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

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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.

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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 decomposi-
tion 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 composi-
tions (Fontaine et al., 2007) and different activities of enzymes produced by both plant roots and microorganisms (Grierson and
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, polysaccharidas, 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 (Huang 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—cellulohydrolase 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) cellulohydrolase 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 rhizobases with nematode-infected 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 rhizobases 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 rhizobases were kept inclined at an angle of 45° so that the roots grew along the lower wall of the rhizobases. 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-celllobioside (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 substrates for each enzyme. The rhizobases 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 membrane soaks 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.
The resulting images were used for further analysis: four roots were segmented as replicates in each nematode-inoculated box, with roots clearly distinguishable from the surrounding soil due to strong contrast between the soil and roots. Therefore, we had a total of 12 roots from 3 nematode-inoculated boxes and another 12 roots from control boxes (without nematode infection). A threshold method in Matlab was used to detect the boundaries of the roots (Chaudhuri et al., 1989). The length and radius of segmented roots were calculated using the Euclidean distance map function in Matlab to calculate overall enzyme activity on root surface.

Hotspots were distinguished from the surrounding area by their contrasting color intensity in digital images. Based on referenced images and of the calibration line, the color intensity of all pixels exceeding the average value (i.e. >0.6) was assigned to hotspots of enzyme activity, represented by red color, with blue indicating lower activities (Hoang et al., 2016). To confirm the boundaries, one-way analysis of variance (ANOVA) was applied to assess the significant differences between independent variables (mean color intensity values of five adjacent pixels, i.e. equal to 0.1 mm). Significant differences between two adjacent groups of 5 pixels were then considered as a boundary for each category of activity (low, medium and hotspot) (Fig. S1) (Hoang et al., 2016). The ANOVA, followed by Tukey HSD test at a probability level of \( p < 0.05 \), confirmed the categories of enzyme activity and also defined the significant difference of one specific enzyme distribution between two treatments (with and without nematodes). Homogeneity of variance and normality were tested by Levene's test and Shapiro-Wilk's W test.

3. Results

3.1. Overall enzyme activities and hotspots

The formation of root-knots and cluster roots by lupine was well pronounced one month after inoculation with *M. incognita* (Fig. 1). Consistently, for all replicates, inoculated plants showed the formation of 3 times more cluster roots, as well as more lateral roots, compared to the non-infected control. The zymograms of individual plants with and without *M. incognita* inoculation demonstrate the distributions of enzyme activities along and across the roots (Fig. 2 and Fig. 3). The percentage contribution of medium activity and hotspots to total activity of cellobiohydrolase decreased strongly (by a factor of 1.5 and 20 times, respectively) with nematode infection (Fig. 2). In contrast, these percentages for phosphatase increased by 1.2 times for medium activity and 6 times for hotspots (Fig. 3). High activity at root knots was a common pattern for both phosphatase and cellobiohydrolase.

Thus, nematode infection not only changed root morphology, with more generation of lateral and cluster roots, but also the overall enzyme activities on the rhizoplane and in the rhizosphere (Figs. 2 and 3).

3.2. Distribution of enzyme activities along and outward from the roots

Both enzymes demonstrated a similar uniform distribution along the roots (Fig. 4). However, cellobiohydrolase activities were 67% lower in infected versus control plants, whereas phosphatase activities were 56% higher.

The rhizosphere extent of non-infected plants varied between enzymes: the phosphatase activity distribution was broader (3–3.5 mm from the root) than for cellobiohydrolase (1–1.5 mm). The rhizosphere extent for both enzymes was strongly affected by nematode infection: phosphatase distribution was broader (4–4.5 mm) and cellobiohydrase distribution was significantly narrower in infected plants (0.5–0.8 mm) than in healthy plants. Altogether, the radial patterns of enzyme activity around the lateral roots of infected plants differed from those for non-infected control plants (Fig. 5).

4. Discussion

*M. incognita* infection strongly affected the root morphology and rhizosphere biochemistry within 3 weeks of infection.

