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Chapter

Protease Activity in the Rhizosphere of Tomato Plants Is Independent from Nitrogen Status

Hannah Holzgreve, Manuela Eick and Christine Stöhr

Abstract

Rhizoboxes were developed in order to analyse root system and corresponding protease activity in the rhizosphere of young tomato plants (*Solanum lycopersicum* cv. Moneymaker). The activity of proteases exuded by tomato roots applying in situ zymography was detected along the entire root system. The corresponding root architecture as well as root and shoot biomasses was determined to correlate protease activity with plant growth parameters under varying nitrogen supplies. With higher nitrate fertilisation, the proteases in the rhizosphere were more active than nitrogen-deficient plants. This may indicate that exuded proteases were not solely a plant response to nitrogen deficiency with the aim to increase nitrogen availability. Instead, they may have different roles, e.g. in root development.

Keywords: rhizosphere, protease, nitrate supply, in situ zymography, exudation

1. Introduction

The rhizosphere is most concisely described as the soil influenced by plant roots [1]. It is considered as a dynamic system of interacting processes with major implications for climate and environmental changes in aspects of greenhouse gas emission, carbon sequestration and soil fertility management for sustainable agriculture [2]. Various biotic interactions among plants and microorganisms occur in the rhizosphere, influencing fluxes between organic and inorganic nutrient pools, plant nutrient availability and plant health [3]. Both positive and negative biotic interactions in the rhizosphere are considered to be vitally mediated by root exudates, deeming them a focus in rhizosphere research [4, 5].

Root exudation is a process of excreting substances from plant roots and assumed to be the main source of organic carbon in the rhizosphere [6, 7]. Exudation can occur by rhizodeposition (sloughing off of cells) and passive or active exudation mechanisms of single compounds [8]. Exudation rate and composition have been suggested to vary between root zones with highest rates in apical regions, declining towards older root parts [9]. Other models, however, suggest an even exudation zone surrounding the whole root system [2].

While carbohydrates may constitute the bulk of root exudates, other components like acids, single ions, allochemicals and proteins are of no less importance for rhizosphere processes [4]. In focus of this study are proteases, hydrolytic enzymes

cleaving peptide bonds which can be found in all cells and organelles of a plant body [10]. Furthermore, proteases have been shown to occur in the rhizosphere [11]. While bacteria and fungi have long been known to excrete proteases among other extracellular enzymes [12], the knowledge of exudation of proteases by plant roots is more recent [13–15].

It is generally assumed that the main function of both microbial and plant-exuded proteases is nutrient cycling, making nitrogen from organic compounds available for consumption [12, 16]. A second function that has been identified for certain proteases is pathogen defence, working in concert with other lytic enzymes such as lipases and collagenases [15, 17]. A third function still sometimes underestimated is the processing of extracellular proteins, regulating cell growth and development, occurring mostly in the cell wall [18].

Modern techniques allow the detection of enzymatic activity in the rhizosphere, using rhizoboxes [19] and in situ zymography to obtain a potentially realistic impression of enzyme activities, their distribution and intensities within the rhizosphere [11]. This study aimed at localising activities of proteases using gelatin as substrate. It has been shown that plants increase their exudation of corresponding compounds to increase nutrient availability under deficiency [9]. Since exuded proteases are supposed to increase N availability [20], the focus was set on protease activity in the rhizosphere of tomato plants grown under different nitrate regimes.

2. Materials and methods

2.1 Plant material

Tomato seeds (*Solanum lycopersicum* cv. Moneymaker) were germinated and cultivated in sand using nutrient solution [21] with different nitrate regimes (0.5, 1.0, 2.0, 5.0, 7.0, 10.0, 15.0, 20.0 mM $Ca(NO_3)_2$ for plants in pots; 0.0, 2.5 or 10.0 mM $Ca(NO_3)_2$ for plants in rhizoboxes). The nutrient solution without nitrogen contained 3.2 mM $CaSO_4$ and 1 mM $CaCl_2$ to maintain osmolarity. The plants were cultivated in a greenhouse (light/dark rhythm 14–10 h and 28–22°C). Seeds were sown in pairs in small pots and transferred individually either to rhizoboxes after 7 days or to medium pots after 10 days. All plants were watered daily: the plants in rhizoboxes with 50 ml of the corresponding solution and the plants in pots using 200 ml nutrient solution for six plants.

