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Micromapping of microbial hotspots and biofilms from different crops using digital image mosaics of soil thin sections

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ABSTRACT

Despite the importance of spatial analysis and its contribution to environmental microbiology, there are few studies examining size, quantity, and distribution patterns of hotspots and biofilms at microscale levels, remaining such an issue as a main challenge within soil ecology. Micro-cartography of digital image mosaics, with high spectral and spatial resolutions, associated to a georeferenced grid, and with several microscopic techniques, may provide an alternate method to study microbiological features in the rhizosphere of several crops systems. Thin sections (7 cm × 5.5 cm), from undisturbed soil samples, were selected in order to show how the methodology works and its potential application. Bacteria were stained in thin sections using CFW (calcofluor white) M2R. Sequential images were obtained with a high-definition digital camera, mounted on petrographic and optic (equipped with mercury lamp for epifluorescence) microscopes, using 2× and 10× objective lens. According to these objective lens, the processed mosaics represented 38.5 cm^2 (81 images with a spatial resolution of 2.61 μm) and 1 cm^2 (216 images with a spatial resolution of 200 nm), respectively. Results indicate that it is possible to quantify and elaborate thematic maps of bacteria colonies (hotspots and biofilms), and to relate these colonies to other soil components, within different plants and cropping systems. Although most of the bacterial hotspots were associated to aggregates or groundmass, differences between crops were observed in the greater colonies. In maize for instance, greater bacteria colonies were related with mineral grains and voids; whereas in alfalfa and in grass, these colonies were associated with exudates of root residues. In addition, biofilms occurred within the rhizosphere of alfalfa and within several bio-pores in grass. Micro-mapping of bacteria through digital mosaics allows researchers to performing "*in situ*" spatial analysis of hotspots and biofilms, and their relation with soil components. The hybridization of different technological tools provides a strategy to effectively assess microbial activity in soils within a multi-scalar frame of reference.

1. Introduction

The quantitative analysis to attain the spatial distribution of soil bacteria (now hotspots) requires locating bacterial cells, which are most quantified accurately at different scales (Nunan et al., 2001). However, despite the importance of spatial analysis within the discipline of environmental microbiology, there are few studies that examine the distribution patterns of bacterial communities at fine scales (Franklin and Mills, 2007; Nunan et al., 2007), in which occur important ecological interactions. The lack of information concerning these scales implies that such interactions cannot be explained by current models describing the development and function of microorganisms in the soil (Nunan et al., 2007).

Furthermore, this quantitative analysis carried out from altered soil samples presents disadvantages of being difficult to know the distribution of soil bacteria, neither its relation with other components of the soil matrix (Li et al., 2004). In consequence, estimating the size of hotspots and the proportion of the total soil volume that they represent becomes a major challenge within soil ecology (Kuzyakov and Blagodatskaya, 2015). Similarly, understanding the interactions between rhizosphere microorganisms still has limitations, because it is difficult to study such processes under near-to-real conditions (Bais et al., 2006).

A direct approach to characterize the distribution "*in situ*" of soil components (microbial habitat) includes the use of soil thin sections, which provide valuable information (Ringrose-Voase, 1991; Li et al., 2003); which therefore, constitute a powerful technique in soil microbiology (White et al., 1994). Yet, the problem remains in assessing bacterial distribution patterns, because they have been studied with individual images using very high microscope magnifications (Chenu et al., 2001; Eickhorst and Tippkötter, 2008). These images represent very small areas, so small that they can reach up to ~0.01 mm^2 (Nunan et al., 2001), which may limit direct observation of

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microorganisms and their relations to other soil components (Li et al., 2004). It is in this sense that the relationships between bacterial communities and their spatial organization within soil structure have not been fully understood (Grundmann, 2004).

In addition, sequential images are necessary to visualize all soil components of interest and high resolution mosaics need to be built (VandenBygaart and Protz, 1999). Mosaics have been elaborated (Nunan et al., 2001; Bruneau et al., 2005); however, the visualization surface is very small (0.282 mm^2), making it hard to establish distribution patterns between soil components at multiple scales. Moreover, without image georeferencing, the overlay of images showing the same feature at nano and micro scale is impossible, losing information about their interactions.

Besides, research studies that use images of greater size, that is, at soil thin section level (Terribile and Fitzpatrick, 1992; Protz and VandenBygaart, 1998; VandenBygaart and Protz, 1999; Aydemir et al., 2004), have low resolution because they are created using a scanner. Other researchers use geographic information system software to process soil images (Aydemir et al., 2004; Tarquini and Favalli, 2010) created with different light sources, such as bright-field, polarized, ultraviolet, and infrared (Protz et al., 1992; Terribile and Fitzpatrick, 1992), with easier feature quantification. Nevertheless, the low resolution of images limits feature identification, and therefore, their delimitation and mapping.

