

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

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

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16

17 **Abstract**

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

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

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

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

37

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

40

## 41 1 INTRODUCTION

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

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

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

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

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

## 98    **2        MATERIALS AND METHODS**

99 **2.1 Plant material and growth chamber conditions**

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

112 **2.2 Bacterial strain and inoculum preparation**

113 The Pst strain DC3000, rifampicin resistant<sup>17</sup> was used in this study. The bacterial strain  
114 was grown in King B medium (KB)<sup>18</sup> with rifampicin added (50 mg ml<sup>-1</sup>; Sigma  
115 Aldrich, San Luis, MO, USA) at 28°C in darkness for 24 h. The bacterial suspension  
116 used for leaves inoculation was obtained from this culture, by resuspending the bacteria  
117 in sterile MgSO<sub>4</sub> (10 mM) solution containing 0.01% of the surfactant Silwet L-77 (Osi  
118 Specialties, Danbury, CT, USA) and adjusted to 5 × 10<sup>5</sup> colony-forming units (CFU)  
119 ml<sup>-1</sup> using spectrophotometer<sup>19</sup>.

120 **2.3 Effect of Cu heptagluconate on plant growth and plant immune system**

121 *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<sup>-1</sup> 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<sub>4</sub> 10 mM. Serial dilutions from these suspensions were  
145 plated in KB medium with rifampicin (50 mg ml<sup>-1</sup>). Cfus were counted after 24 h at 28°  
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 °C  
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<sub>2</sub>O<sub>2</sub>*

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<sup>-1</sup> 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) <sup>20</sup>

### 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 ([<sup>2</sup>H<sub>6</sub>] ABA), deuterated salicylic acid ([<sup>2</sup>H<sub>4</sub>]  
167 SA), dihydrojasmonic acid (dhJA) and propylparaben} was added at 100 ng ml<sup>-1</sup> 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.

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

## 180 2.4 Statistical analysis

181 All experiments were conducted at least two times. Data from different  
182 repetitions was analysed together due to the fact that analysis of variance (ANOVA) did  
183 not show significant differences ( $P > 0.05$ ) between repetitions in each experiment.  
184 Subsequently, for each assessment, ANOVA was performed with DI, DS, plant growth,  
185 H<sub>2</sub>O<sub>2</sub>, hormones or phenolic compound levels as dependent variables and treatment  
186 (plants treated or non-treated with Cu heptagluconate and/or inoculated or non-  
187 inoculated with Pst DC3000 as independent variable). All data of this study were tested  
188 for normality, homogeneity of variances, and residual patterns. Mean values were  
189 compared using the Fisher's protected least significant difference (LSD) test at  $P =$   
190  $0.05^{21}$ . Statistical analyses were performed by using the software Statgraphics Centurion  
191 XVI (Statpoint Technologies, Warrenton, VA, USA).

192

## 193 3 RESULTS

### 194 3.1 Effect of Cu heptagluconate on disease severity, plant growth, and bacterial 195 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<sup>-1</sup> 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  $8.80 \times 10^8$  and  $1.14 \times 10^8$  cfu g<sup>-1</sup> 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

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

### 225 **3.2 Effect of Cu heptagluconate on the levels of H<sub>2</sub>O<sub>2</sub>**

226 In general, the formation of a dark brown insoluble precipitate was observed after leaf  
227 staining in 3,3 DAB for 24 h in all leaf samples tested, indicating H<sub>2</sub>O<sub>2</sub> accumulation.  
228 Nevertheless, ANOVA showed significant differences in the levels of H<sub>2</sub>O<sub>2</sub> between the  
229 four treatments tested ( $P = 0.0002$ ). Samples collected from plants treated with Cu  
230 heptagluconate and inoculated with Pst showed fewer dark brown precipitate than that  
231 observed in samples from positive control plants. This result indicates that treatments  
232 with Cu heptagluconate decrease markedly the levels of H<sub>2</sub>O<sub>2</sub> in treated plants. In fact, the  
233 level of H<sub>2</sub>O<sub>2</sub> in positive control plants was 30% higher than that observed in plants  
234 treated with Cu heptagluconate and inoculated with Pst. Interestingly, the effect of Cu  
235 heptagluconate was also visible in the batch of plants which were treated but non-  
236 inoculated, also showing a reduction of ROS levels  $\approx 30$  times compared with positive  
237 and negative (non-treated and non-inoculated plants) controls (Fig. 3).

