfates. An Acad Bras Cienc 90:2375–2380

Azevedo FA, Chasin AAM (2003) As bases toxicológicas da ecotoxicologia. InterTox, São Paulo, p 322

Barak JD, Vancheva T, Lefeuvre P, Jones JB, Timilsina S, Minsavage GV, Vallad GE, Koebnik R (2016) Whole-genome sequences of *Xanthomonas euvesicatoria* strains clarify taxonomy and reveal a stepwise erosion of type 3 effectors. Front Plant Sci 7:1805

Barbosa JC, Maldonado Junior W (2015) AgroEstat: sistema para análises estatísticas de ensaios agronômicos. FCAV/UNESP, Jaboticabal, p 396

Basim H, Minsavage GV, Stall RE, Wang JF, Shanker S, Jones JB (2005) Characterization of a unique chromosomal copper resistance gene cluster from *Xanthomonas campestris* pv. *vesicatoria*. Appl Environ Microbiol 71:8284–91

Bastos RW, Freitas GJC, Carneiro HCS, Oliveira LVN, Gouveia-Eufrasio L, Santos APN, Moyrand F, Maufrais C, Janbon G, Santos DA (2019) From the environment to the host: how non-azole agrochemical exposure affects the antifungal susceptibility and virulence of *Cryptococcus gattii*. Sci Total Environ 681:516–523

Batista JNG, Ferreira MASV, Quezado-Duval AM (2021) Molecular and phenotypic characterization of *Xanthomonas campestris* pv.

campestris causing black rot in Brassica crops in Brazil. Trop Plant Pathol 46:684–701

Behlau F, Canteros BI, Minsavage GV, Jones JB, Graham JH (2011) Molecular characterization of copper resistance genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*. Appl Environ Microbiol 77:4089–4096

Behlau F, Hong JC, Jones JB, Graham JH (2013) Evidence for acquisition of copper resistance genes from different sources in citrus associated xanthomonads. Phytopathology 103:409–418

Behlau F, Gochez AM, Lugo AJ, Elibox W, Minsavage GV, Potnis N, White FF, Ebrahim M, Jones JB, Ramsubhag A (2017) Characterization of a unique copper resistance gene cluster in *Xanthomonas campestris* pv. *campestris* isolated in Trinidad, West Indies. Eur J Plant Pathol 147:671–681

Behlau F, Scandelai LHM, Silva Junior GJ, Lanza FE (2017) Soluble and insoluble copper formulations and metallic copper rate for control of citrus canker on sweet orange trees. Crop Prot 94:185–91

Behlau F, Gochez AM, Jones JB (2020) Diversity and copper resistance of *Xanthomonas* affecting citrus. Trop Plant Pathol 45:200–212

Bibi S, Weis K, Kaur A, Bhandari R, Goss E, Jones JB, Potnis N (2023) A brief evaluation of a copper resistance mobile genetic island in the bacterial leaf spot pathogen *Xanthomonas euvesicatoria* pv. *perforans*. Phytopathology 113:1394–1398

Boyer C, Lefeuvre P, Richard D, Lobin KK, Pruvost O (2024) Complete genome sequence of a copper-resistant *Xanthomonas campestris* pv. *campestris* strain isolated from Broccoli in Mauritius Suggests Adaptive Gene Gain Through Horizontal Gene Transfer. Phytopathology 114:328–333

Calabrese EJ (2008) Hormesis and medicine. Br J Clin Pharmacol 66:594–617

Calabrese EJ (2016) Preconditioning is hormesis part II: how the conditioning dose mediates protection: dose optimization within temporal and mechanistic frameworks. Pharmacol Res 110:265–75

Calabrese EJ, Baldwin LA (2002) Defining hormesis. Hum Exp Toxicol 2:91–97

Chillappagari S, Seubert A, Trip H, Kuipers OP, Marahiel MA, Miethke M (2010) Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in *Bacillus subtilis*. J Bacteriol 192:2512–24

