Elucidating the mechanism of action of Copper heptagluconate on the plant immune system against *Pseudomonas syringae* in tomato (*Solanum lycopersicum* L)

Running title: Effect of Cu-heptagluconate against *Pseudomonas syringae* in tomato

Ana I González-Hernández[a], Eugenio Llorens[a*], Carlos Agustí-Brisach[b], Begonya Vicedo[a], Teresa Yuste[c], Antonio Cerveró[c], Carlos Ledó[c], Pilar García-Agustín[a] and Leonor Lapeña[a]

[a]Grupo de Bioquímica y Biotecnología, Departamento de Ciencias Agrarias y del Medio Natural, Universitat Jaume I de Castellón, Avenida de Vicent Sos Baynat, s/n, 12071 Castellón de la Plana, Spain.

[b]Grupo Patología Agroforestal, Departamento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales, Edif. C4, 14071 Córdoba, Spain.

[c]Departamento I+D+i, IDAI Nature S.L., C/Moscú, 10 Polígono Industrial Mas de Tous, 46185 La Pobla de Vallbona, Valencia, Spain.

e-mail: ellorens@uji.es

Abstract

BACKGROUND: Phytopathogenic problems caused by the bacterial pathogen *Pseudomonas syringae* in tomato have been becoming more serious by the emergence of resistant strains to classical pesticides. This situation has propelled the research for new formulations with lower environmental problems. One of the most promising alternatives to the use of classical pesticides is the induction of natural plant defences. New formulations based on Cu complexed with heptagluconic acid are able to induce

plant innate defences which could be an alternative to the classical treatments based on inorganic Cu against bacterial speck. In order to study the efficacy of this compound in tomato against *P. syringae*, we tested its systemic effect applied the treatments via radicular and infected tomato leaves.

RESULTS: Treated plants showed lower infection development and lower number of viable bacteria in leaves. We also observed better performance in parameters involved in plant resistance such as antioxidant response and accumulation of phenolic compounds.

CONCLUSION: Results showed that soil drench applications can be highly effective for the prevention and control of bacterial speck in tomato plants, showing a reduction of symptoms around 50%. Moreover, the application of Cu heptagluconate induced accumulation of plant polyphenols caffeic and chlorogenic acids and reduced the amount of ROS in infected plants.

Keywords: Copper heptagluconate, induced resistance, *Pseudomonas syringae*, *Solanum lycopersicum*

1 INTRODUCTION

Pseudomonas syringae is a hemibiotrophic pathogen with a high degree of specialization and host specificity. According to that specificity, the different *P. syringae* strains have been classified in pathovars (pv.) which only can infect a certain number of plant species or a certain plant species[1]. *Pseudomonas syringae* pv. *tomato* (Pst) (Okabe, 1933) Young, Dye & Wilkie, 1978 is considered as one of the most devastating pathogens of tomato plants (*Solanum lycopersicum* L.) over the world. This pathogen causes lesions on the stems, as well as on the petioles and fruits, which could

reduce the production of the plant by 60%. The typical symptoms are black necrotic specks surrounded by a yellow halo. The bacteria are able to survive on the plant leaf in an epiphytic lifestyle and enter into the apoplastic space through natural openings or wounds. If the bacterial population is high enough, the presence of elevated levels of homoserine lactone (AHL) will induce the expression of virulence factors and secondary metabolites that mediate colonization of the host. This coordinated expression of virulence factors depending on the population density is known as quorum-sensing (QS)[2]. As a hemibiotrophic pathogen, *P. syringae* can multiply very fast in a susceptible host under favorable conditions. However, this initial multiplication occurs in the apoplast, with the absence of apparent plant cell death. At the later stage of pathogenesis, plant cells die and infected tissues show necrotic spots[1]. Despite the large number of investigations about the pathogenicity of Pst[1,3], disease control is still based on preventive chemical spray applications. Nowadays, around 100 compounds including strains of non-pathogenic bacteria, plant extracts, essential oils, antibiotics, etc. have been tested to control bacterial diseases. However, only 20 of them have been tested against Pst[4]. But, these alternative compounds are not generalized or allowed in crop protection reducing the number of available compounds mainly to inorganic formulations of copper (Cu), such as Cu-hydroxide or Cu-sulphate, or a mixture of Cu-based and ethylene bis-dithiocarbamates (EBDC; commonly called mancozeb or maneb)[5]. In addition, resistant commercial tomato cultivars to Pst have not been obtained yet[4].

