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Zinc toxicity stimulates microbial production of extracellular polymers in a copiotrophic acid soil

Marc Redmile-Gordon [a], Lin Chen [b, c, *]

[a] Department of Sustainable Soils and Grassland Systems, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK
[b] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[c] Institute of Soil and Water Resources and Environmental Science, Zhejiang University, Hangzhou 310058, China

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ABSTRACT

The production of extracellular polymeric substances (EPS) is crucial for biofilm structure, microbial nutrition and proximal stability of habitat in a variety of environments. However, the production patterns of microbial EPS in soils as affected by heavy metal contamination remain uncertain. Here we investigate the extracellular response of the native microbial biomass in a grassland soil treated with refined glycerol or crude unrefined biodiesel co-product (BCP) with and without $ZnCl_2$. We extracted microbial EPS and more readily soluble microbial products (SMP), and quantified total polysaccharide, uronic acid, and protein content in these respective extracts. Organic addition, especially BCP, significantly stimulated the production of EPS-polysaccharide and protein but had no impact on EPS-uronic acids, while in the SMP-fraction, polysaccharides and uronic acids were both significantly increased. In response to the inclusion of Zn^{2+}, both EPS- and SMP-polysaccharides increased. This implies firstly that a tolerance mechanism of soil microorganisms against Zn^{2+} toxicity exists through the stimulation of SMP and EPS production, and secondly that co-products of biofuel industries may have value-added use in bioremediation efforts to support *in-situ* production of microbial biopolymers. Microbial films and mobile polymers are likely to impact a range of soil properties. The recent focus on EPS research in soils is anticipated to help contribute an improved understanding of biofilm dynamics in other complex systems - such as continuously operated bioreactors.

1. Introduction

Extracellular polymeric substances (EPS) are complex high-molecular-weight mixtures of polymers synthesized by microbial cells. EPS play cardinal roles in nutrient acquisition (Laspidou and Rittmann, 2002), stabilization and protection of biofilm structure (Flemming and Wingender, 2010), microbial adhesion to the habitat matrix (Cammarota and Sant'Anna, 1998; Hong et al., 2013), and impart resistance to toxicity (Henriques and Love, 2007; Joshi et al., 2012). Indeed, stress seems to be a common factor underlying many of the triggers to production of EPS. These stressful triggers can include physical shear, bacteriophage abundance, organic contaminants, biocides and antibiotics (Vu et al., 2009). In the past few decades, the majority of reports on the production and roles of microbial EPS focus on aqueous environments, such as marine (Beech and Cheung, 1995; Zinkevich et al., 1996) and wastewater treatment systems (Sheng et al., 2010; Miao et al., 2015; Wei et al., 2016). Exogenous organic substrate and heavy metal ion concentration are crucial factors influencing microbial EPS production and biofilm formation in these systems. For instance, silver ions and nanoparticles affect the composition of phototrophic biofilm in operated bioreactors (González et al., 2015), and copper and iron concentrations affect the profile of phenolic compounds exuded by marine microalgae (Rico et al., 2013; Lopez et al., 2015).

Zinc (Zn) is a heavy metal of particular concern. Zn^{2+} can be highly damaging for the environment and organisms exposed to it, and is routinely discharged during anthropogenic activity in the mining, chemical, pulp and paper industries (Lu and Chiu, 2006). A recent study of wastewater treatment systems investigated the biological complexation of Zn^{2+} by EPS and found that stretching vibration of O–H, N–H groups and C=O bonds were implicated

Abbreviations: EPS, Extracellular polymeric substances; SMP, Soluble microbial products; BCP, Biodiesel co-product; CER, Cation exchange resin; SOC, Soil organic carbon; iLUC, Indirect land-use change.

* Corresponding author. Institute of Soil Science, Chinese Academy of Sciences, 71 East Beijing Road, Nanjing 210008, China.
E-mail addresses: lchen@issas.ac.cn, linzishike@163.com (L. Chen).