2.2 Rhizoboxes

After 7 days, eight seedlings per nutrient solution (four seedlings only for deionised water) were transferred individually to rhizoboxes made of PTFE (internal dimension 15 × 17 × 1.5 cm, **Figure 1**). The bottom of each rhizobox was perforated to avoid dammed-up water but covered with nylon gauze (pore size 200 μm) to retain sand particles. The removable and transparent front glass pane was mounted by polycarbonate clamps and sealed by O-ring insertion (material NBR70).

For plant cultivation, the rhizobox was filled with sand and moistened with nutrient solution. The pane was removed temporarily to insert the seedling. The pane and the sand surface were covered with pond foil to reduce algal growth. The rhizoboxes were kept inclined by 45° during cultivation with the glass pane pointing downwards (**Figure 1**). Plants were cultivated for another 7 days. This procedure allows the observation of root growth and non-invasive analysis of root exudation.

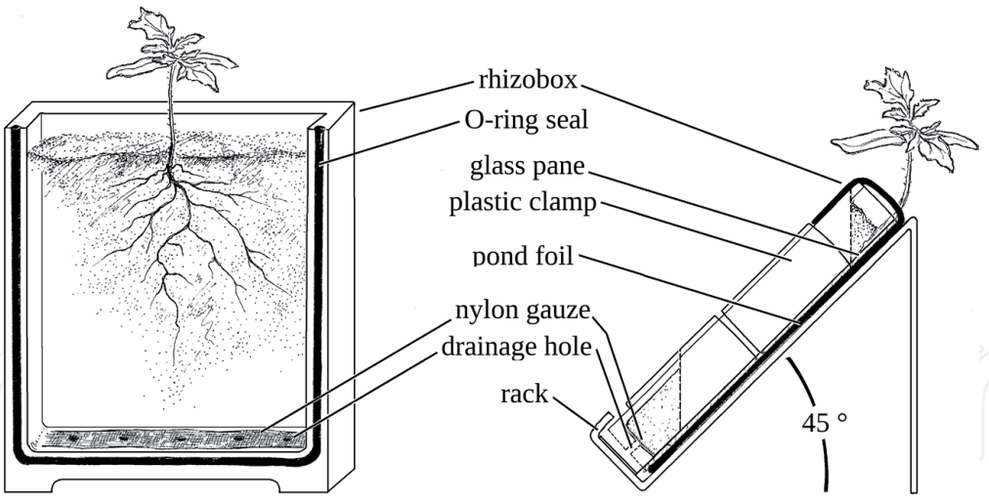

Figure 1.
Design of a rhizobox with removable front lid. Front and side view of a rhizobox with a tomato seedling growing inside the sand-filled box (internal dimensions 15 × 17 × 1.5 cm). The drainage holes at the bottom of the rhizobox were covered by nylon gauze to retain the sand when watering from the top. The front wall was replaced by a glass pane, which was held in place by plastic clamps. An O-ring sealed the gap between the glass and the edges of the rhizobox. The drawing is not to scale.

2.3 In situ soil zymography

2.3.1 Performance of in situ soil zymography

In situ soil zymography was adapted from [11], who used 1% w/v agarose gels with 0.1–0.01% w/v gelatin to determine protease activity. In this study, however, gels with 5% w/v polyacrylamide as matrix and 0.1% gelatin (w/v) as substrate were used. Gelatin was boiled for 10 min to denaturate contaminating enzymes before usage. After polymerisation, gel sizes were adjusted to the inner rhizobox dimensions (16 × 18 × 0.1 cm) and incubated in millipore water for 15 min to remove remaining non-polymerised acrylamide.

Zymographies were performed on four plants per nutrient solution. The rhizoboxes were irrigated with the correspondent solution 2–3 h prior to the experiment. The glass pane was removed carefully, and the root system was documented (Canon PowerShot G7 X) while being illuminated with UV light (365 nm; Blak Ray® B-100 AP, 100 W). Due to the fluorescence of lignified cell walls and phenolic compounds [22], the root system was emphasised (**Figure 2**). Preliminary tests showed no influence on the protease activity due to the irradiation with UV light, as well as the mechanical stress by pushing the glass pane off the root (data not shown).

The gel was placed on top of the root system, locked into position with the pane and wrapped in plastic foil overlaid with a dark cloth. It was incubated for 6 h at 28°C in the growth chamber. After incubation, the pane with the adhering gel was removed, and plants were immediately harvested.

