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Mapping the footprint of nematodes in the rhizosphere: Cluster root formation and spatial distribution of enzyme activities

Bahar S. Razavi [a, *, 1], Duyen T.T. Hoang [b, c, 1], Evgenia Blagodatskaya [b, d], Yakov Kuzyakov [a, b]

[a] Department of Agricultural Soil Science, University of Göttingen, Göttingen, Germany
[b] Department of Soil Science of Temperate Ecosystems, University of Göttingen, Göttingen, Germany
[c] Department of Soil Sciences, Vietnam National University of Forestry, Hanoi, Viet Nam
[d] Institute of Physicochemical and Biological Problems in Soil Science, Pushchino, Russia

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ABSTRACT

Nematodes are among the most important pathogens in agriculture, greatly reducing crop biomass and yield. The direct effects of nematodes on above– and belowground plant parts are well known, but the broad range of indirect effects, especially on carbon (C) and phosphorus (P) cycles underground, remains unknown. For the first time, using soil zymography, we analyzed the indirect effects of *Meloidogyne incognita* cellobiohydrolase and phosphatase. The rhizosphere of lupine (*Lupinus polyphyllus* L.), a species sensitive to pathogens with high P demand, was selected to study the activity, distribution and localization of two enzymes responsible for C and P cycling: The distribution patterns of cellobiohydrolase and phosphatase demonstrated that *M. incognita* induced the formation of knots as well as cluster roots, which corresponded to hotspot locations on zymogram images for both enzymes. Increased C release by nematode–infected roots into the soil led to a decrease in the overall activity of cellobiohydrolase and especially at hotspots (by ~ 20 times). In contrast, the increased P demand of infected plants raised the phosphatase activity, leading to an increase in the rhizosphere extent around the roots and especially of the hotspot area (by 6 times). Remarkably, this 1 mm increase of rhizosphere extent in 2D equals a 2-fold increment in soil volume (3D) for nutrient mobilization.

We conclude that nematode infection not only has direct effects by changing root morphology, but also induces a number of subsequent biochemical changes (e.g. enzyme activities and consequently nutrient mobilization) in the rhizosphere, affecting C and P cycling.

1. Introduction

The complex interactions between plants and microorganisms in the rhizosphere – the soil volume affected by roots – are a compromise between costs and benefits. Microorganisms benefit from rhizodeposition and habitat niches, and plants inherit the available nutrients released by microbial community decomposition of organic matter. However, the C-rich rhizosphere (Bais et al., 2006) also attracts and accommodates pathogens. Among the various pathogens, nematodes – as the most abundant group of soil fauna – are a focus of interest (Ferris et al., 2001). Root-knot nematodes (*M. incognita*) are obligate biotrophic parasites that invade roots (Adam et al., 2014; Taylor and Sasser, 1978) by piercing and rupturing the cell walls with their stylets. This ultimately causes the cytoplasm to expand and become denser (Williamson and Hussey, 1996). Infected plants can be recognized after one month of infection based on the swollen root morphology. After invading the root, the infective juveniles either move down to the root tip, turning around the root apical meristem or migrate up to the root (Williamson and Hussey, 1996). To some extent, the whole root will be affected by a nematode infection, which leads to a change in root exudation (Bais et al., 2006). Root exudates are an important constituent of rhizodeposits, comprising carbohydrates, organic acids and amino acids (Bais et al., 2006; Hirsch et al., 2013). The composition of root exudates, however, is not identical along the entire root affected by nematode invasion (Hallmann et al., 2001). This is reflected in different microbial community compositions (Fontaine et al., 2007) and different activities of enzymes produced by both plant roots and microorganisms (Grierson and

* Corresponding author.
E-mail address: brazavi@gwdg.de (B.S. Razavi).
[1] Two first authors contributed equally to this work.

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Adams, 2000; Blagodatskaya et al., 2009) along and around individual roots. Although the spatial distribution of enzymes is associated with root type (tap roots, fibrous roots) (Razavi et al., 2016), past studies have focused solely on healthy roots. However, the microbial community modified by ruptured plant cells and nematode activity can apparently regulate the activities of the respective enzymes in the rhizosphere. This is important, considering that species of *Meloidogyne* are parasites that secrete more than sixty plant cell wall-degrading enzymes, including cellulases, xylanases, polygalacturonases, pectate lyases and arabinases (Abad et al., 2008). Nonetheless, we lack any knowledge about the gradient or distribution of enzyme activities along and around individual roots in response to nematode attack.

Direct soil zymography – a non-destructive 2D technique – has been used to illuminate enzyme activities in soil (Sanaullah et al., 2016), biopores (Hoang et al., 2016), the rhizosphere (Ge et al., 2017; Razavi et al., 2016) and the detritusphere (Liu et al., 2017; Ma et al., 2017). This study presents quantitative imaging of enzyme activities in soil as a function of distance along and outward from the root to clarify whether 1) nematodes affect the spatial distribution of enzyme activities as a function of distance from the root; 2) this effect is enzyme specific. We conducted experiments on two enzyme activities – cellobiohydrolase and phosphatase – using the zymography technique. Cellobiohydrolase synthesized by microorganisms and nematodes (Williamson and Gleason, 2003) is a specific enzyme catalyzing cellulose degradation by hydrolysis of β-1,4-glycosidic bonds (German et al., 2011). Concurrently, nematode invasion shifts plants into a severe P-stress status (Venkatesan et al., 2013). In response, phosphatase is produced simultaneously by roots and microbes (Nannipieri et al., 2011) to increase P availability to cover the P demands of the plants. We hypothesize that i) cellobiohydrolase activity will be lower after nematode infestation due to more labile C (sugars) in the rhizosphere, whereas we expect a higher activity of phosphatase stimulated by the elevated P demand of plants; and ii) plant roots produce more P-acquiring enzymes in response to their P stress, caused by nematodes, leading to a larger spatial extension of phosphatase than cellobiohydrolase.