### 238 **3.3 Effect of Cu heptagluconate on the enhancement of hormones related to** 239 **plant defence**

240 For all hormones tested, their levels were higher in inoculated plants in both treated and  
241 non-treated lots of plants with Cu heptagluconate, with the exception of SA. Levels of  
242 OPDA were higher in inoculated plants without significant differences ( $P = 0.0858$ )  
243 between positive control ( $437.6 \pm 138.1 \text{ ng g}^{-1}$  of leaf) and treated and inoculated plants  
244 ( $336.7 \pm 12.1 \text{ ng g}^{-1}$  of leaf) (Fig. 4A). On the other hand, the results obtained for SA

245 showed a different pattern to that observed for OPDA. The highest level of SA was  
246 observed in treated and inoculated plants ( $1206.3 \pm 417.0 \text{ ng g}^{-1}$  of leaf) showing no  
247 significant differences between the rest of the treatments tested ( $P = 0.5290$ ). Moreover,  
248 the SA level of the positive control was lower ( $820.9 \pm 281.2 \text{ ng g}^{-1}$  of leaf) than that  
249 observed for treated and non-inoculated plants ( $998.7 \pm 388.5 \text{ ng g}^{-1}$  of leaf), but  
250 ANOVA did not show significant differences in SA level between these two treatments  
251 ( $P = 0.5290$ ) (Fig. 4B). Finally, the levels of ABA were similar in plants from the four  
252 treatments tested without significant differences in ABA level between treatments ( $P =$   
253  $0.8705$ ). Despite the lack of significant differences between treatments in this last case,  
254 ABA levels were higher in both lots of inoculated plants (Positive control:  $3479.6 \pm$   
255  $531.5 \text{ ng g}^{-1}$  of leaf; Treated and inoculated plants:  $3786.6 \pm 349.7 \text{ ng g}^{-1}$  of leaf) (Fig.  
256 4C).

### 257 **3.4 Effect of Cu heptagluconate on the levels of phenolic compounds**

258 The measurement of the accumulation of phenolic compounds in leaves showed that  
259 soil drench treatments with Cu heptagluconate induce the accumulation of caffeic and  
260 chlorogenic acids. Accumulation of caffeic acid was induced in response to the  
261 inoculation with Pst in both plants, treated with Cu heptagluconate ( $18161.0 \pm 2184.9$   
262  $\text{ng g}^{-1}$  of leaf) and nontreated plants ( $17011.2 \pm 2277.1 \text{ ng g}^{-1}$  of leaf), without  
263 significant differences between treatments ( $P = 0.7341$ ). Interestingly, treated and non-  
264 inoculated plants also showed a significantly high level of caffeic acid ( $14798.5 \pm$   
265  $3042.6 \text{ ng g}^{-1}$  of leaf) compared to non-treated plants, but without significant differences  
266 between the two lots of inoculated plants ( $P = 0.6541$ ). (Fig. 5A). On the other hand,  
267 accumulation of Chlorogenic acid was not observed in both lots of non-treated plants  
268 (negative and positive control), whereas the treatment with Cu heptagluconate induced  
269 the accumulation of this phenolic compound in both inoculated ( $5802.4 \pm 3515.5 \text{ ng g}^{-1}$

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

#### 272 4 DISCUSSION AND CONCLUSIONS

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

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

294 populations in the region<sup>4</sup>. 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%<sup>31</sup> to no control in others<sup>32</sup>. 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)<sup>32,33</sup>. 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<sup>34</sup>, 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.*<sup>35</sup> 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<sup>36</sup>. 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<sup>37</sup>. The  
320 production of ROS in plants after *Pseudomonas syringae* infection is induced by the  
321 non-host-specific phytotoxin coronatine (COR)<sup>38</sup>. 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)<sup>38,39</sup>. 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 <sup>40,41</sup>. 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<sup>42,43</sup>. 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<sup>40</sup> which could  
343 indicate that the application of Cu is protecting the plant not only by a direct

