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Changes in mycelia growth, sporulation, and virulence of *Phytophthora capsici* when challenged by heavy metals (Cu^{2+}, Cr^{2+} and Hg^{2+}) under acid pH stress

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ABSTRACT

Phytophthora capsici, an economically devastating oomycete pathogen, causes devastating disease epidemics on a wide range of vegetable plants and pose a grave threat to global vegetables production. Heavy metals and acid pH are newly co-occurring stresses to soil micro-organisms, but what can be expected for mycelia growth and virulence and how they injure the oomycetes (especially *P. capsici*) remains unknown. Here, the effects of different heavy metals (Cu^{2+}, Cr^{2+}, and Hg^{2+}) on mycelia growth and virulence were investigated at different pHs (4.0 vs. 7.0) and the plausible molecular and physiological mechanisms were analyzed. In the present study, we compared the effective inhibition of different heavy metals (Cu^{2+}, Cr^{2+}, and Hg^{2+}) and acid pH on a previously genome sequenced *P. capsici* virulent strain LT1534. Both stress factors independently affected its mycelia growth and sporulation. Next, we investigated whether ROS participated in the pH-inhibited mycelial growth, finding that the ROS scavenger, catalase (CAT), significantly inhibited the acid pH-induced ROS in mycelia. Additionally, because MAPK specially transmits different stress responsive signals in environment into cells, we employed CAT and a p38-MAPK pathway inhibitor to investigate ROS and p38-MAPK roles in heavy metal-inhibited mycelia growth at different pHs (4.0 vs. 7.0), finding that they significantly inhibited growth. Furthermore, ROS and p38-MAPK influenced the heavy metal-induced TBARS content, total antioxidant capacity (TAC), and CAT activity at different pHs, and also reduced the expression of infection-related laccases (*PcLAC2*) and an effector-related protein (*PcNLP14*). We propose that acid pH stress accelerates how heavy metals inhibit mycelium growth, sporulation, and virulence change in *P. capsici*, and posit that ROS and p38-MAPK function to regulate the molecular and physiological mechanisms underlying this toxicity. Although these stresses induce molecular and physiological challenges to oomycetes, much remains to be known the mechanisms dedicated to resolve these environmental stresses.

1. Introduction

Soil contamination by heavy metals released from industrial wastes and agrochemicals now poses a substantial threat to both plants and soil micro-organisms (Maanan et al., 2015; Wu et al., 2016). Normally, these heavy metals exist in many forms, such as soluble metal complexes, free metal ions, and insoluble compounds, and may become toxic if bioaccumulates via an organism's uptake (e.g., roots) or intake (e.g., ingestion as food). Nonetheless, there exist multiple mechanisms by which heavy metals exert their toxicity (Yadav, 2010). In plants, they typically elicit a burst of ROS (reactive oxygen species), increase lipid peroxidation, and trigger programmed cell death (Sytar et al., 2013). In fungi, heavy metals can inhibit growth and cause morphological and physiological changes (Baldrian, 2003), in addition to inducing oxidative stress and antioxidant enzymes as demonstrated in *Phanerochaete chrysosporium* (Zhang et al., 2015). In particular, the proteins related to the encoding of key structures of enzymes are often the main binding targets of heavy metals (Gao et al., 2012), yet heavy metals may also induce a pH decrease that leads to soil acidification which also alters protein function and membrane potential (Speir et al., 1999; Adamczyk-szabela et al., 2015).

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It is known that to survive in a low-pH environment, such as that caused by acidic effluent, organisms must be able to respond to and tolerate this condition (Bakeraustin and Dopson, 2007). Normally, pH is a critical factor in the strategies of fungal attack—namely how to colonize, invade, or kill the host—and a pH decrease often regulates fungal infection and pathogenicity (e.g., *Botrytis cinerea* and *Sclerotinia sclerotiorum*), such that a pathogenic microorganism cannot simply adapt to any pH level set by its host (De et al., 1998; Billon-Grand et al., 2012). Furthermore, the induction of oxidative stress and antioxidant enzymes has been demonstrated in fungi after their exposure to heavy metals and a fluctuating ambient pH (Wang et al., 2009; Zhang et al., 2015). In fact, ROS often act as signaling molecules in the regulation of many biological processes; hence, excess ROS as induced by heavy metals and pH decreases has the potential to cause oxidative damage to cellular proteins, nucleic acids, as well as membranes—a high membrane stability depends upon a negligible increase in plasma membrane permeability and lipid peroxidation (Tarhanen et al., 1999; Sytar et al., 2013). In addition, since disrupting the balance between ROS production and the activity of the antioxidative system determines ROS accumulation, antioxidant enzymes have an important role to play in the oxidative stress resistance of fungal pathogens (Sheng et al., 2015). Catalase (CAT), which catalyzes the degradation of H_2O_2 into water and oxygen, is considered one of the major H_2O_2-scavenging enzymes, being found in nearly all aerobic organisms (Tian et al., 2013). In fungi, while heavy metal-induced oxidative stress can be neutralized by greater enzyme activity, the pH will usually decrease as the concentration of heavy metal(s) increase (Howlett and Avery, 1997; Chen et al., 2014).

