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Copper alters the physiology of tomato rhizospheric isolates of *Papiliotrema laurentii*

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ABSTRACT

In horticulture copper sulphate is utilized for the inhibition of phytopathogenic fungi. However, copper tends to accumulate in soil with a concomitant effect on soil quality and microbial diversity. As part of the biological diversity of soil and the rhizosphere, yeasts have been relatively less characterized than bacteria or filamentous fungi. In this work, we analyzed the presence of yeasts in the rhizosphere of tomato plants and the effect of copper in fungal biological activities of agronomical and ecological interest. Yeasts isolates that were selected for their higher copper resistance were all identified molecularly as *Papiliotrema laurentii*. Results show that all were capable of auxin production, and that copper has a detrimental effect on it. In contrast, ammonification was mainly increased by the metal. Copper also inhibits the growth of the yeasts on D-xylose, cellobiose and phenolic acids, suggesting a negative consequence on the recycling of lignocellulosic degradation products. Laccase and catechol oxidase activities were increased by the metal in *P. laurentii*. Altogether, results presented in this report indicate that *P. laurentii* presents properties of ecological and agronomical interest. The effect of the metal highlights the importance of the analysis of the consequence of copper utilization as fungicide on microbial activities. At the same time, the variability in the yeast response to copper suggests the concern of not only the characterization of biotechnological properties of a specific strain, but also the effect of copper on them.

1. Introduction

In horticultural farming, plants are affected by diverse fungal diseases, including anthracnose produced by *Colletotrichum coccodes*, early blight by *Alternaria solani,* grey leaf spot by *Stemphylium solani*, or leaf mold by *Cladosporium fulvum*, among others. These phytosanitary problems lead the producer to utilize fungicides, many of which are copper based, since this metal is considered to have a medium to low environmental impact (Wuana and Okieimen, 2011). Copper fungicides includes copper sulphate ($CuSO_4$), copper acetate [$Cu(OAc)_2$], copper oxychloride [$Cu_2(OH)_3Cl$] and copper oxide (Cu_2O). Although it has a relatively low toxicity, similar to other heavy metals copper tends to accumulate in soils altering the physiology of both pathogenic and non-pathogenic microorganisms (Edelsteina and Ben-Hur, 2018). For instance, copper increases the respiratory activity, and decreases some enzymatic activities (e.g., alkaline phosphatase, nitrite oxidase and nitrogenase) of microorganisms in soil (Olayinka and Babalola, 2001). At the same time, high levels of copper in soil cause a selective pressure against the more sensitive species resulting in an alteration of the microbial diversity (Keiblinger et al., 2018; Nunes et al., 2016). Up to date, research has mainly focused on the capacity of some microorganisms to bioremediate the metal or the description of the microbial oxidative stress response triggered by copper (Dávila Costa et al., 2011; Irazusta et al., 2013; Villegas et al., 2009). Relatively little is known about the alteration of other aspects of the microbial physiology by copper and its putative consequences on soil quality and plant growth. In particular, the alteration of the physiology of soil and rhizospheric yeasts by copper has remained largely unexplored. Although yeasts are components of the microbial diversity, their qualitative and quantitative contribution to the rhizospheric microbiota of horticultural crops is not well known.

Filamentous fungi and yeasts contribute in a significant manner to the microbial diversity in soil. In particular, yeasts are mainly found in the rhizosphere, the soil in close contact to, and directly influenced by

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plant roots, since the exudates are easy assimilable (Mestre et al., 2011). In the soil, yeasts have important ecological functions related to the transformation of nutrients. For instance, yeasts can assimilate carbohydrates (i.e., L-arabinose, D-xylose or cellobiose) or phenolic compounds (i.e., gallic acid, caffeic acid or vanillic acid), which can be generated after the hydrolysis of lignocellulosic material (Bisaria and Ghose, 1981; Tomme et al., 1995). Yeasts can also nitrify ammonium to nitrate, thereby contributing to the nitrogen cycle in soil (Botha, 2011). Although less studied, yeasts can also play a role in plant growth promotion through phosphate solubilization, the inhibition of phytopathogens or the synthesis of growth regulators including auxins, gibberellins and polyamines (Fu et al., 2016).

As described and discussed below, all the yeast isolates analyzed in this work were identified as *Papiliotrema laurentii* (Syn. *Cryptococcus laurentii*), a species of yeast well known for its prevalence in agricultural soils (Golubtsova et al., 2007; Sláviková and Vadkertiová, 2013). It has been shown that *Papiliotrema* genus can assimilate a wide range of hexoses, pentoses and organic acids, which could explain its ubiquity in soil and its prevalence in the rhizosphere in comparison to other yeast species (Golubtsova et al., 2007; Mestre et al., 2011), since these compounds are constituents of root exudates (Kamilova et al., 2006). However, the ecological function of *P. laurentii* has remained largely unexplored. It is known that *P. laurentii* interacts in a synergistic manner with arbuscular mycorrhizal fungi (e.g., *Funneliformis mosseae*) increasing the uptake of phosphorus and nitrogen, the chlorophyll content, as well as the production of phenolic compounds, which results in a beneficial effect on the vegetal development (Boby et al., 2008; Sampedro et al., 2004). It has also been shown that the symbiosis of *P. laurentii* with Buchu (*Agathosma betulina*), a medicinal shrub, stimulates the photosynthesis through the increase in the utilization of photosynthates (Cloete et al., 2009), and that the yeast interactions with mycorrhizal fungi improves the growth of blue lupin (*Lupinus angustifolius* L.) (Moller et al., 2016).

