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Copper complexes of the 1,3,4-thiadiazole derivatives modulate antioxidant defense responses and resistance in tomato plants against fungal and bacterial diseases

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ABSTRACT

The metallic complexes μ-chloro-μ-[2,5-bis (2-pyridyl)-1,3,4-thiadiazole] aqua chlorocopper (II) dichlorocopper (II) (abbreviated 2PTH-Cu_2-Cl_4); aquabis [2,5-bis (2-pyridyl)-1,3,4-thiadiazole-κ^2N^2,N^3] (trifluoromethane-sulfonato-κO) copper(II) trifluoro metrhanesulfonate (2PTH-Cu-tF) and bis[(2,5-bis(pyridine-2-yl)-1,3,4-thiadiazole-di-azido copper(II)] (2PTH-Cu-Az) were compared for their antimicrobial activities in vitro, and their aptitude to control *Verticillium* wilt and crown gall diseases development of tomato in the greenhouse. Results showed that the complex 2PTH-Cu-Az inhibited drastically the growth of *V. dahliae* in vitro. 2PTH-Cu_2-Cl_4 and 2PTH-Cu-tF did not display any noticeable antimicrobial activity in vitro against all of the pathogens tested. However, *in planta* evaluation revealed that the three complexes protected tomato against crown gall similarly. They also reduced *Verticillium* wilt disease severity, although the complex 2PTH-Cu-Az was the most efficient. When compared to other complexes, 2PTH-Cu-Az triggered only a weak oxidative burst as revealed by H_2O_2 measurement and the activity of ascorbate peroxidase and catalase. These results suggest that the superiority of 2PTH-Cu-Az against *V. dahliae* rely on its direct antifungal activity and its ability to modulate H_2O_2 accumulation.

1. Introduction

Thiadiazoles are an important group of five-membered ring compounds that exhibit extensive diversity of biological activities [1]. Their derivatives possess interesting biological activity conferred by the strong aromaticity of the ring system, allowing good stability and low toxicity. In addition, they are considered as good coordinating ligands, because the donor sites of nitrogen and sulfur atoms are able to coordinate with 3d transition metal ions [2] allowing preparation of complex compounds. The transition metal such as Cu(II) is found in all living organisms and plays essential role in the activity of several enzymes and it is involved in energy metabolism. Control of the number and type of coordinating atoms is crucial to obtain metal complexes that mimic the coordination sphere and reactivity of metal-containing enzymes [3,4]. For such purpose Cu(II) is able to form mono or polynuclear species with the ligand [5]. Moreover, complexes based on pseudohalide ions such as sodium azide have been also reported to exhibit different coordination modes [6]. These metal complexes have a wide variety of applications acting as antimicrobial agents [7]. However, their use in the field of crop protection is limited.

Tomato (*Solanum lycopersicum*) crop is susceptible to several diseases including *Verticillium* wilt caused by the fungus *Verticillium dahliae* and crown gall provoked by *Agrobaterium tumefaciens*. This bacteria triggers modification in the plant genome and induces tumors that are associated with severe changes of plant metabolism [8] and can cause significant losses in crop yield if young plants are affected [9]. *V. dahliae* is also responsible in the reduction of yield as it can severely disturb their growth. Affected plants show V-shaped lesions at the edge of the leaf, vascular streaking in the stems and wilting caused by systemic spreading of the fungus [10]. Although these diseases could be managed through the use of some chemicals such as copper for crown gall [11] and spraying fungicides for controlling *Verticillium* wilt disease [12,13]; the resurgence of resistance to these chemicals has prompted researchers to look for other safety products. For these purposes new generation of plant products were revealed to be efficient in controlling crop diseases by stimulating or potentiating plant defense responses

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after pathogen attack. This includes the *S*-methyl benzo [1,2,3]thiadiazole-7-carbothioate (BTH) [14,15], chitosan [16], fatty acids [17], algal extracts [18] and triterpenic plant derivatives [19].

Recently we have shown that a Nickel complex derivative of the ligand 1,3,4-thiadiazole was able to protect tomato against *Verticillium* wilt [20]. In this work we have synthetized three new complexes harboring copper metal instead of Nickel and tested them as new protective agents against *Verticillium* wilt and crown gall diseases. First, we examined the possible in vitro activity against a broad species of plant pathogens and analyzed their protection against the two diseases in the greenhouse. We also evaluated their effect on the accumulation of accumulation of active oxygen species (AOS) and on the activation of antioxidant defense responses.

2. Material and methods

2.1. Synthesis of chemicals

The heterocyclic ligand 2,5-bis(pyridin-2-yl)-1,3,4-thiadiazole (2PTH) was synthesized as described in Lebrini et al. [21]. Synthesis of the dimeric complex μ-chloro-μ-[2,5-bis(2-pyridyl)-1,3,4thiadiazole] aqua chlorocopper (II) dichlorocopper (II) named 2PTH-Cu_2-Cl_4 (Fig. 1A) was carried out as previously described [5]. Copper chloride dehydrate (1.5 mmol, 0.26 g) was dissolved in 8 mL of hot water and mixed with the ligand 2PTH (0.42 mmol, 0.1 g) dissolved in the same volume of hot ethanol. After filtration the solution was left to stand at ambient temperature. Green crystals were obtained 24 h later, they were washed with water, vacuum dried and used for X-ray structure determination. Characterization data were previously published in [5].