On the other hand, the generalized use of Cu-based compounds has lead the appearance of Cu resistant strains that reduced dramatically the effectiveness of classical treatments[6]. Moreover, the use of combinations of Cu-based compounds with EBDC are restricted to the pre-harvesting period since it can last for up to eight weeks

in tomato crops. Additionally, there are many environmental concerns about the accumulation of both Cu and EBDC in the soil. The extensive use of Cu-based fungicides is widely spread in agriculture reporting contamination not only in tomato fields but also in hop fields, apple and especially in vineyards[7–10]. In order to avoid these environmental problems, there is an increasing interest in developing compounds able to induce the plant natural defences as a potential alternative to chemicals. Likewise, it has been demonstrated that the application of some natural and chemical compounds induces a plant innate resistance that, usually, is enough to overcome the stress and survive[11,12]. Moreover, some resistant inducers showed a persistent effect up to several months, which can contribute to reduce the amount of residues in the vegetables and fruits[13,14].

The study of new compounds based on organic formulations of Cu and its double effect as an inductor of the plant innate defences would provide a valuable alternative to farmers to control pathogens such as *P. syringae*. Cu heptagluconate is a compound characterized by the great Cu absorption and diffusion over the plant, and it is safer for the environment compared with others traditional inorganic Cu-based fungicides[15]. Nevertheless, biochemical studies elucidating the mechanism of action of Cu-heptagluconate against Pst, and its compatibility and lack of phytotoxicity in tomato have not been performed yet. Therefore, the main goal of this study was to elucidate the efficacy of Cu enhancing the plant immune system in tomato against Pst. For this purpose, the pathosystem Pst vs. tomato was set up as a model to study the biochemistry and physiology of plant-microbe-Cu heptagluconate interaction. in order to know the mechanism of action of the treatment, infection development, physiological parameters, and several parameters enrolled in plant immune system were monitored.

2 MATERIALS AND METHODS

2.1 Plant material and growth chamber conditions

Tomato seeds of *Solanum lycopersicum* cv. Ailsa Craig were sowed in individual plastic pots (100 ml) filled with Sphagnum peat as substrate (Jiffy products, Kristiansand, Norway) and maintained in distilled water until the firsts true leaves were developed. Subsequently, seedlings were irrigated with Hoagland`s solution[16]. They were grown and pre-conditioned under growth chamber conditions at temperatures ranging from 18 to 26°C with a 16/8 h day/night photoperiod and 60% relative humidity (RH). Four-week old tomato plants were used for the experiments performed in this study. When the experiments were conducted, plants were placed in humid chambers (plastic containers, 59 × 40 × 35 cm, 80% RH), maintained in the growth chamber, and irrigated with Hoagland`s solution until the end of the experiment. The experiments were conducted in the growth chamber of the Department of Agricultural and Environmental Sciences of Universitat Jaume I (UJI) of Castellon (Spain).

2.2 Bacterial strain and inoculum preparation

The Pst strain DC3000, rifampicin resistant[17] was used in this study. The bacterial strain was grown in King B medium (KB)[18] with rifampicin added (50 mg ml^{-1}; Sigma Aldrich, San Luis, MO, USA) at 28ºC in darkness for 24 h. The bacterial suspension used for leaves inoculation was obtained from this culture, by resuspending the bacteria in sterile $MgSO_4$ (10 mM) solution containing 0.01% of the surfactant Silwet L-77 (Osi Specialties, Danbury, CT, USA) and adjusted to 5×10^5 colony-forming units (CFU) ml^{-1} using spectophotometer[19].

2.3 Effect of Cu heptagluconate on plant growth and plant immune system

2.3.1 Copper heptagluconate, treatments, and bacterial inoculation

122 Liquid Cu heptagluconate (Cu 6.0% p/p = 8.22% p/v, IDAI Cobre) was provided by the
123 company Idai Nature S.L. (La Pobla de Vallbona, Valencia, Spain).

124 Applications of Cu heptagluconate were performed when seedlings were at
125 three-four true leaf stage (four-week old tomato plants) with a solution at 6 ml l^{-1} as
126 recommended by the manufacturer for field applications. Preventive treatments were
127 performed by irrigating 20 ml of the solution per plant 72 h before inoculation, and
128 curative treatments were performed by irrigating 20 ml of the solution per plant, 24 h
129 after inoculation or by spraying 5ml of the solution per plant, 24 h after inoculation.
130 Pathogen inoculation was performed by dipping the third and fourth leaves into the
131 bacterial suspension described above for 3 sec. Additionally, plants treated with Cu
132 heptagluconate and non-inoculated, non-treated and inoculated (positive control), and
133 non-treated and non-inoculated (negative control) were included in the experiment for
134 comparative purposes. During the experiment, all plants were maintained in humid
135 chambers and irrigated as described above. There were 10 replicated plants per
136 treatment (4 treatments), 40 plants in total. The experiment was conducted three times.