Gels were washed with millipore water for 15 min and stained with 0.1% (w/v) Coomassie brilliant blue R-250 (in 50% (v/v) methanol, 7% (v/v) acetic acid) (modified from [23]) for 15 h at room temperature. Gels were destained (25% (v/v) methanol; 7% (v/v) acetic acid) and watered in deionised water for 15 min before documentation (biostep Felix 2000) on a daylight fluorescent plate. To calculate the remaining gelatin calibration, gels ranging from 0.0 to 0.1% (w/v) gelatin were stained and destained alongside the zymographies.

2.3.2 Calibration of zymographical results

The digital images of the zymography gels and calibration gels were adjusted with GNU Image Manipulation Program (GIMP) 2.8.22 to the same pixel amount per cm using the scale within each image. Image analysation was performed with ImageJ (https://imagej.nih.gov/ij/index.html). All images were converted to 8-bit greyscale.

2.3.3 Non-linear calibration

The average grey value of the calibration gels containing 0 and 0.1% (w/v) gelatin was measured and used as minimum and maximum to adjust the limits of the display range of all images including the zymography gels by using the brightness/contrast tool. Afterwards, the average grey value of each calibration gel slice was calculated using areas of at least 100,000 pixels.

Using the calibration tool of ImageJ, the measured grey values were plotted against the corresponding gelatin concentrations to obtain a calibration curve. This non-linear curve was fit using the logistic regression "Rodbard" and applied to the zymography images. The images were coloured in pseudo-colours for visualisation.

This calibration was preferred for evaluation since the resolution is higher at low–medium gelatin contents of 0–450 ng gelatin $(mm^2$ gel area$)^{-1}$, while all higher contents merge to form the background noise.

2.3.4 Linear calibration

The ImageJ tool "histogram" was used to analyse and display the number of pixels per grey value within the image. The grey values were then separated into five classes according to the calibration curve. The classes were defined following the intervals of 200 ng gelatin $(mm^2$ gel area$)^{-1}$ from 0 to 1000 ng gelatin $(mm^2$ gel area$)^{-1}$ and changed to pseudo-colours accordingly.

This calibration method results in a linear scale of remaining substrate per gel area, yet at a resolution of 200 ng gelatin $(mm^2$ gel area$)^{-1}$ only. Therefore, the information of nuances amidst the intervals is lost. However, this method may enable a quantitative analysis of soil in situ zymographies in other experimental set-ups and allows a vivid visualisation (**Figure 2**).

2.4 Analysis of zymographies and root systems

Both zymography images and UV photographies were turned to greyscale pictures for the analysis in WinRHIZO (Pro Version; Upgrade 2016a, Regent Instruments Inc.). Each picture contained a length standard for scaling to real values. The parameters obtained from UV photographies were total root length, projected area and the number of root forks per root system. For the zymographies, only the projected area of proteolytic degradation along the root system was obtained for comparison with the root system itself.

2.5 Harvesting of plant biomass

The plants grown in rhizoboxes were harvested immediately after in situ zymography incubation. Plants grown in pots were harvested 25 days after sowing. For all plants, the roots were rinsed thoroughly to remove all sand before shoot, and roots were separated and dried at 70°C for 12 h.