The study and characterization of *P. laurentii* isolates is of economical and biotechnological interest, considering the putative applications of this species. For instance, the physiology of *P. laurentii* can be utilized in the biodiesel or plant feedstuff production (Castanha et al., 2014; Van Staden et al., 2007). While largely studied for its effects on the microbial ecology, relatively little is known about the impact of copper on the physiology of yeasts from agricultural soils. The aim of this work was to analyze several aspects of ecological and agronomical interest in rhizospheric isolates of *P. laurentii* under the influence of $CuSO_4$.

2. Materials and methods

2.1. Microorganisms and growth conditions

Isolates were obtained from the rhizosphere of tomato plants (*Solanum lycopersicum* var. Elpida) cultivated on a commercial farm in the Northwest of Argentina (26°57′47.86″S - 65°24′14.03″O; Lules, Tucumán). Plants were carefully uprooted with a small shovel avoiding any contact with hands or clothes, and placed individually in sterile plastic bags. Bulk soil was removed and 3–5 lateral roots were placed in microtubes containing PBS buffer composed of: 8.06 g L^{-1} NaCl, 0.22 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KH_2PO_4; pH 7.4. Microtubes were sonicated in an ultrasonic bath for 15 s and serial dilutions were plated onto Yeast Malt (YM) agar containing 100 µg mL^{-1} chloramphenicol to avoid bacterial growth, and 0.05 g L^{-1} Rose Bengal to decrease the growth of filamentous fungi. In addition, 100 µl of samples obtained after sonication were utilized to inoculate flasks containing 10 mL YM broth supplemented with 100 µg mL^{-1} chloramphenicol and incubated aerobically overnight at 25 °C before serial dilutions were plated in YM agar supplemented with chloramphenicol and Rose Bengal. After incubation at 25 °C, all isolated colonies obtained were restreaked until pure cultures were obtained. Unless otherwise stated, isolates were cultured in YM broth or agar at 25 °C, and stored at −80 °C with 20% glycerol as cryoprotectant.

2.2. Determination of copper resistance

Isolates were streaked onto YM agar plates supplemented with 2 mM, 5 mM and 10 mM of $CuSO_4$ (Sigma-Aldrich) and incubated for 5 days at 25 °C. Growth was qualified as absence of growth (−), weak growth (+), medium growth (++) or robust growth (+++), where the latter was equivalent to that observed in medium without copper.

2.3. Molecular characterization

After overnight growth at 25 °C in YM agar, a colony of each isolate was aseptically collected and transferred to a microtube containing 800 µL extraction buffer Tris−HCl 100 mM, SDS 1% (w v^{-1}), Triton X100 2% (v v^{-1}), EDTA 10 mM, NaCl 100 mM). 800 µL Chloroform:isoamyl alcohol:phenol (24:1:25) was added together with a small spoon of glass beads (diameter 1 mm) and cells were disrupted in cycles of 2 min in vortex and 2 min of incubation in ice (Yamada et al., 2002). From supernatants obtained after 15 min centrifugation at 8000 *g*, DNA was precipitated with ethanol following standards procedures (Green and Sambrook, 2016) and redissolved in sterile water. The D1-D2 domain of the LSU rDNA gene, the 5.8 rDNA and the intergenic sequences ITS1 and ITS2 were amplified as a single amplicon with primers ITS1 and NL4 essentially as described elsewhere (Chen et al., 2001), except that Pfu DNA polymerase (PROMEGA) was utilized. Thermocycler parameters: 95 °C for 5 min, followed by 30 cycles at 96 °C for 1 min, 52 °C for 1 min, 72 °C for 2.5 min, followed by a final extension at 72 °C for 7 min. Amplicons were subsequently sequenced through the Macrogen (Korea) sequencing service. Identifications were performed, after edition when required, by comparison with sequences available at GenBank database and by species hypothesis in the UNITE database. After a multiple sequence alignment utilizing MAFFT (Katoh et al., 2017), a phylogenetic tree was constructed with MEGA 6 software (Tamura et al., 2013). Partial LSU rDNA gene sequences of selected *Papiliotrema* and *Cryptococcus* species were retrieved from GenBank. Phylogenetic searches were conducted using the Maximum Likelihood method based on the Tamura-Nei model, as implemented in MEGA 6 software. Bootstrap analysis was performed with 1000 replicates to assess the confidence limits of the branching.

2.4. Utilization of carbohydrates

Overnight cultures of yeast isolates on YM agar were aseptically scrapped off, washed twice and resuspended in sterile water. Cell suspensions were utilized to streak Yeast Nitrogen Base (YNB) agar plates with 1 mM of L-arabinose, D-xylose or cellobiose (Sigma-Aldrich) as the sole carbon source, and supplemented or not with 5 mM $CuSO_4$. Plates were incubated at 25 °C and growth was evaluated periodically for 3 days. Assays were independently repeated at least in triplicate.

2.5. Utilization of phenolic compounds

Cell suspensions were prepared as described for the utilization of carbohydrates. Isolates were streak inoculated on YNB agar medium with and without the addition of 5 mM $CuSO_4$ and supplemented independently with gallic, caffeic and vanillic acid (Sigma-Aldrich) at a final concentration of 1 mM as the sole carbon source. Plates were incubated at 25 °C and growth was evaluated periodically for 3 days. Assays were independently repeated at least in triplicate.

2.6. Auxin synthesis

Yeast isolates were precultured overnight at 25 °C in nutrient broth (NB) composed of 5 g L^{-1} peptone and 3 g L^{-1} yeast extract. 100 mL