Zinc phosphate protects tomato plants against *Pseudomonas syringae* pv. *tomato*

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Abstract

The purpose of this study was to determine whether zinc phosphate treatments of tomato plants (*Solanum lycopersicum* L.) can attenuate bacterial speck disease severity through reduction of *Pseudomonas syringae* pv. *tomato* (*Pst*) growth *in planta* and induce morphological and biochemical plant defence responses. Tomato plants were treated with 10 ppm (25.90 µM) zinc phosphate and then spray inoculated with strain DAPP-PG 215, race 0 of *Pst*. Disease symptoms were recorded as chlorosis and/or necrosis per leaf (%) and as numbers of necrotic spots. Soil treatments with zinc phosphate protected susceptible tomato plants against *Pst*, with reductions in both disease severity and pathogen growth *in planta*. The reduction of *Pst* growth *in planta* combined with significantly higher zinc levels in zinc-phosphate-treated plants indicated direct antimicrobial toxicity of this microelement, as also confirmed by *in vitro* assays. Morphological (*i.e.* callose apposition) and biochemical (*i.e.*, expression of salicylic-acid-dependent pathogenesis-related protein *PR1b1* gene) defence responses were induced by the zinc phosphate treatment, as demonstrated by histochemical and qPCR analyses, respectively. In conclusion, soil treatments with zinc phosphate can protect tomato plants against *Pst* attacks through direct antimicrobial activity and induction of morphological and biochemical plant defence responses.

Keywords *Pseudomonas syringae* pv. *tomato* · Bacterial speck disease · Callose deposition · Integrated pest management · *PIN2* · *PR1b1* · Resistance induction

Introduction

Bacterial speck disease is caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young, Dye & Wilkie (*Pst*) and it represents one of the most important foliar diseases of tomato, as it can cause severe losses under cool and rainy conditions (Yunis et al. 1980; Shanin 2001). It also remains difficult to control. Although tomato genotypes with high or intermediate resistance to *Pst* are available on the market, as the pathogen has been shown to overcome this resistance (Buonauro et al. 1996; Kunkeaw et al. 2010; Lin and Martin

2007; Thapa and Coacker 2016), there is the need for further methods of control.

The European Union (EU) pesticides database (<http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database>) includes methods permitted for the control of plant pathogenic bacteria in the EU. These include microorganisms such as *Aureobasidium pullulans* DSM 14940 and DSM 14941, *Bacillus subtilis* QST 713 and *Streptomyces lydicus* WYEC 108. The bactericidal substances included are aluminium sulphate, benzoic acid, copper compounds, sodium hypochlorite and vinegar. Finally, there are also plant activators included, such as acibenzolar-S-methyl (ASM; also known as benzothiadiazole) and cerevisane (an inert extract obtained from cell walls of *Saccharomyces cerevisiae* LAS 117; Romeo[®], Sumitomo Chemical Italy). However, from among these, only commercial formulations based on the active ingredients of *B. subtilis* QST 713, copper and ASM are registered to date for use on tomato for control of bacterial speck disease.

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As reported in Commission Implementing Regulation (EU) 2016/673, the use of *B. subtilis* and copper compounds is permitted in organic tomato farming in the EU. Both of these have roles in the prevention of infections.

Treatments with copper-based formulations immediately before or after *Pst* infection have been shown to significantly reduce bacterial speck disease severity, both as plant stage and under climatic conditions optimal for disease development (Yunis et al. 1980). However, the effectiveness of these treatments has been reduced due to the appearance of copper-resistant *Pst* strains (Bender and Cooksey 1986; Cha and Cooksey 1991). Moreover, due to eco-toxicological issues, a maximum limit of 4 kg ha⁻¹ year⁻¹ for copper application has been imposed in Europe (Commission Implementing Regulation (EU) 2018/1981). These ecotoxicological aspects, and in particular copper persistence, bioaccumulation and toxicity, mean that copper compounds are now on the list of substances that are ‘candidates for substitution’, according to Regulation (European Commission) 2009/1107.