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

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

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## 368 **REFERENCES**

- 369 1 Xin X-F and He SY, *Pseudomonas syringae* pv. *tomato* DC3000: A Model  
370 Pathogen for Probing Disease Susceptibility and Hormone Signaling in Plants,  
371 *Annu Rev Phytopathol* **51**:473–498, *Annual Reviews* (2013).
- 372 2 Scalschi L, Camañes G, Llorens E, Fernández-Crespo E, López MM, García-  
373 Agustín P, *et al.*, Resistance inducers modulate *pseudomonas syringae* pv.  
374 Tomato strain DC3000 response in tomato plants, *PLoS One* **9**, Public Library of  
375 Science (2014).
- 376 3 Bashan Y and De-Bashan LE, Protection of tomato seedlings against infection by  
377 *Pseudomonas syringae* pv. *tomato* by using the plant growth-promoting  
378 bacterium *Azospirillum brasilense*., *Appl Environ Microbiol* **68**:2637–2643,  
379 American Society for Microbiology (ASM) (2002).
- 380 4 McLeod A, Masimba T, Jensen T, Serfontein K, and Coertze S, Evaluating spray  
381 programs for managing copper resistant *Pseudomonas syringae* pv. *tomato*  
382 populations on tomato in the Limpopo region of South Africa, *Crop Prot* **102**:32–  
383 42 (2017).
- 384 5 Ministerio de Agricultura Alimentación y Medio Ambiente, Registro de  
385 Productos Fitosanitarios - Agricultura - [magrama.gob.es](http://magrama.gob.es).  
386 <http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos->  
387 [fitosanitarios/registro/productos/forexi.asp?e=0&plagEfecto=28&culUso=01040](http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/forexi.asp?e=0&plagEfecto=28&culUso=01040)  
388 [10401010000&ambUti=01&solEsp=](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].
- 389 6 Griffin K, Gambley C, Brown P, and Li Y, Copper-tolerance in *Pseudomonas*  
390 *syringae* pv. *tomato* and *Xanthomonas* spp. and the control of diseases associated  
391 with these pathogens in tomato and pepper. A systematic literature review, *Crop*  
392 *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, Lapeña 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).

- 416 15 Zhang Y, Mao R, Zheng J, Xiao Y, Tang M, and Quan J, Laboratory Bioassay  
417 and Field Evaluation of Copper Gluconate as a New Potential Bactericide  
418 Against *Xanthomonas campestris* pv. *citri* (Hasse) Dye, *J Agric Sci* **5**:23 (2013).
- 419 16 Hoagland and Arnon D, The water-culture method for growing plants without  
420 soil, *Circ Calif Agric Exp Stn* **347** (1950).
- 421 17 Cuppels DA, Generation and Characterization of Tn5 Insertion Mutations in  
422 *Pseudomonas syringae* pv. *tomato*., *Appl Environ Microbiol* **51**:323–327,  
423 American Society for Microbiology (ASM) (1986).
- 424 18 KING EO, WARD MK, and RANEY DE, Two simple media for the  
425 demonstration of pyocyanin and fluorescin., *J Lab Clin Med* **44**:301–307 (1954).
- 426 19 Fernández-Crespo E, Scalschi L, Llorens E, García-Agustín P, and Camañes G,  
427 NH<sub>4</sub><sup>+</sup> protects tomato plants against *Pseudomonas syringae* by activation of  
428 systemic acquired acclimation, *EXBOTJ* (2015).
- 429 20 Llorens E, Agustí-Brisach C, González-Hernández AI, Troncho P, Vicedo B,  
430 Yuste T, *et al.*, Bioassimilable sulphur provides effective control of *Oidium*  
431 *neolycopersici* in tomato, enhancing the plant immune system, *Pest Manag Sci*  
432 (2016).
- 433 21 Steel RGD and Torrie JH, *Bioestadística: principios y procedimientos*, 2nded.,  
434 McGraw-Hill, Mexico (1986).
- 435 22 Yruela I, *Copper in plants: acquisition, transport and interactions*, *Funct Plant*  
436 *Biol* **36**:409, CSIRO PUBLISHING (2009).
- 437 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 of *Monilinia*  
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