Constantin EC, Cleenwerck I, Maes M, Baeyen S, Van Malderghem C, De Vos P, Cottyn B (2016) Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. Plant Pathol 65:792–806

Cui X, Huo M, Chen C, Yu Z, Zhou C, Li A, Qiao B, Zhou D, Crittenden JC (2018) Low concentrations of Al (III) accelerate the formation of biofilm: multiple effects of hormesis and flocculation. Sci Total Environ 634:516–524

Davies JM, Lowry CV, Davies KJ (1995) Transient adaptation to oxidative stressing yeast. Arch Biochem Biophys 317:1–6

Deng Z, Lin Z, Zou X, Yao Z, Tian D, Wang D, Yin D (2012) Model of hormesis and its toxicity mechanism based on quorum sensing: a case study on the toxicity of sulfonamides to *Photobacterium phosphoreum*. Environ Sci Technol 46:7746–7754

Dharma KS, Suryanti S, Widiastuti A (2024) Hormesis in pathogenic and biocontrol fungi: from inhibition to stimulation. Caraka Tani: J Sustain Agric 39:281–296

Di YL, Cong ML, Zhang R, Zhu FX (2016) Hormetic effects of trifloxystrobin on aggressiveness of *Sclerotinia sclerotiorum*. Plant Dis 100:2113–2118

Di YL, Lu XM, Zhu ZQ, Zhu FX (2016) Time course of carbendazim stimulation on pathogenicity of *Sclerotinia sclerotiorum* indicates a direct stimulation mechanism. Plant Dis 100:1454–1459

Drummond LJ, Smith DGE, Poxton IR (2003) Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. J Med Microbiol 52:1033–1038

Duan J, Zhao B, Wang Y, Yang W (2015) Development and validation of a standard area diagram set to aid estimation of bacterial spot severity on tomato leaves. Eur J Plant Pathol 142:665–675

EPPO (2023) PM 7/110 (2) Xanthomonas spp. (Xanthomonas euvesicatoria pv. euvesicatoria, Xanthomonas hortorum pv. gardneri, Xanthomonas euvesicatoria pv. perforans, Xanthomonas vesicatoria) causing bacterial spot of tomato and sweet pepper. EPPO Bullet 53:558–579

Garzón CD, Flores FJ (2013) Hormesis: Biphasic dose-reponse to fungicides in plant pathogens and their potential threat to agriculture. In: Nita M (ed) Fungicides - showcases of integrated plant disease management from around the world. Tech, Rijeka, pp 311–328

Graham JH, Brooks C, Yonce HD (2016) Importance of early season copper sprays for protection of Hamlin orange fruit against citrus canker infection and premature fruit drop. Proc Fla State Hortic Soc 129:74–8

Griffin K, Gambley C, Brown P, Li Y (2017) Copper-tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomonas* spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review. Crop Prot 96:144–150

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171–5

Hsiao YM, Liu YF, Lee PY, Hsu PC, Tseng SY, Pan YC (2011) Functional characterization of *copA* gene encoding multicopper oxidase in *Xanthomonas campestris* pv. *campestris*. J Agric Food Chem 59:9290–302

Jones JB, Bouzar H, Stall RE, Almira EC, Roberts PD, Bowen BW, Sudberry J, Strickler PM, Chun J (2000) Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. Int J Syst Evol Microbiol 50:1211–1219

Jones JB, Lacy GH, Bouzar H, Stall RE, Schaad NW (2004) Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst Appl Microbiol 27:755–762

Kaur A, Minsavage GV, Potnis N, Jones JB, Goss EM (2024) Evolution of copper resistance in *Xanthomonas euvesicatoria* pv. *perforans* population. mSyst 9:e01427-24

Khanal S, Hind SR, Babadoost M (2020) Occurrence of copper-resistant *Xanthomonas perforans* and *X. gardneri* in Illinois tomato fields. Plant Health Prog 21:338–344