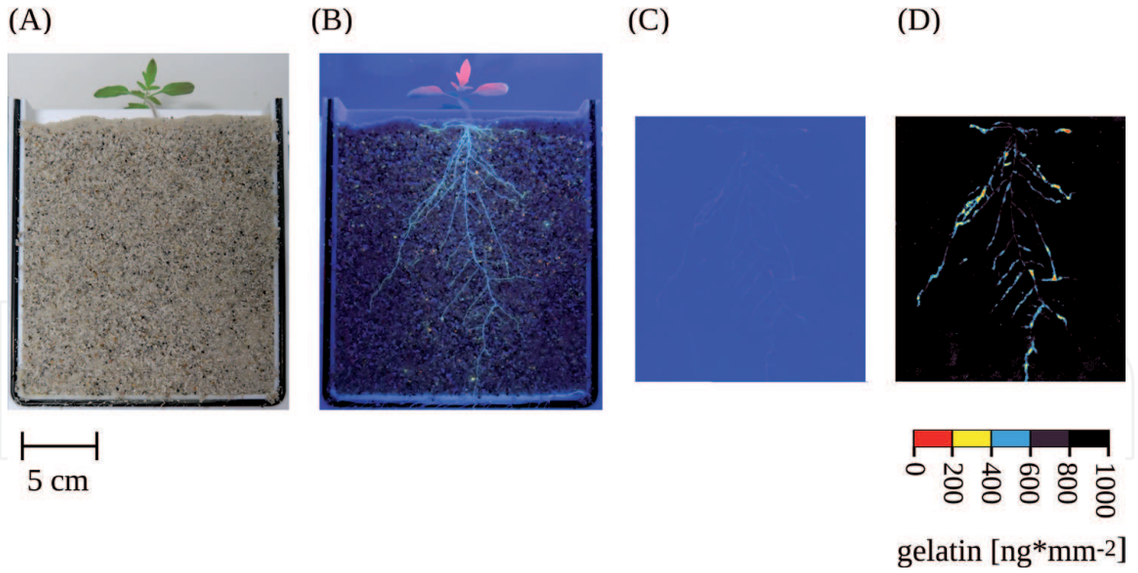

Figure 2.
Tomato root system and corresponding proteolytic activity. (A) Rhizobox rooted by tomato for 7 days and (B) visualisation of the same root system by fluorescence during UV excitation. Corresponding protease activity in the rhizosphere was detected by (C) in situ zymography with gelatin as substrate and Coomassie brilliant blue R-250 staining. (D) The intensity of the proteolytic activity was visualised in pseudo-colours scaled to remaining gelatin [ng mm^{-2}] using a linear calibration.

2.6 Statistical analysis

Experiments were done in randomly blocked design having at least four independent biological samples. ANOVA was performed to reveal statistical significance at 95% confidence level followed by Tukey test for post hoc testing, both using R (Version 3.4.4; 2018, The R Foundation for Statistical Computing) in RStudio (Version 1.1.383; 2009–2017, RStudio Inc.).

3. Results

As in other plants, tomato shoots and roots respond to variation in the nitrogen regime with altered growth and root architecture. To determine the optimum and overload concentration of nitrate for tomato plants, biomass development was analysed after growth in pots and irrigating the plants with nutrient solution containing varying concentrations of $Ca(NO_3)_2$ (**Figure 3**). According to these results, 10 mM nitrate in the nutrient solution was chosen as optimum, and in further experiments, it was more accurately defined as a daily supply of 0.25 mmol nitrate per plant. Excess of nitrate was determined at 20 mM nitrate in the nutrient solution corresponding to 1.0 mmol nitrate per day and plant. Nitrogen deficiency was applied with no additives of nitrate either in nutrient solution or in water. After 7 days cultivation in rhizoboxes (**Figure 2**), nitrogen-deficient plants showed a shoot dry weight that was on average 5 (deionised water) to 8.5 (0 mM nitrate in nutrient solution) times lower than the shoot dry weight of plants with optimum supply (**Figure 4A**). Plants receiving an overload of nitrate showed less shoot dry weight by one third on average than the optimum supply.

The root biomass revealed less difference between the varying cultivation solutions. While the N-deficient plants differed from the optimally supplied plants by a root dry weight 3.7 (deionised water) to 2 (0 mM nitrate in nutrient solution) times less, the root dry weight of plants supplied with a surplus of nitrate did not differ significantly from both optimum- and nitrogen-deficient plants.

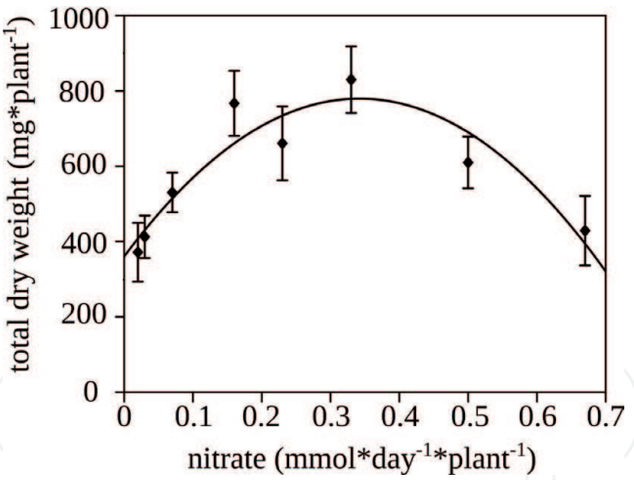

Figure 3.
Effect of nitrate supply on overall dry weight biomass of young tomato plants. The plants were grown in pots and supplied with the correspondent amount of nitrate per day in nutrient solution. The plants were harvested 25 days after sowing. Error bars indicate standard deviations (n = 5 for 0.50 mmol nitrate plant^{-1} day^{-1}, n = 6 for other treatments, p < 0.001).