137 *2.3.2 Disease severity, disease incidence, and plant growth*

138 To determine the disease severity (DS), third and fourth leaves of each plant and
139 treatment were selected at 72 h post-inoculation (pi) and evaluated by visual
140 observations determining the percentage of dark-brown spots developed on the leaf
141 surface. Disease incidence (DI) was determined by counting the cfu of Pst strain
142 DC3000 per gr of leaves developed in KB medium. To this end, three leaves samples of
143 50 mg of each infected treatment were washed with sterile water, ground and
144 resuspended in 50 ml of $MgSO_4$ 10 mM. Serial dilutions from these suspensions were
145 plated in KB medium with rifampicin (50 mg ml^{-1}). Cfu were counted after 24 h at 28º
146 C in darkness.

147 To evaluate the effect of the product on plant growth, total growth was evaluated
148 on non-inoculated plants at 30 days after Cu heptagluconate treatment measuring the
149 plant length from soil surface to the apical shoot. The plant length of non-treated plants
150 (control) was also measured. Moreover, the roots and shoots of the 10 plants of each
151 treatment (Cu heptagluconate and control) were separated and dried in an oven at 65 ºC
152 for 2 days. Subsequently, dried plant tissues were weighed, and the dry weight (DW)
153 was expressed as biomass.

154 *2.3.3 Determination and quantification of H_2O_2*

155 Samples of 10 leaves per treatment were collected for 3′,3-diaminobenzidine (DAB)
156 staining at 48 h pi. Leaves were cut and put immediately in 1 mg ml^{-1} DAB at pH <3
157 for 24 h in darkness and were subsequently discolored in 96% ethanol and rehydrated in
158 distilled water. DAB staining intensities were quantified in micrographies by the
159 number of dark-brown DAB pixels in relation to the total pixels corresponding to plant
160 material using the GIMP program (version 2.6.12) [20]

161 *2.3.4 Evaluation of hormones and phenolic compounds related to plant defense by*
162 *chromatographic analysis*

163 Fresh material (10 leaves per treatment and experiment; 1 leaf per plant in each
164 treatment and experiment) was frozen in liquid N, ground, and freeze-dried. Fresh tissue
165 (0.5 g) was immediately homogenized in 2.5 ml of ultrapure water, and a mixture of
166 internal standard {deuterated abscisic acid ([$^2H_6$] ABA), deuterated salicylic acid ([$^2H_4$]
167 SA), dihydrojasmonic acid (dhJA) and propylparaben} was added at 100 ng ml^{-1} prior
168 to extraction in order to quantify the level of hormones 12-oxo-phyto dienoic acid
169 [OPDA], SA and ABA) and phenolic compounds (Caffeic and chlorogenic acids),
170 respectively. After extraction, a 20 µl aliquot was injected directly into the high-
171 performance liquid chromatography (HPLC) system.

For both hormones and phenolic compounds measurements, analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 mm × 2 mm, i.d. 5 μm; Scharlab, Barcelona, Spain; http://www.scharlab.com). The chromatographic system was interfaced to a Quatro LC (quadrupole–hexapole–quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples[20]

2.4 Statistical analysis

All experiments were conducted at least two times. Data from different repetitions was analysed together due to the fact that analysis of variance (ANOVA) did not show significant differences ($P > 0.05$) between repetitions in each experiment. Subsequently, for each assessment, ANOVA was performed with DI, DS, plant growth, H_2O_2, hormones or phenolic compound levels as dependent variables and treatment (plants treated or non-treated with Cu heptagluconate and/or inoculated or non-inoculated with Pst DC3000 as independent variable). All data of this study were tested for normality, homogeneity of variances, and residual patterns. Mean values were compared using the Fisher's protected least significant difference (LSD) test at $P = 0.05$[21]. Statistical analyses were performed by using the software Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA, USA).