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(Wei et al., 2016). Elsewhere, in agricultural technology, the Zn-tolerant plant-pathogen (*Xylella fastidiosa*) was shown to produce large amounts of EPS-polysaccharide in response to additions of Zn^{2+} to flow cells (Navarrete and De La Fuente, 2014). Indeed there is a growing interest in the responses and roles of microbial exudates in porous media, especially soils, for the purposes of bioengineering and agronomy (e.g. Or et al., 2007) but investigations of native and community-wide EPS responses in soils directly are rare (e.g. Redmile-Gordon et al., 2014b). While easily accessible/labile C is now understood to be a pre-requisite for substantial production of EPS from soil biota (Nunan et al., 2003; Redmile-Gordon et al., 2015a), the influence of any heavy metal contamination on proteinaceous and polysaccharide exudate production by soil native microbial populations *in situ* has not yet been reported.

Besides the knowledge-gap in soil systems, the practical significance of EPS in other environments as a tolerance mechanism against heavy metal contamination suggests there may be further un-explored value in the understanding of soil EPS dynamics such as for more efficient bioremediation of contaminated soils. EPS is also thought to be vital for restoring a range of other important soil ecological and agronomic functions that are related to altered hydraulic dynamics and soil structure (Or et al., 2007). Recently proposed methods to measure EPS in soil (adapted from methods used in aquatic sciences) were applied by Redmile-Gordon et al. (2015a). The authors used ^{15}N isotope probing -and measures of soil ATP- to demonstrate that extraction with cation exchange resin (CER) could be used to contrast changes in total polysaccharide and protein fractions exuded by the native soil microbial biomass. This approach builds upon one of the most frequently used ways to extract EPS in saturated aqueous systems (Frolund et al., 1996). Through the application of this method, biodiesel co-product (BCP) was subsequently shown to be an efficient and sustainable choice of substrate to support EPS production in soil (Redmile-Gordon et al., 2015b). BCP was selected as a C substrate to support microbial metabolism owing to a global and pressing need to reconcile issues of food security and bioenergy through integrated synergies (Redmile-Gordon et al., 2015b; Kline et al., 2016). The growing range of uses for BCP in soils ranges from the capacity to reduce direct N_2O emissions (Alotaibi and Schoenau, 2013) preventing NO_3^- contamination of groundwater (Redmile-Gordon et al., 2014a) and supporting production of EPS via the native soil microbial biomass (Redmile-Gordon et al., 2015b).

Here, we present a laboratory experiment to investigate the responses of microbial EPS production to BCP in a soil contaminated (and not) with Zn^{2+}. The objective of this study was to quantitatively determine how polysaccharide and protein exudate fractions of a heterotrophic soil microbial biomass (utilising BCP as a substrate) was affected by Zn stress. A further objective was to categorise these responses broadly as either belonging to the highly soluble fraction (SMP) or the relatively insoluble but CER extractable fraction: EPS.

2. Materials and methods

2.1. Soil sampling and experimental design

Samples of sandy soil (8.0% clay, a Cambic Arenosol, FAO classification) were collected from the surface horizon (0–23 cm) of a permanent grassland area adjoining plots of the 'Market Garden Experiment' at Rothamsted Experimental Farm (51°59′ N, 0°35′ W), Husborne Crawley, Bedfordshire, UK. The soil contained 16.86 mg g^{-1} organic carbon (SOC), 1.55 mg g^{-1} total nitrogen (N) and a pH of 5.95 (pH as a suspension in boiled, cooled de-ionised water with soil:solution ratio 1:2.5). Twelve moist portions of soil (100 g oven-dry weight equivalent) were placed in glass funnels. These were arranged randomly to compare four treatments with three replicates of each. The treatments compared were: glycerol addition, biodiesel co-product (BCP) addition, BCP plus $ZnCl_2$ addition, and no C addition (control). The Glycerol-C and BCP-C were provided at the start of the experiment at rates of 20 mg C g^{-1} soil. To ensure that the growth of native microbes and EPS throughout the soil were not limited by nutrient availability, ammonium nitrate and monoammonium phosphate were added to all soils at concentrations of 1.50 mg N g^{-1} soil and 0.35 mg P g^{-1} soil, respectively, other nutrients (K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Na^+ and Cl^-) were provided at a concentration of 0.10 mg g^{-1} soil.

