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Effects of soil sterilization and metal spiking in plant growth promoting rhizobacteria selection for phytotechnology purposes

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ABSTRACT

The contamination of the soil with heavy metals (e.g. Zn) is a serious and crosscutting issue worldwide. Phytotechnologies can minimize the negative impact of this problem using plants and microorganisms in soil rehabilitation. However, the efficiency of proper plant-microbe combinations is usually assessed using spiked and/or sterilized soils, which do not mimic the conditions *in situ*, and therefore can lead to outcomes that will not be observed under field situations.

This study aimed to quantify the effect of soil origin and sterilization on the performance of the two plant growth promoting rhizobacteria (PGPR), *Ralstonia eutropha* 1C2 and *Chryseobacterium humi* ECP37, for promoting the growth and metal accumulation of maize plants. A two-experiment approach was applied: the PGPR were inoculated in maize plants growing in (*i*) sterilized soils spiked with Zn (0, 100, 500 and 1000 mg Zn kg^{-1}); and in (*ii*) a field-contaminated soil, under sterilized and non-sterilized conditions (599 mg Zn kg^{-1}). Biomass and Zn accumulation in the root and shoot, and Zn bioavailability in soils were determined. Additionally, lipid peroxidation, activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were assessed in the shoots of plants grown in the field-contaminated soil, as well as the composition of the rhizospheric bacterial community.

Zn in the soils negatively affected maize growth, and its effect was strongest in the field-contaminated soil. Overall, PGPR attenuated the negative effects of Zn by improving plant growth, although less pronounced in non-sterilized soils. Sterilization significantly reduced soil Zn availability and affected its' accumulation in plant tissues. Bioinoculants performance was also different in sterilized soil, i.e., bacteria had no effect in the accumulation of Zn but tended to increase the biomass of maize plants. Despite the higher Zn accumulation in shoot tissues, lipid peroxidation was lower whereas antioxidant enzymes were enhanced in non-sterilized soils, suggesting that plant antioxidant system functioned properly. PGPR tended to decrease the diversity of the rhizospheric community.

This study highlights that while inoculation with PGPR is effective in increasing Zn bioavailability in soil, accumulation in the plant and maize growth in Zn-contaminated soils, the extent of their effect can be different depending on whether the soil is field-contaminated or metal spiked, and on whether is sterilized prior contaminated. Consequently, the effect of bacterial inoculants assessed exclusively in metal spiked soil and/or sterilized soil may be overestimated, and potentially not transferable to field conditions.

1. Introduction

Soil contamination by anthropogenic activities is a critical issue worldwide with implications not only for the sustainability of the ecosystems but also for human health (Science Communication Unit, 2013; Van der Perk, 2013). Among soil pollutants, heavy metals (HM) are elements of particular concern, as most of them do not undergo microbial and chemical degradation, remaining in the soils for long periods of time (Ali et al., 2013). Zinc (Zn) is a HM extensively used in industrial activities, and its increasing levels in the soils have rendered it a widespread contaminant (Wuana and Okieimen, 2011) whose excessive levels are toxic to plants, affecting their growth and

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photosynthetic capacity (Islam et al., 2014a, 2014b; Wang et al., 2009). Furthermore, Zn overexposure causes the production of reactive oxygen species (ROS), such as $O_2\cdot^-$ and H_2O_2 leading to oxidative damages in lipids, proteins and DNA (Islam et al., 2014a, 2014b; Morina et al., 2010). However, plants have developed a complex antioxidant defense system to cope with the excess of ROS, which include the activation of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and antioxidative contents like glutathione and ascorbate (Apel and Hirt, 2004).

The urgent need for soil protection and conservation as well as the development of sustainable technologies to ensure the restoration of soils' environmental functionalities and services are a priority in research programs and legislation worldwide (COM, 2012 46). Phytotechnologies (i.e. the use of plants and microorganisms in the recovery of contaminated ecosystems) represent sustainable alternatives to conventional remediation methods (Kidd et al., 2015). Maize (*Zea mays* L.), a plant with a fast growth rate, tolerance to toxic levels of HM, and easy to cultivate and harvest, gathers the features typical of plants suitable for phytotechnologies (Wuana and Okieimen, 2010). Among microorganisms, plant growth promoting rhizobacteria (PGPR) can also play an important role in land restoration via vegetation cover since they are known for their positive effects in plant growth and health status by decreasing HM phytotoxicity (Ma et al., 2011; Moreira et al., 2016a, 2016b; Nadeem et al., 2014; Pereira et al., 2015a). PGPR are root colonizers capable of producing indole acetic acid (IAA), siderophores and even of presenting ACC-deaminase activity, which can help in the establishment of the host plant in contaminated areas (Glick, 2010). However, once PGPR inoculants are introduced into the soil they are exposed not only to abiotic (e.g. high levels of HM) but also to biotic stressors, as native microorganisms may compete with bioinoculants, interfering with their plant-beneficial features (Martinez-Viveros et al., 2010). Therefore, the diversity and structure of microbial communities present in soils have a crucial role in the effectiveness of recovery/restoration strategies implemented through phytotechnology approaches. Given the complexity of field conditions, in most cases it is unreasonable to approach studies with bioinoculants directly in the field. Therefore, a stepwise process that includes greenhouse assays, with plants and target microorganisms is needed prior to advancing to field trials. Greenhouse studies (i.e. the first step) are often performed using sterilized soil to assess the particular effect of the inoculated bacteria, eliminating the interference of native microbial populations (e.g. Jing et al., 2014; Ma et al., 2017; Marques et al., 2013). However, since the purpose of bioinoculation studies is to evaluate and quantify PGPR effects in plants growing in contaminated environments, it is of utmost importance to understand whether the results obtained in these studies are coherent to the outcomes shown in non-sterilized soils. Moreover, the evaluation of the positive features of PGPR under greenhouse conditions is often conducted in metal-spiked soils (Islam et al., 2014a, 2014b; Ma et al., 2011; Marques et al., 2013; Sangthong et al., 2016). Although providing important steppingstones for future research, these studies may overestimate the potential bacterial performance in plants grown in field-polluted soils, since spiked soils can affect HM availability (Berns et al., 2008).

