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Cross-protection of pepper plants stressed by copper against a vascular pathogen is accompanied by the induction of a defence response

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ABSTRACT

Pepper (*Capsicum annuum* L.) plants stressed by copper showed less disease symptoms after inoculation with *Verticillium dahliae* Kleb. We tested if such protection was accompanied of a defence response induced by copper stress by measurement of peroxidase and chitinase activity, phenolics and the expression of four genes related to plant defence. Peroxidase activity, but not chitinase, increased in roots, stem and leaves of copper-stressed plants. However, treating the plants with an ethylene perception inhibitor (MCP) before applying the copper stress, caused a synergic enhancement of both enzymes in stem and cotyledons. Phenolic compounds were also induced by copper but downregulated by MCP in stem. The expression of a peroxidase gene (*CAPO1*), a sesquiterpene cyclase gene (*CASC1*), a PR1 gene (*CABPR1*) and a β-1,3-glucanase (*CABGLU*) was highly upregulated by copper stress, but MCP neither suppresses nor enhances such an effect. Globally, copper stress causes an induction of defence mechanisms that may partially explain tolerance to Verticillium wilt.

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1. Introduction

Heavy metals are sometimes present in phytotoxic amounts in soils as a result of agricultural and industrial activities [1]. Excessive uptake of such metals by the plants may eventually affect different physiological processes. For example, copper sometimes reaches high levels in the soil because of mining activities or the prolonged application of copper-based fungicides, such as Bordeaux mixture, in old orchards and vineyards [1,2]. In plants, copper stress inhibits photosynthesis, respiration and nitrogen fixation, and causes the alteration of membrane integrity, the formation of active oxygen species and the subsequent enhancement of lipid peroxidation [2–4]. Alteration of processes at the cellular level leads to several macroscopic symptoms in plants suffering from copper stress, such as stunted growth, necrosis, leaf epinasty, chlorosis and red-brownish discoloration [5].

Heavy metals are not the only cause of plant stress. In nature, plants have to cope with various environmental conditions that differ from optimal conditions and they have to respond to different biotic and abiotic signals by adapting their development. Indeed, the exposure of plants to heavy metals may lead to protection against pathogens [6]. There are several explanations for this effect. Firstly, heavy metals are themselves toxic to pathogens, therefore metal accumulation by the plant may suppress pathogen infection. Secondly, heavy metals can act as elicitors of plant defence mechanisms [6,7].

Plants possess structural and biochemical mechanisms for defence against pathogens. One of the structural barriers that prevent plant colonization by pathogens is lignin, which is synthesized by peroxidases from cinnamyl alcohols [8]. Peroxidases and lignification are induced in plants by heavy metal stress [9–11] as well as after infection by pathogens [12]. Plants may also defend themselves against pathogens through the so-called "biochemical" defences, which normally include secondary metabolites (phytoanticipins and phytoalexins) and defence proteins. Many phytoanticipins and phytoalexins are phenolics or isoprenoids, and some of them are accumulated in response to heavy metal stress [13]. Likewise, other defence proteins such as PR proteins are induced by heavy metal stress [14,15].

Responses to both biotic and abiotic stress are mediated by low-molecular weight molecules, such as reactive oxygen species (ROS), salicylic acid, jasmonic acid, abscisic acid and ethylene [7,16,17]. These signals regulate the protective responses of plants against different stresses via synergistic and antagonistic actions, which are referred to as signalling crosstalk [16]. There is evidence

Abbreviations: MCP, 1-methylcyclopropene; MeOH, methanol; PCR, polymerase chain reaction; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; SAR, systemic acquired resistance.

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of such crosstalk between ROS and jasmonic acid or other oxylipins in biotic and heavy metal stress [7,13]. A well-known signal regulating the so-called systemic acquired resistance (SAR) against pathogens, salicylic acid, has been reported to alleviate the negative effect of cadmium on barley [18] and maize plants [19]. However, the actual role of salicylic acid in response to plant abiotic stress is still unresolved [17]. Jasmonic acid and ethylene have also been related to response to heavy metal stress [20–22]. In fact, heavy metal stress caused by copper stimulates the biosynthesis of ethylene [23–25], which may act as an endogenous signal triggering the plant response to such stress. Ethylene is released from the plant in the response of plant to both biotic and abiotic stress [26]. In summary, plant response to pathogens and to abiotic stress, particularly the one caused by heavy metals, employ a lot of common signals and a cross-protection would be possible.

In a previous report, we showed that an excess of copper causes stress in pepper plants, inducing several physiological responses [9]. In the present study, we investigated the ability of copper stress to protect pepper plants against a plant disease, Verticillium wilt, as well as some of the plant defence mechanisms against pathogens that could be triggered by the exposure to such stress.

2. Materials and methods

2.1. Plant material, growth conditions and treatment procedures

Seeds of pepper (*Capsicum annuum* L.) were germinated in perlite, and seedlings were grown for 3–4 days after emergence and then used in the experiments described below. For all experiments, plants were grown at 25 °C under a 16-h photoperiod (Lamps OSRAM L 18W/765; 228 μmol m^{-2} s^{-1} PAR).