Inorganic salts represent a promising tool for both integrated and organic management of plant diseases, with some of them already commonly used in the food industry (Deliopoulos et al. 2010; Kuepper et al. 2001). According to the US Environmental Protection Agency (EPA 2018), inorganic salts such as potassium salts (*e.g.*, bicarbonate, dihydrogen phosphate, silicate) can be termed ‘biochemical pesticides’; *i.e.* substances found in nature (*i.e.* in animals, microbes, minerals, plants) that are effective for plant disease protection. Inorganic salts such as sodium bicarbonate are permitted in organic farming in Europe (Commission Implementing Regulations (EU) 2016/673, 2018/1584). The effectiveness of treatments with inorganic salts for the control of phytopathogenic oomycetes and fungi is well documented, as summarised in the review of Deliopoulos et al. (2010), while only limited data available on the protective effects of inorganic salts against phytopathogenic bacteria (Norman et al. 2006; Lin et al. 2008; Wen et al. 2009; Yaganza et al. 2014).

In this study, we tested the effects of zinc phosphate salt on bacterial speck disease severity, *Pst* growth *in planta* and *in vitro*, and on its induction of morphological and biochemical defence responses in tomato plants (*i.e.* callose deposition, accumulation of defence gene transcripts).

Materials and methods

Plant treatments, bacterial growth and plant inoculation

Tomato plants (*Solanum lycopersicum* L.) cv. Rio Grande were grown in peat for 4 weeks (to second to third true

leaf stage) in a growth chamber at 22 °C to 24 °C, with 240 µE m⁻² s⁻¹ illumination, and a 12-h light period. The plants were then transplanted into pots (324 cm³, one plant per pot) that contained peat (Traysubstrat, Klasmann-Deilmann GmbH) without (negative control) or with addition of 0.01 g kg⁻¹ (10 ppm, 25.90 µM) zinc phosphate [Zn₃(PO₄)₂; Sigma-Aldrich, MO, Saint Louis, USA]. As reported by Gurmani et al. (2012), in tomato plants cv. Rio Grande application of zinc phosphate to soil (in a range from 0 to 0.015 g Kg⁻¹) exerted its highest effect on plant growth and yield at the dose of 0.01 g Kg⁻¹, the same used in our treatments. Plants were then kept in a growth chamber under the above-indicated conditions.

Zinc phosphate was chosen here since both zinc and phosphorus-based salts are known to induce disease resistance in plants (Alexandersson 2016; Orober et al. 2002; Tuzun and Bent 2006). Due to the poor solubility of zinc phosphate, we chose to incorporate it in soil for prolonging its eventual induced resistance capability. This zinc phosphate dose (10 ppm) was the most effective for reduction of bacterial speck disease severity while remaining non-phytotoxic, as we determined in preliminary experiments (Quaglia et al. unpublished data). ASM was used as the positive control (Bion 50 WG-50% wettable granule formulation, 50% active ingredient; Syngenta Crop Protection, Monthey SA, Switzerland). This was applied as a soil drench (*i.e.* 50 mL per pot) from a water solution at 2.1 × 10⁻⁴ mol L⁻¹. This dose is reported in the literature as non-phytotoxic to tomato seedlings and plants, and protective against root-knot nematodes (Molinari 2014) and *Pst* (Scarponi et al. 2001). Moreover, it is similar to the dose that activates defence responses in tomato plants (Soylu et al. 2003).

After transplanting, each plant was irrigated with 50 mL water every 2 days until the end of the trial, and kept in a climatic chamber under the conditions reported above. At 14 days post-treatment (dpt), the tomato plants were spray inoculated with *Pst* DAPP-PG 215 race 0 (from the collection of the Department of Agricultural, Food and Environmental Sciences, University of Perugia, Italy). The inoculum was prepared by growing *Pst* on nutrient agar medium (NA, Thermo Scientific Fisher, Germany) at 27 ± 2 °C for 48 h. These were then suspended in sterile distilled water and spectrophotometrically adjusted to 10⁸ CFU mL⁻¹.

In the growth chamber, the plants were covered with plastic bags for the first 48 h. Disease symptoms were recorded 15 dpi in the youngest (third, fourth and fifth) inoculated leaves of each plant, by evaluation of the levels of chlorosis and/or necrosis per leaf area (%), and counting of the number of necrotic spots on digital photos. For the purpose, the Assess software (Image Analysis Software for Plant Disease Quantification; APS Press, St. Paul, MN, USA; Lamari 2002) was used. Each experiment was

independently repeated four times, with four replicates (plants) per treatment.