4.1. Root morphological response to *M. incognita* invasion

*M. incognita* infection had a clear effect on the host plant through root morphological changes and the appearance of root knots. The widespread appearance of knots along the main roots of
Fig. 2. Above: Zymogram of cellobiohydrolase activity: A) control (normal root) and B) infected with nematodes. Bottom: a. cellobiohydrolase hotspot percentage: control, b. cellobiohydrolase hotspot percentage: infected root. The color bar corresponds to enzyme activity (nmol cm\(^{-2}\) h\(^{-1}\)). Asterisks (*) indicate significant differences in hotspot percentage between control and infected plants at \(p < 0.05\) after Tukey’s HSD test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Above: Zymogram of phosphatase activity: A) control (normal root) and B) infected with nematodes. Bottom: a. phosphatase hotspot percentage: control, b. phosphatase hotspot percentage: infected root. The color bar corresponds to enzyme activity (nmol cm\(^{-2}\) h\(^{-1}\)). Asterisks (*) indicate significant differences in hotspot percentage between control and infected plants at \(p < 0.05\) after Tukey’s HSD test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
lupine is a clear symptom of nematode attack (Seinhorst, 1961). In contrast, an increased number of lateral roots and cluster-root formation are adaptations of the plant for nutrient acquisition (Neumann et al., 2000; Shane and Lambers, 2005). The interactions between roots and pathogenic nematodes lead to the reformation of root structure as a "signaling language" (Baron and Zambrysky, 1995; Mathesius, 2003). One of the first signs of nematode invasion is the local induction of lateral root growth at the feeding site (Karczmarek et al., 2004). In fact, the site of lateral root emergence is also a preferable site of penetration by nematodes (Bird and Kaloshian, 2003). Thus, the appearance of lateral roots is beneficial in terms of nutrient and water absorption by plants in response to nematode damage, but it also increases the possibility of pathogen invasion. Along with more lateral roots, cluster roots became more abundant in infected than in healthy plants in all replicates—this was the second signal of nematode infestation. Generally, cluster roots are stimulated by P starvation (Neumann et al., 1999; Gilbert et al., 1999). Our agriculturally used Luvisol is not P-deficient, so the generation of cluster roots was a nematode effect to increase the efficiency of phosphorus uptake by nematode-infested, P-demanding plants (Oteifa, 1952; Widdowson et al., 1972). Accordingly, cluster root presence supported the plant to deal with nutrient deprivation due to nematodes (Oteifa, 1952) by increasing the chemical mobilization of nutrients in the rhizosphere (Neumann and Martinoia, 2002) and by increasing its surface area for nutrient uptake (Marschner et al., 2002).

Fig. 4. Distribution of enzyme activities: Left: cellobiohydrolase and Right: phosphatase, along the normal and infected lateral roots.

![Fig. 4](image)

**Fig. 5.** Profile of enzyme activity distribution as a function of distance from the control (normal root) and infected lateral root center to the surrounding soil: a. cellobiohydrolase, b. phosphatase. Each line refers to the mean values of four sampled roots. Error bars are omitted to improve visualization. Bands indicate the rhizosphere boundary (extent) for each enzyme.

Fig. 5.

**a. Cellobiohydrolase**

**b. Phosphatase**

4.2. Hotspot distribution associated with roots, root-knot location and enzyme specifics

The differences in enzyme activities between infected and healthy roots demonstrated the quantitative and qualitative effects...
of nematodes on rhizodeposition components, which is in line with findings by Haase et al. (2007). Our results consistently supported the first hypothesis that nematode infection decreases cellobiohydrolase activities but increases phosphatase activities (Figs. 2 and 3).

In the infected-plant rhizosphere, the enzymes are produced not only by roots and microorganisms, but also by nematodes (Abad et al., 2008). The multiple interactions among these organisms may restructure enzyme systems by changing C and nutrient availability near roots. The nematode effects were enzyme specific: decrease of cellobiohydrolase activities (cellulose hydrolysis) but increase of phosphatase activities (organic P hydrolysis). High and medium activities of cellobiohydrolase accounted for 30% of total enzyme activity in nematode-infected roots as compared to 45% in the controls (Fig. 2 bottom). The increased C leakage from infested roots (Yeates et al., 1998, 1999; Bonkowski et al., 2000), especially at the injury sites, diminished the production of enzymes involved in the C cycle. This is an energy-saving strategy of microorganisms (Denton et al., 1998; Bardgett et al., 1999; German et al., 2011). In contrast, the root knots are considered to be metabolic sinks (Wallace, 1974; Back et al., 2002), which attract fungi and bacteria. The colonization of plants by additional pathogens may lead to nutrition competition with the nematodes and enhance enzyme expression. High enzyme activities, therefore, were typical for both cellobiohydrolase and phosphatase at knot locations.

The production of cell-wall-degrading enzymes, namely cellulase (Williamson and Gleason, 2003), is the first step for nematodes to enter roots. This enzyme is also produced by microorganisms to hydrolyze cellulose in sloughed-off root cap cells. Therefore, hotspots of cellobiohydrolase (amounting to 0.1% of total activity around infested roots) represent the combined enzyme synthesis of nematodes and other microorganisms. These hotspots correspond mainly to the nematode knots (high color intensity in Fig. 2). Compared to healthy roots, cellobiohydrolase activities along the main root were lower, as indicated by lower color intensity. This is due to the accumulation of photosynthates at the nematode feeding sites (Bird, 1974; Bird and Loveys, 1975; Melakeberhan and Ferris, 1989) instead of being homogeneously distributed along the main root. High cellobiohydrolase activity at root knots was in line with the metabolic sinks proposed by Bird (1974) and Wallace (1974).