3 RESULTS

3.1 Effect of Cu heptagluconate on disease severity, plant growth, and bacterial populations

196 The preventive treatment with Cu heptagluconate by soil drench reduced markedly the
197 development of black necrotic specks triggered by Pst in tomato leaves (Fig 1). Plants
198 treated by Cu heptagluconate develop only a few symptoms of the disease on leaves 72
199 h pi with Pst strain DC3000 (Fig. 1B) in comparison with the visible damage observed
200 in leaves from non-treated and inoculated plants (positive control) (Fig. 1A). Significant
201 differences in Pst infection (%) and viable Pst populations (cfu mg^{-1} of leaf) were
202 observed between the positive control, and treated and inoculated plants (P = 0.0003
203 and P=0.0022, respectively). At 72 h pi, leaves from plants treated with Cu
204 heptagluconate showed 31.7% of leaf surface covered by black necrotic specks, whereas
205 the positive control had affected leaf surface > 70.51%, which represents a reduction in
206 the percentage of lesions by 55.1% by the use of Cu heptagluconate (Fig. 2A). The
207 control obtained with the preventive treatment is higher than the observed with curative
208 applications which showed a reduction of symptoms around 25% (supplementary fig1).
209 In the same way, the preventive treatment also significantly reduced the viable Pst
210 populations in tomato leaves compared with those from the positive control (Fig. 2B).
211 The analysis showed a bacterial population around 8.80×10^8 and 1.14×10^8 cfu g^{-1} of
212 leaf for inoculated treated and non-treated plants (control), respectively. This result
213 indicates that preventive soil drench applications with Cu heptagluconate are reduces
214 the presence of viable bacteria populations *in planta* by 85.9%, which supports the
215 reduction observed in the percentage of lesions on leaf surface. Due to the fact that the
216 preventive treatment showed better results reducing the bacterial symptoms, we focused
217 the analysis of the mechanism of action in this treatment.

218 **Physiological parameters**

219 Even though plants treated with Cu heptagluconate showed higher values of plant
220 height, and shoot and root dry weight than control plants, ANOVA did not show

significant differences (Plant height: $P = 0.5239$; Shoot dry weight: $P = 0.3804$; Root dry weight: $P = 0.4112$) between both lots of plants. This indicates that the uptake of Cu by the plant is not causing phytotoxicity so it does not compromise the development of the plant.

3.2 Effect of Cu heptagluconate on the levels of H_2O_2

In general, the formation of a dark brown insoluble precipitate was observed after leaf staining in 3,3 DAB for 24 h in all leaf samples tested, indicating H_2O_2 accumulation. Nevertheless, ANOVA showed significant differences in the levels of H_2O_2 between the four treatments tested ($P = 0.0002$). Samples collected from plants treated with Cu heptagluconate and inoculated with Pst showed fewer dark brown precipitate than that observed in samples from positive control plants. This result indicates that treatments with Cu heptaglunate decrease markedly the levels of H_2O_2 in treated plants. In fact, the level of H_2O_2 in positive control plants was 30% higher than that observed in plants treated with Cu heptagluconate and inoculated with Pst. Interestingly, the effect of Cu heptagluconate was also visible in the batch of plants which were treated but non-inoculated, also showing a reduction of ROS levels ≈30 times compared with positive and negative (non-treated and non-inoculated plants) controls (Fig. 3).

3.3 Effect of Cu heptagluconate on the enhancement of hormones related to plant defence

For all hormones tested, their levels were higher in inoculated plants in both treated and non-treated lots of plants with Cu heptagluconate, with the exception of SA. Levels of OPDA were higher in inoculated plants without significant differences ($P = 0.0858$) between positive control (437.6 ± 138.1 ng g^{-1} of leaf) and treated and inoculated plants (336.7 ± 12.1 ng g^{-1} of leaf) (Fig. 4A). On the other hand, the results obtained for SA

showed a different pattern to that observed for OPDA. The highest level of SA was observed in treated and inoculated plants (1206.3 ± 417.0 ng g^{-1} of leaf) showing no significant differences between the rest of the treatments tested (P = 0.5290). Moreover, the SA level of the positive control was lower (820.9 ± 281.2 ng g^{-1} of leaf) than that observed for treated and non-inoculated plants (998.7 ± 388.5 ng g^{-1} of leaf), but ANOVA did not show significant differences in SA level between these two treatments (P = 0.5290) (Fig. 4B). Finally, the levels of ABA were similar in plants from the four treatments tested without significant differences in ABA level between treatments (P = 0.8705). Despite the lack of significant differences between treatments in this last case, ABA levels were higher in both lots of inoculated plants (Positive control: 3479.6 ± 531.5 ng g^{-1} of leaf; Treated and inoculated plants: 3786.6 ± 349.7 ng g^{-1} of leaf) (Fig. 4C).