Lai Y-R, Lin C-H, Chang C-P, Ni H-F, Tsai W-S, Huang C-J (2021) Distribution of copper resistance gene variants of *Xanthomonas citri* subsp. *citri* and *Xanthomonas euvesicatoria* pv. *perforans*. Plant Protect Sci 57:206–216

Li J, Wang N (2014) Foliar application of biofilm formation–inhibiting compounds enhances control of citrus canker caused by *Xanthomonas citri* subsp. *citri*. Phytopathology 104:134–142

Li X, Shi J, Sun H, Lin Z (2020) Hormetic dose-dependent response about typical antibiotics and their mixtures on plasmid conjugative transfer of *Escherichia coli* and its relationship with toxic effects on growth. Sci Total Environ 205:11130

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci U S A 103:19484–9

Longhi TV, Robaina RR, Leite Júnior RP, Balbi-Peña MI (2022) Sensibility of *Xanthomonas vasicola* pv. *vasculorum* to chemicals and efficiency of the chemical control of bacterial leaf streak on corn plants. Acta Sci 44:54952

Lu X, Zhang R, Cong M, Li J, Zhu F (2018) Stimulatory effects of flusilazole on virulence of *Sclerotinia sclerotiorum*. Plant Dis 102:197–201

Marco GM, Stall RE (1983) Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis 67:779–781

Marin TGS, Galvanin AL, Lanza FE, Behlau F (2019) Description of copper tolerant *Xanthomonas citri* subsp. *citri* and genotypic comparison with sensitive and resistant strains. Plant Pathol 68:1088–1098

Martins D, McKay GA, English AM, Nguyen D (2020) Sublethal paraquat confers multidrug tolerance in *Pseudomonas aeruginosa* by inducing superoxide dismutase activity and lowering envelope permeability. Front Microbiol 11:576708

Migliore L, Rotini A, Thaller MC (2013) Low doses of tetracycline trigger the *E. coli* growth: a case of hormetic response. Dose Response 11:550–7

Mo LY, Wang J, Qin LT, Yang YL, Liang N (2023) Mechanism of time-dependent toxicity of quinolone antibiotics on luminescent bacteria *Vibrio qinghaiensis* sp.-Q67. Ecotoxicol Environ Saf 255:114784

Morinière L, Burlet A, Rosenthal ER, Nesme X, Portier P, Bull CT, Lavire C, Fischer-Le Saux M, Bertolla F (2020) Clarifying the taxonomy of the causal agent of bacterial leaf spot of lettuce through a polyphasic approach reveals that *Xanthomonas cynarae* Trébaol et al. 2000 emend. Timilsina et al. 2019 is a later heterotypic synonym of *Xanthomonas hortorum* Vauterin et al. 1995. Syst Appl Microbiol 43:126087

Osdaghi E, Jones JB, Sharma A, Goss EM, Abrahamian P, Newberry EA, Potnis N, Carvalho R, Choudhary M, Paret ML, Timilsina S, Vallad GE (2021) A centenary for bacterial spot of tomato and pepper. Mol Plant Pathol 22:1500–1519

Peng J, Triplett LR, Sundin GW (2021) Activation of metabolic and stress responses during subtoxic expression of the type I toxin hok in *Erwinia amylovora*. BMC Genomics 22:74

Pohronezny K, Sommerfeld M, Raid R (1994) Streptomycin resistance and copper tolerance among strains of *Pseudomonas cichorii* in celery seedbeds. Plant Dis 78:150–3

Qi L, Li H, Zhang C, Liang B, Li J, Wang L, Du X, Liu X, Qiu S, Song H (2016) Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in *Acinetobacter baumannii*. Front Microbiol 12:7–483

Quezado-Duval AM, Gazzoto Filho A, Leite Júnior RP, Camargo LEA (2003) Sensibilidade a cobre, estreptomicina e oxitetraciclina em *Xanthomonas* spp. associadas à mancha-bacteriana do tomate para processamento industrial. Hortic Bras 21:670–675