In contrast, hotspots and medium activities of phosphatase represented 42% of total activity in affected roots versus 35.5% in control soil (Fig. 3). This increase is explained by the 3-fold increase in plant P demand under nematode infestation (Widdowson et al., 1972). The P stress stimulates the rhizosphere microorganisms and roots (Taraifar and Claassen, 1988; Miller et al., 2001) to produce more phosphatase along the whole root and especially at cluster roots. Such a proliferation of cluster roots increases the P uptake by 50% in white lupine (Neumann et al., 1999). Accordingly, high enzyme activity illustrated by high color intensity on zymograms, was associated with roots (Asmar et al., 1994; Marinari et al., 2014), nematode knots, and especially cluster roots. The phosphatase hotspots in infected plants had six-fold higher activities than those of healthy roots. Consequently, nematode infection triggered stress on the plant and heightened demand for P. In conclusion, the visualization of these two enzymes indicates the sites of root injury and the diverted translocation of C and P under nematode attacks.

4.3. Extent of enzyme activity along and across the root after nematode infection

The homogeneity of enzyme patterns observed for lupine corresponded to the pattern in lentil — another N₂ fixing plant (Razavi et al., 2016). We report new details about enzyme patterns in both healthy and infected roots.

Enzymes were continuously and uniformly distributed along the lateral roots (without knots or cluster roots) in infected plants and healthy plants (Fig. 4). This homogeneity is associated with the rhizodeposition pattern along the root (Neumann and Romheld, 1999) and suggests that nematodes do not change the pattern as such, but homogeneously increase exudation along the entire length of lateral roots. Furthermore, the homogenous distribution of enzyme activities along lateral roots reflects the nutrient acquisition strategy of plants (Clarkson, 1991; Hinsinger et al., 2011).

The 2D-images (Figs. 2 and 3) as well as the enzyme activities along and across the roots (Figs. 4 and 5) consistently supported our hypothesis that the effect of nematodes is enzyme-specific. Cellobiohydrolase activities along the infected roots were 50% lower than in healthy plants. The increased availability of glucose and other sugars deterred microorganisms from producing enzymes involved in carbohydrate decomposition. Similarly, the distribution of cellobiohydrolase activity from the root center was narrower in contaminated versus healthy plants. Nonetheless, both infested and healthy roots showed the same decreasing pattern away from the root center, which steeply declined within 1 mm, becoming relatively stable outward into bulk soil. A 1 mm radius around the root belongs to that part of the rhizosphere with intensive exudation and rhizodeposition and is therefore a favorable habitat for microorganisms. The proliferation of bacteria and fungi induced the production of enzymes in this restricted area. Nonetheless, the narrower activity distribution of cellobiohydrolase (functioning in cellulose degradation) under nematode attack than in healthy plants supports earlier reports on the alteration of root exudate composition. The exudates of nematode-infected roots contained more water-soluble C (Van Gundy et al., 1977) and excessive sugars (Wang and Bergeson, 1974; Poll et al., 2007), which are available for microbial uptake. In this case, microbial communities were regarded as being economic units that maximize their productivity by allocating resources to extracellular pools of C-releasing enzymes, depending on substrate quality and nutrient availability (Sinsabaugh and Moorhead, 1994).

In contrast to the cellobiohydrolase distribution, infected roots showed a wider extension of phosphatase (4–4.5 mm) than non-infected roots (3–3.5 mm). Our interpretation is that, in response to higher P demand due to nematode infestation, the plants enhanced phosphatase production in their lateral roots. Thus, the infested roots, rather than microorganisms, play a pivotal role in producing phosphatase. Importantly, this 1 mm increment of rhizosphere extent in 2D equals a 2-fold increase in soil volume (3D) for nutrient mobilization.

Finally, as a next experimental step, we propose manipulating nematode infection as a tool to alter C fluxes and enzyme production to further our understanding of rhizosphere microbial activities.

5. Conclusions

Root infection by the nematode Meloidogyne incognita induced morphological and physiological changes in root tissues and thus biochemically altered the rhizosphere. These biochemical alterations involved increased C input due to root cell-wall rupture and increased plant demand for P. The result was profound changes in enzyme activities and localization (Fig. 6). The effect of the nematodes was enzyme specific: the increased P demand increased phosphatase activity, but the increased C supply decreased cellobiohydrolase activity. Moreover, the response of hotspots was also enzyme specific: nematode infection decreased the area and activity of cellobiohydrolase hotspots by a factor of 20. In contrast, the
phosphatase hotspots increased 6-fold. This correspondingly modified the rhizosphere shape of the enzyme activities across and along the roots. Using zymography to map the footprint of nematodes in the soil, we conclude that nematode infection not only has direct effects by changing root morphology, but also induces a number of subsequent biochemical changes in the rhizosphere, affecting C and P cycling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.08.027.

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