We installed rhizoboxes with nematode-inoculated and nematode-free lupine. Substrate-soaked membranes were applied to map the distribution of enzyme activities in the rhizoplanes. Image processing in Matlab was used to localize and evaluate enzymatic hotspots associated with root and/or nematode effects.

2. Materials and methods

2.1. Sample preparation

Soil samples were taken from an arable loamy Haplic Luvisol located on Campus Klein-Altendorf (50° 37′ N, 6° 59' E), south-west of Bonn, Germany. The soil consisted of 7% sand, 87% silt, 6% clay, with a bulk density of 1.4 g cm^{-3}, a water content of 30% at field capacity, a pH of 6.5, total C of 12.6 g C kg^{-1}, and total N of 1.3 g N kg^{-1} (Kramer et al., 2013; Pausch et al., 2013).

Eight lupine (*Lupinus polyphyllus* L.) plants were grown, each in a separate rhizobox with inner dimensions of 21.2 × 10.8 × 3.3 cm. The rhizoboxes were placed horizontally with front side open and soil was slowly and continuously poured into them through a 2 mm sieve to achieve a uniform soil packing and to avoid soil layering. The front side was then closed, the samples were turned to a vertical position, and they were gently shaken to achieve bulk density of 1.4 g cm^{-3} and a stable soil packing. The seeds were germinated on filter paper for 72 h. Then, one seedling was planted in each rhizobox at a depth of 5 mm.

Four lupines were inoculated– with the same level of inoculum–by aqueous suspension containing 50 *Meloidogyne incognita* after 10 days of plant growth and were incubated for 21 days before harvest. During the 31 days of growth, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew along the lower wall of the rhizoboxes. Plants were kept in a climate chamber with a controlled temperature of 20 ± 1 °C and a daily light period of 16 h with a photosynthetically active radiation intensity of 300 μmol m^{-2} s^{-1}. During the growth period, the soil water content was maintained at 60% of the water holding capacity by irrigating the soil from the bottom with distilled water.

2.2. Direct zymography under influence of nematodes

After cultivating lupine for 31 days, zymography was applied as an *in situ* technique to study the spatial distribution of enzyme activities around the roots. We followed the optimized direct zymography method after Razavi et al. (2016). Enzyme activities were visualized using membranes saturated with 4-methylumbelliferone (MUF) substrates. Cellobiohydrolase activity was detected with 4-methylumbelliferyl-β-D-cellobioside (MUF-C), and acid phosphatase activity with 4-methylumbelliferyl-phosphate (MUF-P). Each of these substrates was separately dissolved to a concentration of 10 mM in MES buffer (Koch et al., 2007) (Sigma-Aldrich, Germany). Polyamide membrane filters (Tao Yuan, China) with a diameter of 40 cm and a pore size of 0.45 μm were cut to fit the rhizobox. The membranes were saturated with the substrates for each enzyme. The rhizoboxes were opened from the lower, rooted side and the saturated membranes were applied directly to the soil surface (Sanaullah et al., 2016; Razavi et al., 2016). After incubation for 1 h (incubation time was determined in preliminary experiments), the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers.

Quantification of zymogram images requires a standard calibration that relates the activities of various enzymes to the gray-value of zymogram fluorescence (i.e. of the membrane). The calibration function was obtained by zymography of 2 × 2 cm membranes soaked in a solution of MUF with concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1, 3, 6, 8, and 10 mM. The amount of MUF on an area basis was calculated from the solution volume taken up by the membrane and its size. The membranes used for calibration were imaged under UV light and analyzed in the same way as the samples.

2.3. Image processing and analysis

Fluorescence of the zymograms under UV light shows the areas in which the substrate has been enzymatically hydrolyzed. The intensity of fluorescence is proportional to the activity of the enzyme. To obtain quantitative information, we processed the zymograms in Matlab according to Razavi et al. (2016). Briefly, zymograms were transformed into 16-bit grayscale images as matrices and corrected for light variations and camera noise. Then the zymograms were referenced based on the grayvalue received from a reference object embedded in all the zymograms. We used the grayvalue obtained from the blank sides (outside the membrane) of each image as the referencing point for all images. After referencing the zymograms, we calculated an average background grayvalue through the zymograms of calibration lines at zero concentration and subtracted this value from all the zymograms. Note that the same filters were applied to all of the images, including both the zymograms of the roots and the calibration baseline. The pixel-wise grayvalues from zymography were converted to enzyme activities using the calibration function obtained for both enzymes.