3.4 Effect of Cu heptagluconate on the levels of phenolic compounds

The measurement of the accumulation of phenolic compounds in leaves showed that soil drench treatments with Cu heptagluconate induce the accumulation of caffeic and chlorogenic acids. Accumulation of caffeic acid was induced in response to the inoculation with Pst in both plants, treated with Cu heptagluconate (18161.0 ± 2184.9 ng g^{-1} of leaf) and nontreated plants (17011.2 ± 2277.1 ng g^{-1} of leaf), without significant differences between treatments (P = 0.7341). Interestingly, treated and non-inoculated plants also showed a significantly high level of caffeic acid (14798.5 ± 3042.6 ng g^{-1} of leaf) compared to non-treated plants, but without significant differences between the two lots of inoculated plants (P = 0.6541). (Fig. 5A). On the other hand, accumulation of Chlorogenic acid was not observed in both lots of non-treated plants (negative and positive control), whereas the treatment with Cu heptagluconate induced the accumulation of this phenolic compound in both inoculated (5802.4 ± 3515.5 ng g^{-1}

of leaf) and non-inoculated (5720.9 ± 5431.9 ng g^{-1} of leaf) plants, without significant differences between them ($P = 0.4625$) (Fig. 5B).

4 DISCUSSION AND CONCLUSIONS

Copper-based fungicides are among the oldest pesticides used in agriculture. Since the discovery of the "Bordeaux mixture" in the nineteen century, the use of copper against plant diseases have been maintained until nowadays. However, the excessive use of inorganic formulations for the lasts 140 years has brought with it a number of problems. The first of these problems is the toxic levels of Cu due to anthropogenic activities[22,23]. The excessive use of Cu based pesticides to control plant diseases has resulted in Cu accumulation in the surface layer of agricultural soils[24,25]. In Europe, continuous spray of Cu based pesticides, such as Bordeaux mixture has drastically increased the Cu pollution of soils. In agricultural soils, normal Cu concentration varies from 5 to 30 mg kg^{-1} depending on soil type, but the soils of vineyards, which are commonly sprayed with this kind of pesticides, contain Cu that ranges from 200 to 500 mg kg^{-1} [26]. The excess of Cu may cause toxicity to the environment and for the crops. Is well known that photosynthetic reactions, both photochemical and biochemical ones, are usually inhibited many heavy metals and in particular Cu[27]. This excess of metal in the soil can result in decreasing final fruit number, dry root weight, and plant height[28].

On the other hand, the excessive use of copper in agriculture leads to the appearance of resistant pests. Since 1986, an increasing number of studies have reported Cu resistance in Pst[29] and other related pathogens such as *Xanthomonas* spp.[6,30] and the subsequently reduced efficacy of Cu-based products for controlling these pathogens. In this way, it has been demonstrated that the application of Cu is ineffective in managing tomato bacterial speck in South Africa, probably due to the presence of Cu resistant Pst

294 populations in the region[4]. Therefore, alternative management strategies must be
295 developed in order to combat those diseases and minimize the damage caused by the
296 excessive use of Cu.

297 In this study, we evaluated the effectiveness of a formulation of Cu complexed with
298 heptagluconic acid, which is characterized by high assimilability by the plant. The
299 results obtained showed that, although the formulation of this compound is absorbed
300 highly by the plant, there were no symptoms of phytotoxicity. A single treatment prior
301 to bacterial inoculation was able to reduce disease symptoms and the number of viable
302 bacteria in leaves by more than 50%. This control is higher than observed in treatments
303 with Cu hydroxide or other inorganic formulations in tomato, which can range from
304 40%[31] to no control in others[32]. The lack of control of regular inorganic formulations
305 has forced to the use of alternatives or combinations of compounds such as Cu with
306 mancozeb or Cu with Acibenzolar-s-methyl (ASM)[32,33]. Moreover, the content of
307 copper in the classical formulations can range from 20% of Cu in the Bordeaux mixture
308 to 50% of Cu in copper oxychloride formulations[34], whereas the content of copper in the
309 Cu heptagluconate formulation is only 6%. According to the recommended doses of
310 application, this reduction of Cu in the formulation would represent a reduction between
311 40 and 65% compared with classical formulations. In this way, under field conditions,
312 Dagostin *et al*.[35] demonstrated that heptagluconic acid controlled *P. viticola* at the same
313 levels than copper hydroxide reducing the amount of metallic copper per ha per year by
314 40% in comparison to the control treatments.