Plants grown under nitrogen deficiency or total deficiency (deionised water) differed significantly in their shoot/root ratio from plants grown under optimum amount and surplus of nitrogen, while no difference was visible within those two groups (**Figure 4B**).

The root architecture of young tomato plants was analysed after 7 days growth in rhizoboxes. With varying supply of nitrogen from deficiency to optimum and overload of nitrate, tomato plants differed highly in root branching (**Figure 5**). When considering the number of root forks per plant (**Figure 5A**), both shortage and surplus of nitrate revealed lower fork counts than optimally supplied plants. While the plants supplied with deionised water did not differ significantly in fork number from the nitrogen-deficient plants, which showed on average three times less forks than plants with optimum nitrate concentration, the plants grown with a surplus of nitrate showed nearly twofold less forks on average than optimally

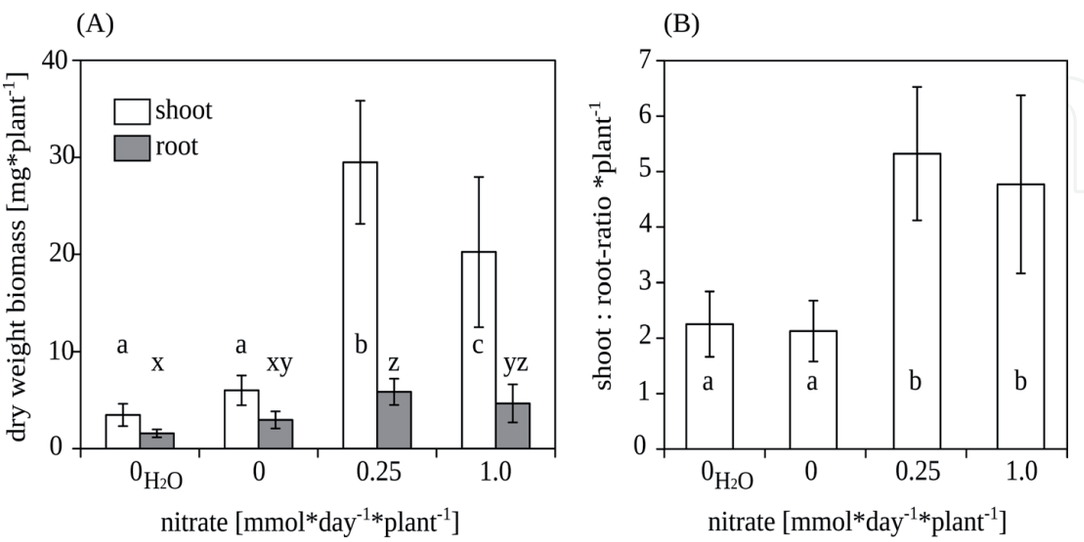

Figure 4.
Effect of nitrate supply on the biomass of young tomato plants. Plants were provided with the correspondent amount of nitrate per day in either deionised water (0_{H_2O}) or nutrient solution and transplanted to rhizoboxes after 1 week of cultivation. The plants were harvested 2 weeks after sowing. (A) Dry weights of shoot (white bars) and root (grey bars) per plant as well as their proportions (B) are shown. Letters indicate group differences at a p-value of <0.001 (n = 4 for deionised water, n = 8 for other nutrient solutions).