Effects of zinc phosphate on *Pseudomonas syringae* pv. *tomato* growth *in planta* and *in vitro*

The effects of the zinc phosphate and ASM (positive control) treatments on *Pst* growth *in planta* were determined at 6 dpi. This time-point was chosen as we previously reported that a decrease in *Pst* growth starting from 6 dpi in ASM-treated tomato plants (Scarponi et al. 2001). Three leaf disks (diameter, 10 mm) from three leaflets of the third true leaf (where symptoms were more evident) were ground in a mortar with sterile deionised water. Leaf homogenates were diluted ten-fold, and 100 µL drops of each dilution were streaked onto NA plates. After 24 h incubation at 27 ± 1 °C, the number of *Pst* colonies was counted. Each experiment was independently repeated twice, with four replicates (plants) per treatment.

The effects of zinc phosphate on *in vitro* *Pst* growth were determined using ELISA 96-well plates that contained 160 µL King's B medium (King et al. 1954), 20 µL bacterial suspension (10^8 CFU mL⁻¹) and increasing concentrations of zinc phosphate (0, 25, 40, 50, 60, 75 ppm) in a final volume of 200 µL. To obtain the zinc phosphate concentration series, a 100 ppm stock solution of zinc phosphate in 2% HCl was diluted as necessary. The effects of the increasing final HCl concentrations (0.5%, 0.8%, 1.0%, 1.2%, 1.5%) on *Pst* growth were also determined. The plates were incubated at 27 °C in a microplate reader (Thermo Scientific Multi-Skan EX; Thermo Fisher Scientific, Waltham, MA, USA) set for 5 s of shaking every minute, with the reading of the OD₆₃₀ every hour for 24 h. The data obtained from the HCl effects were subtracted from those obtained from the zinc phosphate samples. The effective concentrations (EC) were calculated in the exponential phase of the bacterial growth (15 h), as reported in the paragraph Statistical analysis.

Determination of zinc and phosphorous levels in tomato leaves

At 28 days post-treatment (dpt), three true leaves of the tomato plants (third, fourth, fifth) treated with water (negative control), zinc phosphate or ASM were oven-dried at 60 °C for 48 h, and homogenised. The total zinc concentrations were determined following US-EPA Method 3052B (USEPA 1996). Briefly, 0.25 g of each sample was microwave-digested (Ethos One high-performance microwave digestion system; Milestone Inc., Sorisole, Bergamo, Italy) in the presence of ultrapure concentrated nitric acid (65% w/w) and 2 mL hydrogen peroxide (30% w/w), for 30 min at 1,000 W and 200 °C. After cooling, the samples were diluted to 20 mL with Milli-Q water (18.2 MΩ) and

filtered (pore size, 0.22 µm). The zinc concentrations were determined using an atomic absorption spectrophotometer (AA-6200; Shimadzu, Tokyo, Japan). A range of zinc concentrations (0.1–0.5 mg L⁻¹) was used as standards, and the zinc concentrations were determined for each sample by comparison with the standard curve. This method was accurately validated using a recovery test ($n=3$) by adding a zinc standard solution (4 mg L⁻¹) into a mixture of a zinc-enriched sample and nitric acid prior to digestion in tubes and after appropriate dilution, according to US-EPA Method 3052B. Once diluted, the same nitric acid solutions reported above were analysed spectrophotometrically for phosphorus content using a molybdic reagent, according to the method of Murphy and Riley (1962). Each experiment was independently repeated three times with three replicates (bulks of 10 plants each) per treatments.

Callose quantification in tomato leaves

At 48 h post-treatments (hpt) with water, zinc phosphate or ASM, small pieces of the leaves (5 × 5 mm) were collected from the first leaflet of the third true leaf of the tomato plants and cleared in 96% ethanol at 80 °C for 10 min. These were then stained with aniline blue (Carlo Erba Reagents S.r.l., Italy), as reported by Luna et al. (2011).

Callose quantification of the stained samples was performed by epifluorescence microscopy, equipped with UV filters (excitation, BP 365–395; barrier, LP 420) and using the ImageJ software (Quaglia et al. 2017). Each experiment was independently repeated three times with three plants and three leaf pieces per treatment, and a photograph was taken at random for each piece, for a total of nine replicates per treatment.