Mosaics, at different magnifications (spatial scales), using for instance, cartographic techniques similar to those found in GIS applications, is what have spawned into a new recent proposal for mapping the soil micro-environment at different scales (from millimeters to microns), including the quantification and distribution of several soil features (Gutiérrez-Castorena et al., 2015), without the use of a microscope and without losing the spatial reference (georeference) of the original soil sample. In addition, mosaics representing all the thin section surface, show soil features, including soil structure "*in situ*", and with the aid of fluorescent microscopy, microorganisms may be observed (Li et al., 2003; Altemüller and van Vliet-Lanoe, 1990; White et al., 1994; Fisk et al., 1999; Nunan et al., 2001) directly in contact with other soil components; thus hotspots and biofilms of bacteria can be quantified in an easier way.

Therefore, fusion of these techniques might increase image resolution and microorganism identification, making it possible to mapping soil components at micro scales, quantifying several features using different light sources, and creating bacterial hotspots maps.

The objective of this research was to determine the distribution and quantification *in situ* of hotspots and biofilms at the rhizosphere in three different crops, using mosaics of high spectral and spatial resolutions, at different zoom levels in soil thin sections, and with fluorescent microscopy and cartographic tools.

2. Materials and methods

2.1. Study area

The present research was carried out in "Los Insurgentes" Irrigation District, located in Teoloyucan, State of Mexico, at 20 km north from Mexico City. It is geographically bounded by North latitudes 19°43′11″ and 19°47′11″, and West longitudes 99°43′15″ and 99°12′57″, with an average altitude of 2250 m.

Soils in the area of study have been irrigated through flooding with wastewater coming from Mexico City's metropolitan zone. Additionally, these soils were formed from lacustrine sediments dredged from the Zumpango lagoon, and from alluvial sediments extracted from the "Santo Tomás" channel. Soils have been classified as Terric Hydragric Anthrosols (Reséndiz-Paz et al., 2013); although some instances in the region have been classified as Hortic Hydragric Anthrosols, because of their biological activity (IUSS-WRB Working Group, 2007).

2.2. Field work

From a previous soil survey carried out by Reséndiz-Paz et al. (2013) in a 450 ha agricultural site belonging to the previously mentioned irrigation district, a total of 64 (unaltered and altered) soil samples were collected for laboratory and micromorphological analysis from 16 representative soil profiles. However, in the experimental part, only six undisturbed soil samples, taken directly in the rhizosphere of different crops, were selected in order to show how the methodology works and its potential applications. Undisturbed soil samples were collected using a Kubiena tin in maize (*Zea maize* L.), alfalfa (*Medicago sativa* L.) and forage grass (*Lolium multiflorum* Lam Husnot).

2.3. Laboratory analysis

Bulk soil samples were air-dried at room temperature, ground, and sieved with a 2 mm mesh. Physical and chemical analyses were carried out according to Van Reeuwijk (2002). Analyses performed for describing basic soil properties included texture (pipette method), color for both dry and wet samples (Munsell color chart), bulk density (clod in paraffin), organic matter (Walkley and Black), soil reaction (pH in 1:2.5 soil:water ratio), cation exchange capacity (CEC), by the ammonium acetate method, and base saturation from the sum of exchangeable cations.

2.4. Soil thin sections preparations

Undisturbed samples were air dried prior to impregnation with polyester resin and monostyrene (1:1 ratio); then soil thin sections (7 cm × 5.5 cm × 20 μm) were elaborated according to the method of Murphy (1986). Soil components or features such as aggregates, roots, and organic matter were described following the concepts and terminology of Bullock et al. (1985) and Stoops (2003).

Soil thin sections, without cover glass, were stained applying sigma Calcofluor white (Sigma-Aldrich Switzerland) according to the procedure described by Altemüller and Vorbach (1987), and Postma and Altemüller (1990); however, a slight modification was made to such procedure, consisting in that soil thin sections were only immersed in a recipient with fluorochrome for 5 min, and washed with distilled water, prior to their drying and covering with cover-glass.

2.5. Culturable bacteria in soil

To determine the individual colonies of bacteria number or colony forming units (CFU), a rhizosphere soil sample of each crop was spread or poured uniformly on a surface of an agar plate, incubated (for 48 h), and then, colonies formed were counted following the methodology of Breed and Dotterrer (1916).

2.6. Digital mosaic construction and image digital analysis

Acquisition of digital images, their geo-referencing, and projection to individual images until thematic maps elaboration are summarized in the Fig. 1.

Digital sequential images (DSI) were obtained manually through a screen (23″) using a Canon EOS Rebel 35OD digital camera, mounted on an Olympus BX51 petrographic microscope, with a 2× objective lens, and with a Plane Polarized Light (PPL). Each image had a resolution-size of 4272 × 2848 pixels and an overlap of 1 mm horizontally and vertically. In addition, the raster mosaic was integrated by 81 images (9 × 9), equivalent to an area of 38.5 cm^2. The final dimensions of the mosaic were 29,194 × 19,956 pixels, a resolution of 2.6 μm and a size of 1.83 GB.

From the 2× mosaic a portion of 1 cm^2 was selected in an elongation zone where high density of root exudates occurred, according to Lagos et al. (2015). A new set of DSI was taken using the same digital camera