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Bioaugmentation of thiabendazole-contaminated soils from a wastewater disposal site: Factors driving the efficacy of this strategy and the diversity of the indigenous soil bacterial community☆

Evangelia S. Papadopoulou [a], Savvas Genitsaris [a, 1], Michalis Omirou [b], Chiara Perruchon [a], Anastasia Stamatopoulou [a], Ioannis Ioannides [b], Dimitrios G. Karpouzas [a, *]

[a] University of Thessaly, Department of Biochemistry and Biotechnology, Lab of Plant and Environmental Biotechnology, 41500 Larissa, Greece
[b] Agricultural Research Institute of Cyprus, Nicosia, Cyprus

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ABSTRACT

The application of the fungicide thiabendazole (TBZ) in fruit packaging plants (FPP) results in the production of effluents which are often disposed in adjacent field sites. These require remediation to prevent further environmental dispersal of TBZ. We assessed the bioaugmentation potential of a newly isolated TBZ-degrading bacterial consortium in a naturally contaminated soil (NCS) exhibiting a natural gradient of TBZ levels (12000, 400, 250 and 12 mg kg^{-1}). The effect of aging on bioaugmentation efficacy was comparatively tested in a soil with similar physicochemical properties and soil microbiota, which was artificially, contaminated with the same TBZ levels (ACS). The impact of bioaugmentation and TBZ on the bacterial diversity in the NCS was explored via amplicon sequencing. Bioaugmentation effectively removed TBZ from both soils at levels up to 400 mg kg^{-1} but failed at the highest contamination level (12000 mg kg^{-1}). Dissipation of TBZ in bioaugmented samples showed a concentration-dependent pattern, while aging of TBZ had a slight effect on bioaugmentation efficiency. Bioaugmentation had no impact on the soil bacterial diversity, in contrast to TBZ contamination. Soils from the hotspots of TBZ contamination (12000 mg kg^{-1}) showed a drastically lower α-diversity driven by the dominance of β- and γ-proteobacteria at the expense of all other bacterial phyla, especially Actinobacteria. Overall, bioaugmentation with specialized microbial inocula could be an effective solution for the recovery of disposal sites contaminated with persistent chemicals like TBZ.

1. Introduction

Thiabendazole (*2-(thiazol-4-yl) benzimidazole*, TBZ), is a fungicide widely used in fruit packaging plants (FPP) to control fungal infestations during storage (Errampalli et al., 2006). It is applied to fruits via dipping, drenching, spraying, or waxing with its recommended dose in the treatment solutions reaching to 2 g L^{-1} (EC, 2013). The extensive use of TBZ in FPP has resulted in its frequent detection in water resources in fruit production areas (Castillo et al., 2000; Masia et al., 2013). This has raised serious environmental concerns associated mainly with its high environmental persistence ($DT_{50field\_soil}$ = 833–1444 days) (US EPA, 2002), and toxicity to aquatic organisms (i.e. EC_{50} *Onchorynchus mykiss* 0.55 mg L^{-1}) (EC, 2013).

To minimize the environmental risk associated with its use, TBZ was granted authorization by the European Commission (EC) under the clause that "particular attention should be placed to control the wastewater produced by its use and where appropriate risk mitigation measures (i.e. treatment of wastewater prior to their environmental release) should be implemented" (EC, 2000; EC, 2017). In response to this, several wastewater treatment methods including SiO_2-assisted photocatalysis (Jiménez et al., 2015), Fenton oxidation either alone (Carra et al., 2015), or in combination with membrane biological processes (Sánchez Peréz et al., 2014) were developed. Despite their high depuration efficiency these methods were not scaled-up due to problems associated (i) with the

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* Corresponding author. University of Thessaly, Department of Biochemistry and Biotechnology, Laboratory of Plant and Environmental Biotechnology, Viopolis, Larissa 41500, Greece.
E-mail address: dkarpouzas@bio.uth.gr (D.G. Karpouzas).
[1] Current address: Aristotle University of Thessaloniki, Department of Biology, Thessaloniki 54124, Greece.

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production of oxidized intermediates of unknown toxicity, and (ii) the fact that their efficiency was shown at low TBZ concentrations that falls below the levels of TBZ in wastewaters. This gap has forced FPP to dispose their effluents either in municipal sewage treatment plants or in adjacent field sites. The latter practice is expected to result in the buildup of high fungicide levels in such soil disposal sites. These could act as sinks for the lateral environmental dispersal of TBZ, threatening the integrity of adjacent natural water resources. Bioaugmentation is considered a promising approach for the clean-up of soil sites contaminated with recalcitrant chemicals, like TBZ, that tend to resist biodegradation by the indigenous microbial community (Cea et al., 2010). To date little is known about the biodegradation of TBZ. Only recently Perruchon et al. (2017) isolated the first bacterial consortium, composed of α-, β- and γ-proteobacteria, able to rapidly degrade TBZ.

The success of bioaugmentation strategies depends, amongst other factors, on the bioavailability of the target pollutant and the capacity of the inoculum to survive in the soil environment (Schultz-Jensen et al., 2016; Cycon et al., 2017; Horemans et al., 2017). To date most bioaugmentation tests with pesticides have been performed in artificially contaminated soil microcosms (Silva et al., 2004; Wang et al., 2014; Dai et al., 2015). Such experimental approaches do not reflect the real conditions and challenges that exogenous microbial inocula are confronted in naturally contaminated field sites, where pesticides bioavailability becomes limited with time, as a result of aging (Alexander, 2000; Silva et al., 2015).