RNA extraction and qRT-PCR analysis

The fifth leaf of the tomato plants treated with water (negative control), zinc phosphate or ASM was collected at 24, 48 and 96 hpt and snap-frozen in liquid nitrogen. Total RNA was extracted using PureLink RNA mini kits (Ambion, Life Technologies, Carlsbad, CA, USA), and DNase treatments were performed using the PureLink DNase set (Ambion, Life Technologies, Carlsbad, CA, USA). RNA concentrations were measured using a fluorimeter (Qubit 2.0; Thermo Fisher Scientific, BioRad, Hercules, CA) and assay kits (Qubit RNA BR). Five-hundred ng of total RNA was reverse transcribed into the first strand cDNA using the iScript cDNA synthesis kits (Bio-Rad Laboratories Inc., Hercules, CA, USA). Then, 5 µL (20 ng) 1:20 cDNA (diluted in RNase-free water) was mixed with 10 µL SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA); 0.4 µM specific primers for the salicylic-acid-responsive tomato 'pathogenesis-related protein 1b1' (*PR1b1*) gene

(Goyal et al. 2016) and for the jasmonate-mediated ‘protease inhibitor 2’ (*PIN2*) gene (Castagna et al. 2007) (Supplementary Table S1) were added, in a 20 µL reaction volume. The relative gene expression levels were normalised to actin (Goyal et al. 2016) and elongation factor 1- α (*EF1- α*) (Fowler et al. 2009), as the housekeeping genes (Supplementary Table 1). All of these reactions were carried out using a PCR machine (CFX96 Real-Time PCR Detection System; Bio-Rad, Hercules, CA, USA), where the PCR programme comprised: 98 °C for 2 min, 40 cycles at 98 °C for 3 s and 56 °C for 30 s, 95 °C for 10 s, cooling at 65 °C for 1 min, and finally an increase to 95 °C at a 0.2 °C increase every 10 s, with measurement of fluorescence. A dissociation curve was included at the end of the qPCR programme to monitor for potential primer-dimers and non-specific amplification products. Threshold cycles (*C_q*) were used to quantify the relative gene expression according to the method of Pfaffl (2001), and as modified in Vandesompele et al. (2002), to account for the two reference genes. *C_q* differences were calculated by adopting the *C_q* values for water application at 24 h as the treatment control. Normalised expression was log + 1 transformed before plotting and analysis. Each experiment was repeated independently twice, with three biological and technical replicates.

Statistical analysis

The data relating to the effects of the treatments on bacterial speck disease severity, *in planta* *Pst* growth, callose deposition, *PR1b1* and *PIN2* gene expression, and zinc and phosphorous levels were separately submitted to one-way (treatment) analysis of variance (ANOVA), with significant differences compared using Duncan multiple range tests, using the Excel software macro DSAASTAT (Onofri and Pannacci 2014). Details of the experimental settings are described in the Figure legends. The data related to the *Pst* *in vitro* growth were analysed using a nonlinear regression dose–response model proposed by Streibig et al. (1993), according to Eq. (1):

$$Y = C + \frac{D - C}{1 + \exp\{b[\log(X) - \log(a)]\}} \quad (1)$$

where *Y* is the bacterial growth reduction as a function of zinc phosphate concentration, *D* is the upper asymptote (positive control response), *C* is the lower asymptote (response of highest concentration tested), *a* is the zinc phosphate concentration that gives the intermediate response between the upper and the lower asymptotes, and *b* is the slope at the point of inflection.

The resulting dose–response curve was used to calculate the effective concentrations as *EC*₁₀, *EC*₅₀ and *EC*₉₀, which

indicate the concentrations of zinc phosphate corresponding to bacterial growth reductions of 10%, 50% and 90%, respectively, with respect the untreated control (Pestemer and Günther 1995).

The data fitting to the nonlinear regression model was evaluated using lack-of-fit F-tests (Ritz and Streibig 2005). Statistical analysis was performed using the Excel software macros BIOASSAY97 and DSAASTAT (Onofri and Pannacci 2014).

Results

Effect of zinc phosphate and ASM treatments on tomato plants protection from *Pseudomonas syringae* pv. *tomato* inoculation

The leaves of the tomato plants grown in the soil with added 10 ppm zinc phosphate were systemically protected against *Pst* attack. Indeed, the treatments with zinc phosphate significantly reduced the *Pst* disease severity at 14 dpi, as expressed by the diseased areas of the leaves (%) and numbers of bacterial spots per leaf (Fig. 1a, b). The significant reduction in the mean diseased area for the zinc phosphate treatment (0.69%) was not significantly different to that for the ASM treatment (0.81%) (Fig. 1a). Instead, a significant reduction in the mean number of spots per leaf with 10 ppm zinc phosphate (17.02 spots per leaf) was more marked for the 2.1×10^{-4} mol L⁻¹ ASM treatment (3.22 spots per leaf) (Fig. 1b).