After 24 h of C and nutrient addition, 20 mL of 0.01 M $CaCl_2$ was applied to the soil surface in each funnel. This step was repeated each day thereafter to simultaneously re-moisten the soil, remove excess substrate C from soil pores, and redistribute solutes as would occur in a more natural system exposed to weather in an open environment (Redmile-Gordon et al., 2015a). Dilute $CaCl_2$ is commonly used in preference to deionized water in soil laboratory studies as a surrogate for rainwater owing to osmotic similarity (Jalali and Rowell, 2003). A contrasting three portions of 0.01 M $CaCl_2$ were spiked with $ZnCl_2$ to deliver 300 μg Zn^{2+} g^{-1} soil. These were added to three replicates of the BCP-amended soils each day and allowed to drain freely. This daily addition of Zn^{2+} is similar to the difference in Zn concentration between contaminated and uncontaminated soils taken from the same site described previously by Chander and Brookes (1991) with 'uncontaminated' soil yielding a concentration of 107 μg Zn g^{-1} soil by digestion (4:1 (v/v) HCl:HNO_3).

All treatments were incubated in the dark at 25 °C for 7 days. While 10 days has previously been given for development of EPS in these conditions (e.g. Redmile-Gordon et al., 2014b) 7 days was chosen in the present study for analytical convenience. Importantly, it is around this time that EPS responses are likely to be detectable because EPS production is typically greatest around the transition between 'log' and 'stationary' growth phases (Wagner et al., 2003). From our previous observations of inflection points for cumulative CO_2 release curves this point appears to occur sometime between 4 and 12 days in the conditions specified above. At the end of the 7 day incubation period, excess pore-water was removed by applying a 40 cm of mercury-equivalent vacuum to the funnel-outlet and the mesocosms were destructively sampled.

2.2. SMP and EPS extraction, quantification, and statistical analysis

The SMP and EPS extraction protocols were followed as described in the open access article by Redmile-Gordon et al. (2014b). Accordingly, the residual SMP fraction was extracted from moist subsamples (2.5 g dry weight equivalent) placed in 50 mL polypropylene centrifuge tubes (manufactured by Greiner) on an end-end shaker set to 2 cycles per second at 4 °C using 0.01 M $CaCl_2$ at a 1:10 soil:solution ratio. Extracts were then centrifuged at 3200×*g* for 30 min, the SMP solution was decanted and frozen for subsequent analyses. EPS was then extracted from the remaining pellet by re-suspending in new tubes containing 25 mL of EPS extraction buffer. Buffer was prepared in 18 MΩ H_2O to: 2 mM $Na_3PO_4 \cdot 12H_2O$ (0.760 g L^{-1}), 4 mM $NaH_2PO_4 \cdot H_2O$ (0.552 g L^{-1}), 9 mM NaCl (0.526 g L^{-1}), 1 mM KCl (0.0746 g L^{-1}), adjusted to pH 7 with 1 M HCl and cooled to 4 °C. Sufficient CER (Dowex 'Marathon C' sodium form, strongly acidic, 20–50 mesh) was prewashed twice in the above buffer and then added as an amount equal to 178 mg per mg organic carbon in the untreated soil (Redmile-Gordon et al., 2014b), therefore, 7.50 g CER per 2.5 g soil sample in the present case. This was shaken at the same speed as for SMP removal but for 2 h at 4 °C. Samples were then centrifuged at 4000×*g* for 30 min and the supernatant transferred into new tubes. These were frozen