For the experiments of inoculation with *Verticillium dahliae* Kleb., a control group of plants was grown in perlite soaked in a nutrient solution composed of 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, 20 μM EDTA and 20 μM $Fe(NH_4)_2(SO_4)$. A second group was grown in the same nutrient solution but supplemented with 50 μM $CuSO_4$. The solutions were periodically renewed and aerated. Four days after these treatments, roots of plants were washed in distilled water and plants were challenge-inoculated with *V. dahliae* (isolate UDC53Vd) by dipping the roots in a suspension of 10^6 conidia ml^{-1} for 45 min [27]. The control groups were inoculated with water. Following inoculation, the plants were transferred to pots containing a mixture of perlite and potting soil (1:2, v/v). The severity of Verticillium wilt symptoms was estimated both by the reduction of the length of the stem in relation to non-inoculated plants and the percentage of wilted leaves per plant. Both parameters were monitored weekly until 28 days after inoculation. The experiments were performed three times.

In a second set of experiments, a group of plants was exposed to 1-methylcyclopropene (MCP), an inhibitor of ethylene perception. Plants were exposed to MCP at a final concentration of 0.2 μL L^{-1} in a sealed container [28]. Control plants were kept in a container with no chemical added. Containers were opened after 8 h and plants were then treated with the control nutrient solution or with the 50 μM $CuSO_4$ supplemented solution described above. Samples of cotyledons, stems and roots were taken at 96 h after the beginning of the copper stress treatment and stored at −80 °C for further analysis. The experiments were performed at least twice for each parameter analysed (enzymes, gene expression, phenolics).

2.2. Enzyme extraction and assays

Cotyledons, stems or roots from 20 plants (0.3–1 g) were homogenised at 4 °C in 50 mM Tris–HCl buffer (pH 7.5) with the addition of 0.05 g polyvinylpolypyrrolidone (PVPP) per gram of fresh weight. Crude extracts were centrifuged at 10,000 × g at 4 °C for 20 min. Supernatants were desalted in a PD-10 column (GE Healthcare) and the eluate analysed for enzyme activity. Peroxidase activity was determined according to [27] and chitinase activity was determined by the method reported in [29]. Proteins were determined as in [27].

2.3. Extraction and determination of soluble phenolics

Stems from 20 plants (0.2–0.4 g) were homogenised in 2.5 ml of 80% MeOH. The homogenised sample was incubated for 15 min at 70 °C and then filtered. The residue in the filter was washed with 2.5 ml of 80% MeOH to optimise the extraction. The final volume was adjusted to 5 ml and used immediately for phenolic determination.

Total soluble phenols were determined with Folin–Ciocalteu reagent as described in [27]. The content of the soluble phenols was calculated from a standard curve obtained with different concentrations of gallic acid.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen samples with the Aurum™ Total RNA Mini Kit (BioRad), according to the manufacturer's instructions. RNA quantity was measured spectrophotometrically and its integrity was checked by 1.2% agarose-formaldehyde gel electrophoresis. First strand cDNA was synthesized from 100 ng total RNA with the iScript cDNA Synthesis Kit (BioRad) and following the protocol supplied by the manufacturer.

2.5. Real-time RT-PCR assay

The expression of several genes related to defence against pathogens was studied. The genes were a peroxidase gene (*CAPO1*), a sesquiterpene cyclase gene (*CASC1*), a PR1 gene (*CABPR1*) and a β-1,3-glucanase (*CABGLU*). An actin gene (AY572427) was used as a constitutively expressed endogenous control, whose expression levels were essentially constant in the Cu conditions assayed. All the primers and gene accessions are described in [30]. Real-time PCR was performed in 50 μl of reaction mixture composed of 2.5 μl of cDNA, 1× iQ SYBR Green Supermix (BioRad) and 0.3 μM of each gene-specific primer, with an iCycler iQ system (BioRad). The thermal cycling conditions consisted of initial denaturation at 95 °C for 2 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final step at 72 °C for 5 min. The specificity was tested by identification of only one peak in the melting curve analysis. A fivefold series of dilutions of reverse transcribed total RNA concentrations was used to calculate the PCR reaction efficiency as described by Pfaffl [31]. This method defines the efficiency as the slope of the line formed by representation of the cycle thresholds (Ct) versus concentrations of the serial dilutions. The relationship between slope and efficiency is given by the equation: $E = 10^{-1/\mathrm{slope}}$. The relative expression level of each gene used here depends on this efficiency and is described as the difference between the studied gen (target) Ct of the control and that of the sample, and later normalization with the reference gen (actin). The difference in Ct is the number of cycles that the amount of sample RNA needs to equal the amount of control RNA, therefore the relative expression is defined as follows: relative expression $= E^{\Delta \mathrm{Ct}_{\mathrm{target}}(\mathrm{Control-sample})} / E^{\Delta \mathrm{Ct}_{\mathrm{reference}}(\mathrm{Control-sample})}$. The relative expression is, therefore, the number of times that the amount of RNA template sample is higher or lower than the amount in the control and therefore, this level must be relative to the control level, taken as a standard value "1". Each experiment was repeated twice and each measurement was performed in duplicate.

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