For these tomato plants that were treated with zinc phosphate, at 6 dpi the reduction in disease severity was accompanied by a significant (7.5%) reduction, from the control, of the *in planta* growth of *Pst*, with the ASM treatment showing a similar but greater reduction (10.3%) (Fig. 1c).

The *in vitro* effects of different zinc phosphate concentrations on *Pst* growth were expressed as OD₆₃₀ relative to the control (%), and these fitted well to the log-logistic curve given in Eq. (2) (Fig. 2):

$$y = 100 / \{1 + \exp [7.58 (\log (x) - \log (45.9))]\} \quad (2)$$

On the basis of this curve, the calculated effective concentrations for the zinc phosphate treatments are 34.3 ± 3.53 ppm (*EC*₁₀), 45.9 ± 1.95 ppm (*EC*₅₀) and 61.3 ± 5.55 ppm (*EC*₉₀).

Effect of zinc phosphate treatments on the induction of callose deposition and defence gene expression

A marked and significant increase in callose deposition was observed at 48 hpt for the leaves of the tomato plants treated with zinc phosphate (10 ppm; 7.8-fold control), and the effect was comparable to that of the ASM treatment (2.1×10^{-4} mol L⁻¹; 7.0-fold control) (Fig. 3).

Fig. 1 Effects of zinc phosphate on bacterial speck disease severity and *Pst* growth *in planta*. Effects of the soil treatments of the tomato plants with water (control), zinc phosphate [$\text{Zn}_3(\text{PO}_4)_2$; 10 ppm (25.90 μM)] and acibenzolar-S-methyl (ASM; 2.1×10^{-4} mol L^{-1}) on bacterial speck disease severity expressed as diseased areas of the leaves **a** and numbers of spots per leaf **b** at 14 dpi, and on *Pseudomonas syringae* pv. *tomato* growth *in planta* at 6 dpi, as cell numbers per cm^2 leaf **c**. Data are means $\pm$ SE of 3 independent experiments and 4 replicates. Columns with different letters are significantly different ($p \leq 0.01$, **a**, **b**; $p \leq 0.05$, **c**; Duncan's multiple range tests). CFU, colony forming units

Expression of the salicylic-acid-responsive *PR1* gene was significantly induced for zinc phosphate- and ASM-treated tomato plants at 24, 48 and 96 hpt (Fig. 4a), which was maximal for 48 hpt (3.5-fold, 6.5-fold, respectively). Also, a significant 50% reduction in the expression of the jasmonate-mediated *PIN2* gene was observed for the zinc phosphate-treated plants at 24 hpt, but not for the ASM treatment (Fig. 4b).

Effect of zinc phosphate treatments on zinc accumulation in the tomato leaves

Significant zinc accumulation to 2.3-fold the control was detected in the leaves of the zinc phosphate-treated plants at 28 dpt (Fig. 5a), while no differences were seen for the phosphorus levels (Fig. 5b).

Fig. 2 Effects of zinc phosphate on *in vitro* *Pst* growth. Dose-response curve for the growth of *Pseudomonas syringae* pv. *tomato* (OD₆₃₀, relative to control [%]) versus concentration of zinc phosphate ($\text{Zn}_3(\text{PO}_4)_2$) added. Closed circles represent the observed data ($n=8$), and line is the log-logistic fitted curve $y = 100 / \{1 + \exp[7.58 (\log(x) - \log(45.9))]\}$. The standard error of the mean is 0.0109

Fig. 3 Effects of zinc phosphate on callose deposition. Top: Callose deposition, expressed as percentage of digitalised photos covered by blue pixels, in leaves of tomato plants treated with water (control), zinc phosphate [$\text{Zn}_3(\text{PO}_4)_2$; 10 ppm (25.90 μM)] or acibenzolar-S-methyl (ASM; 2.1×10^{-4} mol L^{-1}) at 48 hpt. Each column represents the mean of 27 replicated photos per treatment $\pm$ SE. Columns with different letters are significantly different ($p \leq 0.01$; Duncan's multiple range tests). Bottom: Representative images for callose deposition (10 $\times$ magnification), as shown by blue pixels