Upon their release in soil, the exogenous microbial inocula should compete with the indigenous microbial community for space and nutrients. Microcosm studies with artificially pesticide-contaminated soils showed that bioaugmentation with bacterial inocula imposed consistent, transient or no effects on the soil microbial community (Morgante et al., 2010; Dai et al., 2015; Chang et al., 2015). In those studies microbial diversity was determined via molecular fingerpinting, which could identify only the dominant members of the soil microbial community (Muyzer and Smalla, 1998). In contrast, novel high-throughput amplicon sequencing approaches provide adequate sequencing depth to characterize the top 99.99% of the soil microbiota (Lynch and Neufeld, 2015). Application of these tools in naturally polluted soils subjected to bioaugmentation would provide an in-depth view of the diversity of the microbial communities in heavily polluted soils and their dynamics during bioaugmentation.

The main aims of our study were (a) to explore the bioaugmentation potential of a TBZ-degrading consortium for the decontamination of naturally contaminated soils exhibiting a gradient of TBZ contamination levels, (b) to investigate the impact of aging on the bioaugmentation potential of the consortium, and (c) to explore the impact of bioaugmentation and TBZ contamination levels on the composition of the soil bacterial community using high-throughput amplicon sequencing.

2. Materials and methods

2.1. Chemicals

An analytical standard of TBZ (99.8% purity, Fluka, Switzerland) was used for analytical purposes. A commercial formulation of TBZ (Hykeep 30 EC) was used in the soil bioaugmentation experiment.

2.2. Soils

Two soils were used in this study. The first was collected from a field site in Limmasol, Cyprus which was used for the systematic disposal of wastewaters from a nearby FPP regularly using *ortho*-phenylphenol and TBZ (Naturally Contaminated Soil, NCS). Wastewaters were discharged in the field through a pipe. The field was divided into four 2-m transects at 0–2, 2–4, 4–6, and 6–8 m distance from the pipe, which were named transects A, B, C, and D, respectively (Supplementary Fig. 1). HPLC analysis of triplicate soil samples from each transect showed the presence of a natural gradient of TBZ concentration levels in transects A (12000 mg kg^{-1}), B (400 mg kg^{-1}), C (250 mg kg^{-1}), and D (12 mg kg^{-1}).

The second soil had no record of exposure to TBZ (Artificially Contaminated Soil, ACS), and it was collected from a potato-cultivated field site in the region of Thiva, Greece. Topsoil samples (0–20 cm) were collected from five selected points of the field site following the W non-systematic pattern of sampling, according to the ISO (2002a,b). Samples were mixed to provide a bulk soil sample which was stored at 4 °C until further analysis. Soil analysis demonstrated that the two soils had similar physicochemical properties (Table 1).

2.3. TBZ-degrading consortium

The TBZ-degrading consortium used in the study was isolated from a soil from a wastewater disposal site. The consortium was composed of α- (*Sphingomonas*, *Oligotropha, Shinella*), β- (*Hydrogenophaga, Thiobacillus*), and γ- (*Hydrocarboniphaga*) proteobacteria (Perruchon et al., 2017). The *Sphingomonas* and *Hydrogenophaga* phylotypes were the dominant members of the consortium contributing more than 20% of the total number of sequences in the preliminary assembled metagonenome of the consortium (Vasileiadis S., unpublished data). The consortium was routinely grown in a minimal selective medium (MSMN) (Karpouzas and Walker, 2000a) supplemented with TBZ (25 mg L^{-1}) where the pesticide constituted the sole C source.

2.4. Preparation of bacterial inoculum

An actively growing culture of the consortium in MSMN + TBZ (25 mg L^{-1}) was used for the inoculation of fresh MSMN + TBZ (25 mg L^{-1}) where the degradation of TBZ was followed as described below. In addition, we determined the total bacterial growth during degradation of TBZ by (i) q-PCR using bacterial universal primers 338f (Lane, 1991) and 518r (Muyzer et al., 1993), as described by Perruchon et al. (2016) (ii) OD_{600} measurements and (iii) dilution plating on LB (Supplementary Fig. 2). In parallel, the dynamics of the *Sphingomonas* phylotype, known to drive the degradation of TBZ in the consortium, was also determined by q-PCR using bacterium-specific primers (Perruchon et al., 2017). These tests were essential to determine the consortium growth kinetics, and the final inoculum density for the bioaugmentation study.

An aliquot (6.25 mL) of the latter culture was used to inoculate a fresh culture (250 mL) of the consortium which was grown on a shaking incubator at 25 °C and 180 rpm. Bacterial inoculum was harvested at 50 h, which coincided with near complete degradation of TBZ and the maximum density of both the *Sphingomonas* phylotype and the total bacterial community (Supplementary Fig. 2). Cells were washed three times in sterile ddH_2O before re-suspended to appropriate volumes of ddH_2O which were used for the inoculation of soil samples aiming to a final inoculum density of 6.5×10^6 cells g^{-1} soil (on a dry weight basis).

2.5. Bioaugmentation study

ACS (4 Kg) was autoclaved at 120 °C for 30 min to eradicate its indigenous soil microbiota. The autoclaved ACS was then inoculated with 5% (w/w) of the NCS. Inoculated samples were mixed