Rana R, Jaiswal G, Bansal K, Patil PB (2023) Comparative genomics reveals the emergence of copper resistance in a non-pigmented *Xanthomonas* pathogen of grapevine. Environ Microbiol 6:716–726

Ranieri MR, Whitchurch CB, Burrows LL (2018) Mechanisms of biofilm stimulation by subinhibitory concentrations of antimicrobials. Curr Opin Microbiol 45:164–169

Revitt-Mills SA, Robinson A (2020) Antibiotic-induced mutagenesis: under the microscope. Front Microbiol 11:585175

RStudio T (2020) RStudio: Integrated development for R. RStudio, PBC, Boston Available in: http://www.rstudio.com/. Accessed 2024

Shaner G, Finney RE (1977) The effect of nitrogen fertilization on the expression of slow mildewing resistance in Knox wheat. Phytopathology 67:1051–1056

Sharma A, Sharma D, Verma SK (2019) Physiological and molecular plant pathology in silico identification of copper-binding proteins of *Xanthomonas translucens* pv. *undulosa* for their probable role in plant-pathogen interactions. Physiol Mol Plant Pathol 106:187–195

Silva EL, Freitas-Lopes RL, Lopes UP (2018) Fungicide-induced hormesis in phytopathogenic fungi and oomycetes: current status and research perspectives. Revisão Anual de Patologia de Plantas 26:69–80

Silva EL, Garzón CD, Lopes UP (2024) Hormetic-like effect and management of thiophanate-methyl resistance in *Lasiodiplodia theobromae* isolates. Trop Plant Pathol 49:936–942

Stebbing AR (1982) Hormesis - the stimulation of growth by low levels of inhibitors. Sci Total Environ 3:213–234

Strayer-Scherer A, Liao Y-Y, Abrahamian P, Timilsina S, Paret M, Momol T, Jones J, Vallad GE (2019) Integrated management of bacterial spot on tomato in Florida. University of Florida 6:8

Sun H, Zhang Y, Wang J, Ren LF, Tong D, Wang J, Tang L (2023) Time-dependent hormetic effects of polypeptide antibiotics and two antibacterial agents contribute to time-dependent cross-phenomena of their binary mixtures. Sci Total Environ 892:164343

Teixeira EC, Franco de Oliveira JC, Marques Novo MT, Bertolini MC (2008) The copper resistance operon *copAB* from *Xanthomonas axonopodis* pathovar *citri*: gene inactivation results in copper sensitivity. Microbiology 154:402–412

Timilsina S, Iruegas-Bocardo F, Jibrin MO, Sharma A, Subedi A, Kaur A, Minsavage GV, Huguet-Tapia JC, Klein-Gordon J, Adhikari P, Adhikari TB, Cirvilleri G, de la Barrera LBT, Bernal E, Creswell TC, Doan TTK, Coutinho TA, Ege DS, Félix-Gastélum R, Francis DM, Kebede M, Ivey ML, Louws FJ, Luo L, Maynard ET, Miller SA, Nguyen NTT, Osdaghi E, Quezado-Duval AM, Roach R, Rotondo F, Ruhl GE, Shutt VM, Thummabenjapone P, Trueman C, Roberts PD, Jones JB, Vallad GE, Goss EM (2025) Diversification of an emerging bacterial plant pathogen; insights into the global spread of Xanthomonas euvesicatoria pv. perforans. PLoS Pathogens 21:e1013036

Voloudakis AE, Reignier TM, Cooksey DA (2005) Regulation of resistance to copper in *Xanthomonas axonopodis* pv. *vesicatoria*. Appl Environ Microbiol 71:782–789

Yue Y, Liu H, Xia H, Chu Z (2023) Double- or triple-tiered protection: prospects for the sustainable application of copper-based antimicrobial compounds for another fourteen decades. Int J Mol Sci 24:108933

Zhang S, Fu Y, Mersha Z, Pernezny K (2017) Assessment of copper resistance in *Pseudomonas syringae* pv. *phaseolicola*, the pathogen of halo blight on snap bean. Crop Prot 98:8–15