Fig. 4 Effects of zinc phosphate on defence gene induction. Relative expression levels of pathogenesis-related protein (*PR1b1*) **a** and tomato proteinase inhibitor 2 (*PIN2*) **b** genes in tomato (cv. Rio Grande) leaves at 24, 48 and 96 hpt with water (control), zinc phosphate [$Zn_3(PO_4)_2$; 10 ppm (25.90 μM)] or acibenzolar-S-methyl (ASM; 2.1×10^{-4} mol L^{-1}). Data are means of 9 replicates (3 biological, 3 technical) $\pm$ SE. Columns with different letters are significantly different ($p \leq 0.01$; Duncan's multiple range tests)

Discussion

Zinc is one of the first elements that was discovered, and it is one of the main components of the Earth's crust (Duffy 2007). Zinc has a pivotal role in all living organisms, including animals, plants and microorganisms (Cabot et al. 2019). In plants, zinc is the sole metal that is associated with all the six enzyme classes (Coleman 1998), and it also has structural functions in proteins (Cabot et al. 2019). Zinc is involved in plant cell multiplication, water and nutrient uptake, phytohormone activities and plant responses to biotic and abiotic stresses (Cabot et al. 2019; Duffy 2007).

However, the role of zinc, applied to the soil (Grewal et al. 1996; Machado et al. 2018) or to the diseased peach shoots (Li et al. 2016), in plant defence responses is not unique, and instead varies in relation to the host–stress-factor combination (Cabot et al. 2019; Duffy 2007). Thus, in potato plants, zinc has been reported to reduce incidence and severity of powdery scab caused by *Spongiospora subterranea* subsp. *subterranea* J.A. Toml., but it is ineffective against common scab caused by *Streptomyces scabiei* (Thaxt.) Lambert and Loria (Basu Chaudhary 1967; Falloon et al. 1995). Furthermore, zinc reduces the incidence and severity of crown

Fig. 5 Effects of zinc phosphate treatment on zinc and phosphorous levels in tomato leaves. Zinc **a** and phosphorus **b** contents (mg kg^{-1} dry weight) in tomato (cv. Rio Grande) leaves 28 dpt with water (control), zinc phosphate [$Zn_3(PO_4)_2$; 10 ppm (25.90 μM)] or acibenzolar-S-methyl (ASM; 2.1×10^{-4} mol L^{-1}). Data are means of 3 independent experiments $\pm$ SE. Columns with different letters are significantly different ($p \leq 0.01$; Duncan's multiple range test). d.w., dry weight

and root rot caused by *Fusarium graminearum* Schwabe on wheat (Sparrow and Graham 1988), while it does not have the same efficacy towards *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato (Duffy and Défago 1999).

Here, we have demonstrated that tomato plants grown in soil supplied with zinc phosphate are protected against *Pst* attack, and that this protection was observed as reduced diseased areas and numbers of spots per leaf. This protection is also comparable to that provided by the resistance inducer ASM, although this was observed only for diseased areas of the leaves. In contrast, when expressed as numbers of spots per leaf, the protective effect of zinc phosphate was less marked with respect to that of ASM. This would indicate therefore that in the zinc phosphate-treated plants the diameters of the spots were lower than those for the ASM-treated plants. Similar data were obtained by Elsharkawy et al. (2018) in the same pathosystem, who used zinc oxide nanoparticles as a spray treatment for the leaves.

Pst growth was also repressed in the zinc phosphate-treated plants here. Similar findings have been reported for *Pst* in zinc-supplied plants (Elsharkawy et al. 2018; Fones and Preston 2013) and for other pathogenic or beneficial bacteria (Othman et al. 2017; Zhang et al. 2015). This