315 On the other hand, our results also showed a reduction of ROS in treated and infected
316 plants. Generation of ROS during pathogen attack is part of the defensive response and
317 is involved in the stimulation of hypersensitive cell death[36]. However, the accumulation
318 of ROS produced by the disruption of the cellular homeostasis under pathogen attacks

319 also produces oxidative damage in membrane lipids, proteins, and nucleic acids[37]. The
320 production of ROS in plants after *Pseudomonas syringae* infection is induced by the
321 non-host-specific phytotoxin coronatine (COR)[38]. COR is a toxin formed by the
322 coronafacic acid (CFA) and coronamic acid (CMA) which acts as a structural and
323 functional analog of plant signal molecules, such as jasmonic acid (JA)[38,39]. Presence of
324 COR promotes stomatal aperture, which facilitates the entry of the bacteria into the
325 mesophilic, and induce the JA pathway and antagonize SA-mediated defence responses
326 during Pst infection, preparing the plant for a successful pathogen colonization. Our
327 results showed that levels of OPDA are lower in plants treated with Cu than those
328 observed in untreated controls, and therefore, the SA is not repressed. This lack of
329 hormonal alteration expected by the presence of COR after *Pseudomonas* inoculation
330 and the reduced levels of ROS may indicate that the application of Cu via soil drench
331 could cause a delay or impairment in the pathogenesis machinery or an induction of
332 plant defensive responses. In order to ascertain if the application of the treatment is
333 inducing the plant natural defences we measured the phenolic compounds. These
334 metabolites have been found throughout the plant kingdom and possess antioxidant
335 activity and its induction has been described as part of the active defence of the plant
336 [40,41]. Besides its direct antimicrobial activity, phenolic compounds are also able to
337 inactivate microbe produced enzymes that are involved in pathogenesis or inhibit the
338 synthesis of specific toxins[42,43]. Our results show the application of Cu in soil drench
339 induces accumulation of chlorogenic and caffeic acids in absence of inoculation. In this
340 way, previous studies showed that the application of resistance inducers or priming
341 agents such as acibenzolar-S-methyl, isonitroacetophenone, lipopolysaccharide,
342 flagellin or chitosan resulted in the accumulation of phenolic compounds[40] which could
343 indicate that the application of Cu is protecting the plant not only by a direct

antimicrobial activity but also by the induction of plant defences. Previous studies about the effect of resistance inducers demonstrated that the application of priming compounds is able to affect the behavior of the bacteria in the plant. Scalschi *et al*.[2] observed that the reduction of bacterial population provoked by the resistance inducer application prevent bacteria from reaching the quorum sensing, delaying in this way the production of COR and the subsequent colonization of the plant[2].

As conclusion of this work, the application of Cu heptagluconate by irrigation manages to activate the defence mechanisms of the plant reducing the incidence of *P. syringae* by more than 50%, by the direct antimicrobial activity of Cu, together with the increase of the phenolic compounds an activation of this mechanism against the pathogen. It should be noted that the activation of plant innate immunity combined with the direct effect of Cu against the pathogen, could prevent the bacteria from reaching the quorum sensing and, thus, reduce the amount of COR produced by the bacteria with the subsequent reduction of oxidative damage and avoiding the hormonal balance disruption.

ACKNOWLEDGEMENTS

This research was financially supported by the 'Instituto Valenciano de Competitividad Empresarial' (IVACE) Ref. IFINOA/2014/46 and the Spanish Ministry of Science and Innovation (AGL2013-49023-C3-2-R). Ana I. González-Hernández is the holder of a fellowship by the "Programa de formacio del personal investigador (PREDOC/2016/27)" and C. Agustí-Brisach is the holder of a 'Juan de la Cierva-Incorporación' fellowship from MINECO. The authors are grateful to the 'Serveis Centrals d'Instrumentació Científica' (SCIC) from 'Universitat Jaume I' (UJI, Castellón, Spain).

REFERENCES

1 Xin X-F and He SY, *Pseudomonas syringae* pv. *tomato* DC3000: A Model Pathogen for Probing Disease Susceptibility and Hormone Signaling in Plants, Annu Rev Phytopathol **51**:473–498, Annual Reviews (2013).

2 Scalschi L, Camañes G, Llorens E, Fernández-Crespo E, López MM, García-Agustín P, *et al.*, Resistance inducers modulate pseudomonas syringae pv. Tomato strain DC3000 response in tomato plants, PLoS One **9**, Public Library of Science (2014).

3 Bashan Y and De-Bashan LE, Protection of tomato seedlings against infection by Pseudomonas syringae pv. tomato by using the plant growth-promoting bacterium Azospirillum brasilense., Appl Environ Microbiol **68**:2637–2643, American Society for Microbiology (ASM) (2002).

4 McLeod A, Masimba T, Jensen T, Serfontein K, and Coertze S, Evaluating spray programs for managing copper resistant Pseudomonas syringae pv. tomato populations on tomato in the Limpopo region of South Africa, Crop Prot **102**:32–42 (2017).