Ralstonia eutropha 1C2 and *Chryseobacterium humi* ECP37 are PGPR isolated from an industrially contaminated site. Both strains have shown strong positive effects in the growth and development of maize plants in spiked Cd-contaminated soils (Moreira et al., 2014), rendering them as encouraging candidates for microbial assisted phytoremediation. In the present study, we aimed to test the efficiency of these bacterial strains to promote growth and Zn accumulation of maize plants grown in: i) sterilized Zn-spiked soil and ii) Zn field-contaminated soil, under sterilized and non-sterilized conditions. With this twofold approach we aimed to evaluate the transferability of results from the simplest to more complex environmental conditions and determine whether soil conditions influences the performance of the bioinoculants. We hypothesize that i) bioinoculants should maintain their positive effects promoting the growth of plants in field-contaminated soil, but ii) sterilization should affect bioinoculants' performance and metal accumulation due to the removal of microorganisms native to soil and due to changes in metal availability. A secondary goal of this study was to evaluate the plant defense response and the changes in rhizosphere bacterial community due to bacterial inoculation in the field-contaminated soil.

2. Materials and methods

2.1. Bacterial inocula

The bacterial strains *R. eutropha* 1C2 (B1) and *C. humi* ECP37 (B2) were isolated from sediment samples recovered from a HM-contaminated area (Estarreja Chemical Complex). In these sediments Zn appears as one of the main contaminants (Pereira et al., 2015b; Pires et al., 2017), reaching values of 3620 mg kg^{-1} (Marques et al., 2007). These bacteria presented in vitro PGP traits, being able to produce IAA, hydrogen cyanide, ammonia and siderophores, show ACC-deaminase activity, and were able to increase growth and nutrient uptake in maize grown in metal-contaminated soils (Moreira et al., 2014). Also, these strains have revealed resistance to Zn up to 1000 mg L^{-1} in solid media (Pereira et al., 2015b; Pires et al., 2017).

2.2. Experiment 1 – efficiency of bacterial inoculation in sterilized Zn-spiked soils

The soil for Zn spiking was randomly collected from a depth of 0–20 cm in an agricultural land from northern Portugal (41°10′N, 8°33′W; NW Portugal). The soil properties were as follows: soil type Cambisol; pH 6.71 ± 0.08 (IUSS, 2015); organic matter content (%) 3.1 ± 0.2; cation exchange capacity (meq 100 g^{-1}) 13.6 ± 0.05; texture sandy loam (sand 75%, silt 16%, clay 9%); total N (mg kg^{-1}) 1735 ± 50; total P (mg kg^{-1}) 2600 ± 23; extractable K (mg kg^{-1}) 106 ± 12; total Zn (mg kg^{-1}) 32 ± 4; total Cd (mg kg^{-1}) < 1.8 (Limit of Detection (LOD)). Soils were autoclaved (120 °C for 70 min in two consecutive days), and dried in an oven at 40 °C for 4 days. Zn spiking was carried out by supplementing soil with zinc chloride to achieve concentrations of 100, 500 and 1000 mg Zn kg^{-1}. Soils were irrigated for 1 week by adding deionized water to maintain 60% of the water holding capacity; the soil was then dried in the greenhouse for approximately 2 weeks. The amended soil was subjected to 3 cycles of wet and dry processes and mixed once a week enabling Zn to disperse in the soil (Blaylock et al., 1997).

The greenhouse experiment consisted of a factorial design with four Zn treatments: non-contaminated soil (control), 100, 500 and 1000 mg Zn kg^{-1} spiked soil, and with three bacterial treatments: B0 (uninoculated – no bacteria), B1 (*R. eutropha* 1C2) and B2 (*C. humi* ECP37) with 4 replicates each.

Maize seeds var. Aveline (Lusosem, Portugal) were surface sterilized with 0.5% (v/v) NaOCl for 10 min and rinsed several times with deionized-sterilized water. Six seeds, placed at 2 cm depth, were sowed in plastic pots (8 cm diameter and 10 cm height) with 400 g of the tested soil. After germination, seedlings were thinned to 4 per pot.

Pure cultures of both bacterial strains were grown in Tryptic Soy Broth (TSB) medium overnight at 30 °C, after which were washed and resuspended in sterilized saline solution (0.85%) in order to obtain a final concentration of 10^8 CFU ml^{-1}.

Bacterial inoculation (10 mL per pot) was performed by spraying soil surfaces two days after seedling emergence as in Moreira et al. (2016a, 2016b). After bacterial inoculation, pots were randomized on the greenhouse (12 h photoperiod, 450 µmol m^{-2} s^{-1} photosynthetically active radiation, 18–21 °C temperature range, 50–60% relative humidity range), process that was repeated every two weeks. The soil was moistened to 60% water-holding capacity and maintained