reduction of *Pst* growth *in planta*, combined with the significantly higher levels of zinc in the zinc phosphate-treated plants in comparison with controls suggest direct antimicrobial activity of this microelement (Cabot et al. 2019) even if we cannot exclude that a systemic acquired tolerance could be activated as the decrease of *Pst* population does not fully justify the reduction of symptoms. Similar findings were reported by Block et al. (2005) in tomato-*Pst* pathosystem. Zinc toxicity towards *Pst* was ascertained *in vitro* using several concentrations of zinc phosphate. Similar results were reported by Elsharkawy et al. (2018) using zinc oxide nanoparticles and the agar disk diffusion technique. In particular, the EC₅₀ of 46 ppm zinc phosphate determined here *in vitro* was similar to the zinc concentration reached in the treated tomato plants (50 ppm), which was more than twice that of the control plants. The zinc levels detected in our control plants (22.4 ppm) are supported by those reported for healthy crop species (Marschner 1995), including tomato (Karagiannidis et al. 2002; Ozores-Hampton et al. 1994). In preliminary experiments, we have observed no toxic effects of zinc at the concentrations applied and reached in our tomato plants. These results agree with those of Vijayarengan et al. (2013) and Cabot et al. (2019). In particular, Vijayarengan et al. (2013) reported that zinc exerted its toxic effect on tomato plants starting from a soil supply dose of 150 mg Kg⁻¹, that is 15 times higher than those used in our treatments. Furthermore, in bioremediation experiments, the shoots chlorophyll content, an indicator of phytotoxicity, was not affected by zinc level higher than those reached in our tomato plants (Yamudna Devi et al. 2020). Of note, a zinc enrichment of plants could be useful in food and feed (Udechukwu et al. 2016) even if, high zinc doses (more than 50 mg day⁻¹, World Health Organization <https://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=4197>) can cause adverse effects in humans and animals. This dose is compatible with that found in our tomato plants and in tomato fruits (Salam et al. 2011).

Phosphorus is the main nutrient that interacts with zinc in plants, including tomato plants. In particular, a negative relationship between these two nutrients has been documented during both their uptake and their transport and signalling (Bouain et al. 2014; Mousavi et al. 2012; Parker et al. 1992; Sainju et al. 2003; Soltangheisi et al. 2014). In agreement with that demonstrated by Mousavi (2011) and Mousavi et al. (2012), we observed that zinc applied to tomato plants reduces uptake and accumulation of phosphorus. Phosphate salts have been reported to induce resistance against pathogens in many plants, including solanaceous plants (Alexandersson 2016; Orober et al. 2002; Tuzun and Bent 2006). As the phosphorus levels *in planta* were not different between the control and zinc phosphate-treated tomato plants, we can speculate that the protective effects observed here were due to the zinc.

We have also demonstrated that zinc is involved in the induction of plant defence responses, in terms of callose apposition and *PR1b1* gene expression. In particular, the increased *PR1b1* gene expression observed in the zinc phosphate- and ASM-treated plants is in agreement with Elsharkawy et al. (2018) and Huang and Vallad (2018). For the *PIN2* gene expression, in the three biological replicates we found that its expression was unchanged for both the zinc phosphate and ASM treatments, except for the reduction in the zinc phosphate-treated plants at the early treatment time (24 hpt). These data are in agreement with Herman et al. (2007) for ASM treatment in tomato cv. Rio Grande.

It should be noted that callose apposition was induced in this zinc phosphate- and ASM-treated tomato plants. Although callose apposition after zinc phosphate or ASM treatments has been documented in several plant species (Benhamou and Bélanger 1998; Feigl et al. 2015; Kohler et al. 2002; Peterson and Rauser 1979), to the best of our knowledge, this is the first report of zinc phosphate induced callose apposition in tomato plants.

Conclusions

We have shown here that soil treatments with zinc phosphate inorganic salt protects tomato plants against *Pst* attack, through the induction of morphological and biochemical plant defence responses and direct antimicrobial activity. In conclusion, zinc phosphate applied to the soil especially for greenhouse-cultivated tomatoes represents a valid means to support the plant defences against bacterial pathogens, particularly in organic farming, where ASM use is not permitted and the use of copper is being progressively restricted. Moreover, the use of molecules that can have both nutritional and protective roles matches the desire for reduced chemical input for crop production during eco-friendly agriculture.

Supplementary file 1 (DOCX 22 kb)Supplementary Information The online version contains supplementary material available at (<https://doi.org/10.1007/s41348-021-00444-z>) .

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Authors contributions Mara Quaglia, Chiaraluce Moretti and Roberto Buonaurio were involved in methodology, writing—review and editing, funding acquisition. Benedetta Orfei helped in methodology, data curation, writing first draft. Marika Bocchini contributed to methodology, data curation, software. Roberto D’Amato helped in data curation. Franco Famiani was involved in review and editing, funding acquisition.

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Declaration

Conflict of interest The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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