The MAPK (mitogen-activated protein kinase) is a widely distributed type of enzyme that is highly conserved in eukaryotic cells, where it functions in the sensory detection of and response to stimuli and adaptation to environmental change. In *Fusarium oxysporum*, *Botrytis cinerea*, *Metarhizium robertsii*, and *Phytophthora sojae*, the MAPKs are known to regulate abiotic stresses and pathogenesis (Gao et al., 2015; Chen et al., 2016; Luque et al., 2016). For *Sclerotinia sclerotiorum*, its MAPK-regulated sclerotial development is linked to fluctuating pH (Chen et al., 2004). Three subfamilies of the MAPKs have been clearly identified: the JNKs (c-Jun NH_2-terminal kinases) and p38-MAPKs are activated by environmental stimuli, while the ERK1/2 (extracellular responsive kinases) pathway mainly responds to growth and mitogens (Kefaloyianni et al., 2005). The p38-MAPK pathway functions in the stress response and innate immunity of the nematode *Caenorhabditis elegans* (Inoue et al., 2005), and later work suggested its involvement in the stress response and survival of conidiospores of *Aspergillus nidulans* (Balloy et al., 2008).

In the last few decades, the excessive release of heavy metals and heavy metal-induced soil acidification, as well as pH decreases caused by acidic effluent in soils, have become harmful to microorganisms susceptible to stress-induced physical and molecular changes (Yadav, 2010; Zhang et al., 2015). This toxicity is normally accompanied by more oxidative stress in plants and by the inhibition of mycelial growth, colonization, invasion, and host mortality rates in fungi. However, there remains surprisingly little known about this toxicity in oomycetes. The oomycete plant pathogen *Phytophthora capsici* is a virulent, hemibiotrophic attacker of commercial vegetable crops upon which it inflicts heavy, destructive losses around the world. In the last decade, the simultaneous occurrence of heavy metals and ambient acid pH stress in the soil environment has been noted. These two coexisting stresses are especially dangerous to the plants and soil micro-organisms, but the exact molecular and physiological mechanisms by which co-occurring pH and heavy metals induce injury to oomycetes (especially *P. capsici*) remains unknown. The objective of the present study was to investigate the interactive effects of heavy metals (Cu^{2+}, Cr^{2+}, and Hg^{2+}) and acid pH stress at varying levels on *P. capsici* mycelia growth, sporulation, and virulence, with the aim to better elucidate the possible molecular and physiological mechanisms underlying changes in these fungal responses.

2. Materials and methods

2.1. P. capsici strains and culture conditions

The *P. capsici* strain LT1534, which has been genome-sequenced and normally used as a model virulence strain, was maintained on 10% V8 juice agar medium at 25 °C in the dark for 7 days (Iribarren et al., 2015) and then mycelia discs (5 mm) were transferred onto 10% V8 juice agar medium. Radial growth was measured on day 5. The assays were repeated three times. The zoospores induction of *P. capsici* were conducted as previously described (Liu et al., 2016). The 9–10 days *P. capsici* mycelia were washed three times with 30–40 ml of sterile distilled water and then an additional 20–30 mL of sterile distilled water was added to induce sporangia at 25 °C for 24 h. Zoosporangia was induced by 4 °C for 30–40 min and then room temperature for 1 h. The number of zoosporangia was counted and the mean of three duplications was used as the result of one replicate.

2.2. Effect of heavy metals and acid pH stress on mycelial growth and sporulation of P. capsici

Heavy metals (Cu^{2+}, Cr^{2+} and Hg^{2+}) were obtained from their chlorine salt and they were dissolved in sterilized distilled water to obtain stock solutions of 1 M/L. For the growth inhibition of heavy metals, 0, 0.1, 0.5 and 1 mM Cu^{2+} (Cr^{2+} and Hg^{2+}) were added to 10% V8 juice agar (10% V8 juice filtered through four layers of cheesecloth, 1% $CaCO_3$, and 1.5% agar). For the growth inhibition of acid pH stress, different acid pH levels (range: 3.0–7.0) were added. For the growth inhibition of heavy metals and acid pH stress, different acid pH levels (4.0 and 7.0) and 0.5 mM heavy metals Cu^{2+} (Cr^{2+} and Hg^{2+}) were added. The mycelia growth and zoospores induction of *P. capsici* were conducted as previously described (Liu et al., 2016). The assays were repeated three times.

2.3. Effect of ROS and p38-MAPK on heavy metals inhibited mycelial growth at different pH

To analysis the effect of ROS on heavy metals and pH inhibited mycelia growth, 5 mM H_2O_2 and 1500 U/ml CAT (ROS scavenger, Sigma, Ca#C1345) were applied. To investigate the effect of p38-MAPK on heavy metals and pH inhibited mycelia growth, 20 μm p38 (Sigma, Ca#SB202190) were applied. The mycelia growth and zoospores induction of *P. capsici* were conducted as previously described (Liu et al., 2016). The assays were repeated three times.

2.4. Lipid peroxidation assay

Mycelium was homogenized (1:10 w/v) with 50 mM sodium phosphate buffer (pH = 7.0) and a TBA solution (29 mM TBA dissolved in 8.75 M acetic acid) was prepared. Then, a 2 ml mixed solution of homogenate and TBA (1:1 v/v) was heated for 60 min at 95 °C. After cooling, 3.5 ml of n-butanol was added and the tubes were vigorously shaken. Next, the organic layer was collected by centrifugation, and then measured at k = 531 nm (excitation) and k = 553 nm (emission). Protein content in the mycelium was maintained as described (Bradford et al., 1976) and the level of lipid peroxides was expressed in nM of TBARS per mg of proteins (Paraszkiewicz et al., 2010). The assays were repeated three times.