The monomeric complex aquabis[2,5-bis(2-pyridyl)-1,3,4thiadiazole-κ^2N^2,N^3](trifluoromethane-sulfonato-κO)copper(II) trifluorometrhanesulfonate named 2PTH-Cu-tF (Fig. 1B) was synthetized according to Bentiss et al. [22]. $Cu(O_3SCF_3)_2$ (1.5 mmol, 0.54 g) in 8 mL of water was added to (0.42 mmol, 0.1 g) of 2PTH, dissolved in 8 mL of ethanol. The solution was filtered and after 24 h, the blue compound crystallized at room temperature. Crystals were washed with water and dried under vacuum. X-Ray structure and chemical characterization were previously reported [22]. The dimeric complex bis[(2,5-bis(pyridine-2-yl)-1,3,4-thiadiazole-di-azido copper(II)] named 2PTH-Cu-Az (Fig. 1C) was synthetized according to Laachir et al. [23]. 0.1 mmol, (24 mg) of 2,5-bis(2-pyridyl)-1,3,4-thiadiazole was dissolved in 20 mL of CH_3CN and placed carefully in glass test tube onto the $CuCl_2$,$2H_2O$ (0.05 mmol, 8.5 mg) and NaN_3 (0.4 mmol, 26 mg) aqueous solution (15 mL) layer. The test tube was sealed with parafilm and left to stand at room temperature. After 3 weeks, diffusion between the metal salt solution and ligand produced green crystals, X-ray structure is published in Laachir et al. [23].

2.2. In vitro assays and antimicrobial activities

The inhibitory effects of the three chemicals 2PTH-Cu_2-Cl_4, 2PTH-Cu-tF and 2PTH-Cu-Az were assessed against the fungus *V. dahliae* and several phytopathogenic bacteria (Table 1). *V. dahliae* was cultured on potato dextrose agar (PDA). Strains of *A. tumefaciens* were cultured on Luria-Bertani agar medium while those of *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tabaci* and *Erwinia amylovora* were performed on King B medium.

For antifungal in vitro assays, chemicals dissolved in 0.2% DMSO were used to amend PDA medium at the concentrations of 50 and 100 μg mL^{-1}. Five-mm of agar plugs were removed from the edge of actively growing cultures of the strain SE and SH of *V. dahliae* and placed on the center of PDA plates. As control PDA plates were amended only with 0.2% DMSO. Mycelia growth was recorded after incubation of petri plates in the dark at 26 °C during 7 days. The percentage of growth inhibition was determined as described [20].

The antibacterial activity of chemicals was evaluated using cellulose discs of 6 mm diameter soaked with 100 μL of extracts as described [24]. Discs imbibed with 100 μL of chemicals were placed on solid medium previously inoculated with a bacterial suspension of 3×10^8 CFU mL^{-1}. After 24 h incubation at 28 °C, the antibacterial activity was measured as the diameter of zone inhibition appearing around the disc and compared to control discs soaked with 100 μL of water.

2.3. Protection assays

Protection assays were carried out against *V. dahliae* and *A. tumefaciens* on the variety 'Campbell' of tomato seedlings grown in the greenhouse at 26 °C with a 12 h photoperiod and 70% relative humidity. The aerial parts of four-week old seedlings were sprayed to runoff with DW, or chemicals at 50 μg mL^{-1} two times at 7 and 3 days before pathogen inoculation. One lot of plants was inoculated with the strain SH of *V. dahliae* by dipping their roots in spore suspension adjusted to 10^7 conidia mL^{-1}during 10 min. For mock inoculation roots were dipped in sterile DW. Wilt disease severity was determined by assessing foliar alteration index, stunting index and browning index at 20 days after inoculation as described [20]. Another batch of treated plants was inoculated with the strain C58 of *A. tumefaciens* as reported [25]. Bacteria suspension (3×10^6 CFU mL^{-1}) supplemented with 2% sucrose and 0.1 mM acetosyringone were inoculated after stem wounding. Inoculated plants were scored for tumors 8 weeks after by recording the diameter and weight of lesions.

2.4. Biochemical analyses

DW or chemicals were sprayed to run-off on the aerial part of seedlings. They were subjected to a second treatment 4 days later, and their roots were inoculated with *V. dahliae* (10^7 conidia mL^{-1}) or with DW (mock inoculation). Different plants collected at 0, 0.25, 1, 2, 4, 7, 9, 11 and 15 days after the first treatment were used for biochemical analyses.

H_2O_2 content was determined from 500 mg of leaves according to the method of Alexieva et al. [26]. Enzymes were extracted at 4 °C by grinding 400 mg of harvested leaves with 3 mL of 50 mM phosphate buffer, pH 7.5, containing 0.01% (*v*/v) Triton X-100, 1 mM of polyethylene glycol and 8% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 16,000 × *g* for 20 min at 4 °C, and the supernatant was used for enzymatic activities. Catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) were determined as described in Faize et al. [27]. Polyphenol oxidase (PPO) (EC 1.10.3.1) activity was measured as described by Masia et al. [28].

2.5. Statistical analysis

For protection assays plants were arranged in a randomized complete block design with three blocks and four plants per treatment in each block (12 plants per treatment).

In vitro antifungal and antibacterial data as well as diseases parameters were analyzed using one-way ANOVA followed by Tukey's HSD ($P < 0.05$).

3. Results

3.1. Antimicrobial activities of copper complexes

We examined whether or not the three copper complexes possess antibacterial activity by analyzing the diameter of inhibition induced against *P. syringae pv. tabaci*, *P. syringae* pv. *syringae*, *E. amylovora* and against three strains of *A. tumefaciens* (Table 2). 2PTH-Cu_2-Cl_4 slightly inhibited the growth of *P. syringae pv. syringae* while inhibition was more noticeable with 2PTH-Cu-Az, mainly at 100 μg mL^{-1} and no significant inhibition was obtained with 2PTH-Cu-tF. For the rest of the