5 Ministerio de Agricultura Alimentación y Medio Ambiente, Registro de Productos Fitosanitarios - Agricultura - magrama.gob.es. http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/forexi.asp?e=0&plagEfecto=28&culUso=0104010401010000&ambUti=01&solEsp= [accessed 12 September 2017].

6 Griffin K, Gambley C, Brown P, and Li Y, 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 (2017).

393 7 Schramel O, Michalke B, and Kettrup A, Study of the copper distribution in
394 contaminated soils of hop fields by single and sequential extraction procedures,
395 Sci Total Environ **263**:11–22 (2000).

396 8 Li W, Zhang M, and Shu H, Distribution and Fractionation of Copper in Soils of
397 Apple Orchards (5 pp), Environ Sci Pollut Res - Int **12**:168–172, Ecomed (2005).

398 9 Adriano DC, Copper, Trace Elements in Terrestrial Environments, Springer New
399 York, New York, NY, pp. 499–546 (2001).

400 10 Komárek M, Čadková E, Chrastný V, Bordas F, and Bollinger J-C,
401 Contamination of vineyard soils with fungicides: A review of environmental and
402 toxicological aspects, Environ Int **36**:138–151 (2010).

403 11 Scalschi L, Vicedo B, Camanes G, Fernandez-Crespo E, Lapena L, Gonzalez-
404 Bosch C, *et al.*, Hexanoic acid is a resistance inducer that protects tomato plants
405 against Pseudomonas syringae by priming the jasmonic acid and salicylic acid
406 pathways, Mol Plant Pathol **14**, 2013/01/03:342–355 (2013).

407 12 Llorens E, Vicedo B, López MM, Lapeña L, Graham JH, and García-Agustín P,
408 Induced resistance in sweet orange against Xanthomonas citri subsp. citri by
409 hexanoic acid, Crop Prot **74** (2015).

410 13 Llorens E, García-Agustín P, and Lapeña L, Advances in induced resistance by
411 natural compounds: Towards new options for woody crop protection, Sci Agric
412 **74** (2017).

413 14 Llorens E, Scalschi L, Fernández-Crespo E, Lapeña L, and García-Agustín P,
414 Hexanoic acid provides long-lasting protection in 'Fortune' mandarin against
415 Alternaria alternata, Physiol Mol Plant Pathol **91**:38–45 (2015).

15 Zhang Y, Mao R, Zheng J, Xiao Y, Tang M, and Quan J, Laboratory Bioassay and Field Evaluation of Copper Gluconate as a New Potential Bactericide Against Xanthomonas campestris pv. citri (Hasse) Dye, J Agric Sci **5**:23 (2013).

16 Hoagland and Arnon D, The water-culture method for growing plants without soil, Circ Calif Agric Exp Stn **347** (1950).

17 Cuppels DA, Generation and Characterization of Tn5 Insertion Mutations in Pseudomonas syringae pv. tomato., Appl Environ Microbiol **51**:323–327, American Society for Microbiology (ASM) (1986).

18 KING EO, WARD MK, and RANEY DE, Two simple media for the demonstration of pyocyanin and fluorescin., J Lab Clin Med **44**:301–307 (1954).

19 Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, and Camañes G, NH 4 + protects tomato plants against Pseudomonas syringae by activation of systemic acquired acclimation, EXBOTJ (2015).

20 Llorens E, Agustí-Brisach C, González-Hernández AI, Troncho P, Vicedo B, Yuste T, *et al.*, Bioassimilable sulphur provides effective control of Oidium neolycopersici in tomato, enhancing the plant immune system, Pest Manag Sci (2016).

21 Steel RGD and Torrie JH, Bioestadistica: principios y procedimientos, 2nded., McGraw-Hill, Mexico (1986).

22 Yruela I, Copper in plants: acquisition, transport and interactions, Funct Plant Biol **36**:409, CSIRO PUBLISHING (2009).

23 Micó C, Recatalá L, Peris M, and Sánchez J, Assessing heavy metal sources in

438 agricultural soils of an European Mediterranean area by multivariate analysis,
439 Chemosphere (2006).

440 24 Mackie K, Müller T, and Kandeler E, Remediation of copper in vineyards–a mini
441 review, Environ Pollut (2012).

442 25 Michaud A, Bravin M, Galleguillos M, and Hinsinger P, Copper uptake and
443 phytotoxicity as assessed in situ for durum wheat (Triticum turgidum durum L.)
444 cultivated in Cu-contaminated, former vineyard soils, Plant Soil (2007).

445 26 Brun L, Maillet J, Richarte J, and Herrmann P, Relationships between extractable
446 copper, soil properties and copper uptake by wild plants in vineyard soils,
447 Environmental (1998).

448 27 Küpper H, Götz B, Mijovilovich A, Küpper FC, and Meyer-Klaucke W,
449 Complexation and toxicity of copper in higher plants. I. Characterization of
450 copper accumulation, speciation, and toxicity in Crassula helmsii as a new copper
451 accumulator., Plant Physiol **151**:702–714, American Society of Plant Biologists
452 (2009).

453 28 Sonmez S, Kaplan M, Sonmez NK, Kaya H, and Uz I, High level of copper
454 application to soil and leaves reduce the growth and yield of tomato plants, Sci
455 Agric **63**:213–218, Scientia Agricola (2006).

456 29 Bender CL and Cooksey DA, Indigenous plasmids in Pseudomonas syringae pv.
457 tomato: conjugative transfer and role in copper resistance., J Bacteriol **165**:534–
458 541, American Society for Microbiology (ASM) (1986).

459 30 Behlau F, Hong JC, Jones JB, and Graham JH, Evidence for Acquisition of
460 Copper Resistance Genes from Different Sources in Citrus-Associated

461 Xanthomonads, Phytopathology **103**:409–418, Scientific Societies (2013).

462 31 Iacobellis NS, Collmer A, Hutcheson SW, Mansfield JW, Morris CE, Murillo J,
463 *et al.*, Pseudomonas syringae and related pathogens : biology and genetic,
464 Springer Science+Business Media (2003).

465 32 Ji P, Campbell HL, Kloepper JW, Jones JB, Suslow TV, and Wilson M,
466 Integrated biological control of bacterial speck and spot of tomato under field
467 conditions using foliar biological control agents and plant growth-promoting
468 rhizobacteria, Biol Control **36**:358–367 (2006).

469 33 Huang C-H, Vallad GE, Zhang S, Wen A, Balogh B, Figueiredo JFL, *et al.*,
470 Effect of Application Frequency and Reduced Rates of Acibenzolar- *S* -Methyl
471 on the Field Efficacy of Induced Resistance Against Bacterial Spot on Tomato,
472 Plant Dis **96**:221–227, The American Phytopathological Society (2012).

473 34 Ferreira ICFR, Barros L, Soares ME, Bastos ML, and Pereira JA, Antioxidant
474 activity and phenolic contents of Olea europaea L. leaves sprayed with different
475 copper formulations, Food Chem **103**:188–195, Elsevier (2007).

476 35 Dagostin S, Schärer H-J, Pertot I, and Tamm L, Are there alternatives to copper
477 for controlling grapevine downy mildew in organic viticulture?, Crop Prot
478 **30**:776–788 (2011).

479 36 Apel K and Hirt H, Reactive oxygen species: metabolism, oxidative stress, and
480 signal transduction, Annu Rev Plant Biol **55**, 2004/09/21:373–399 (2004).

481 37 Mittler R, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci **7**,
482 2002/09/18:405–410 (2002).

483 38 Ishiga Y, Uppalapati SR, Ishiga T, Elavarthi S, Martin B, and Bender CL,
484 Involvement of coronatine-inducible reactive oxygen species in bacterial speck
485 disease of tomato., Plant Signal Behav **4**:237–239, Taylor & Francis (2009).

486 39 Uppalapati SR, Ayoubi P, Weng H, Palmer DA, Mitchell RE, Jones W, *et al.*,
487 The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone
488 pathways in tomato, Plant J **42**:201–217 (2005).

489 40 Hammerschmidt R, Chlorogenic acid: A versatile defense compound, Physiol
490 Mol Plant Pathol **88**:iii–iv (2014).

491 41 Baker CJ, Mock NM, Smith JM, and Aver'yanov AA, The dynamics of apoplast
492 phenolics in tobacco leaves following inoculation with bacteria., Front Plant Sci
493 **6**:649, Frontiers Media SA (2015).

494 42 Bostock RM, Wilcox SM, Wang G, and Adaskaveg JE, Suppression ofMonilinia
495 fructicolacutinase production by peach fruit surface phenolic acids, Physiol Mol
496 Plant Pathol **54**:37–50 (1999).

497 43 Wojciechowska E, Weinert CH, Egert B, Trierweiler B, Schmidt-Heydt M,
498 Horneburg B, *et al.*, Chlorogenic acid, a metabolite identified by untargeted
499 metabolome analysis in resistant tomatoes, inhibits the colonization by Alternaria
500 alternata by inhibiting alternariol biosynthesis, Eur J Plant Pathol **139**:735–747,
501 Springer Netherlands (2014).

502