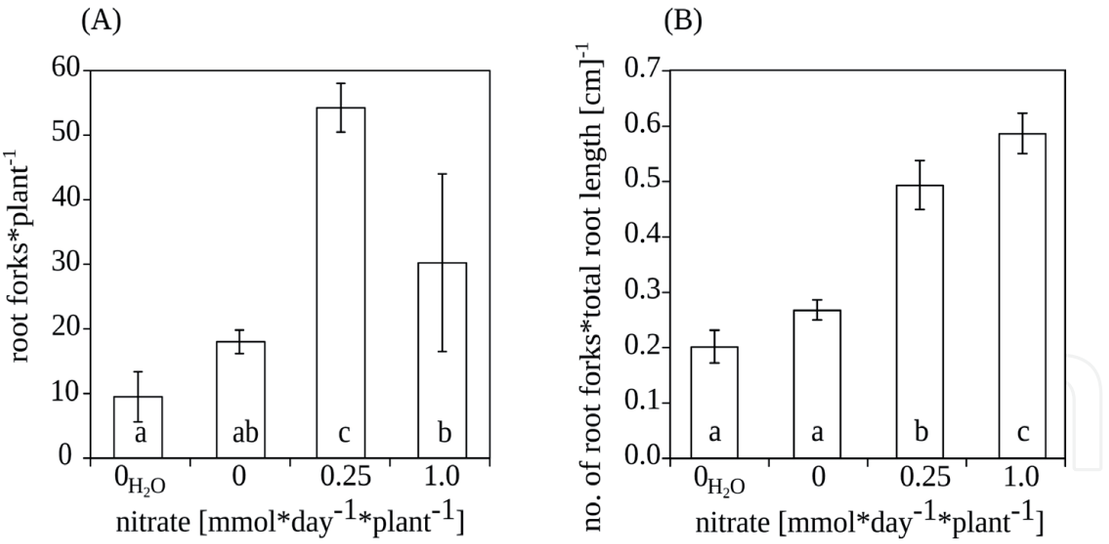

Figure 5.
Effect of nitrate supply on the root architecture of young tomato plants. The plants were supplied with the correspondent amount of nitrate per day in either deionised water (0_{H_2O}) or nutrient solution and transplanted to rhizoboxes after 1 week of cultivation. Harvesting and analyses of the root system were performed after 1 more week. Root lengths were analysed using UV photographies and the software WinRHIZO, and the forks were counted visually. (A) The number of root forks per root system and (B) the number of root forks in relation to root length. Letters indicate group differences at a p-value of <0.001 (n = 4).

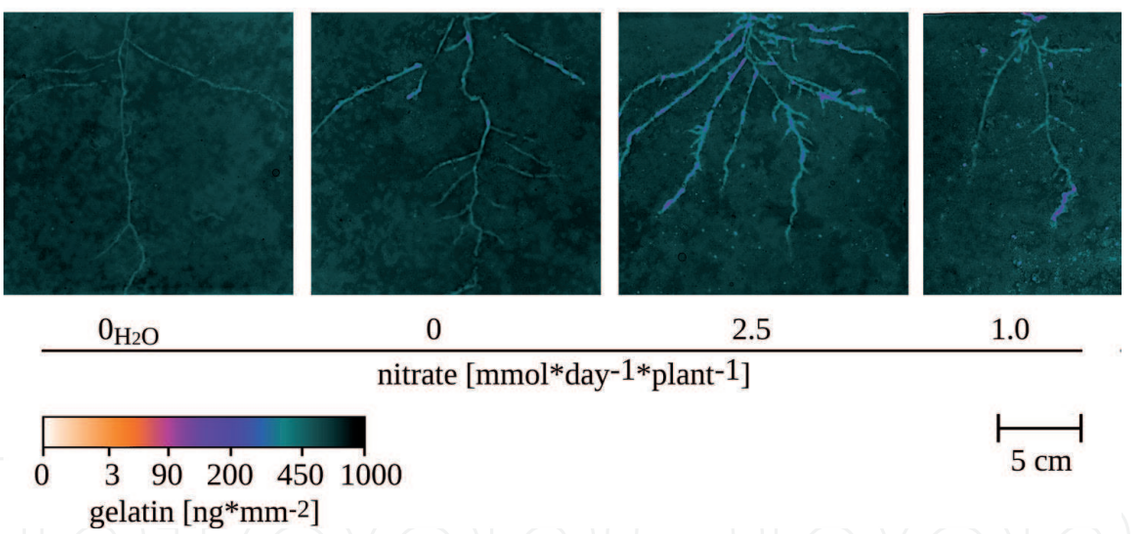

Figure 6.
Effect of nitrate supply during plant growth on protease activity in the rhizosphere of young tomato plants. Exemplary non-linearly calibrated in situ zymographies of root systems of plants grown on deionised water (0_{H_2O}) or different nutrient solutions were converted to pseudo-colours to reveal between different protease activities.

supplied plants. When considering the number of forks in relation to root length, however, plants with both total deficiency and nitrate deficiency showed the lowest number of forks per cm root length, while plants with a surplus of nitrate showed a higher number of forks per cm root length than the optimally supplied plants (**Figure 5B**).

Protease activity in the rhizosphere in relation to plant nitrate supply was visualised along the root system using in situ zymography (**Figure 6**). The area of gelatin degradation was evaluated as proteolytic activity and converted to pseudo-colours to reveal the intensity of proteolytic degradation. This data was used to estimate the area of proteolytic gelatin degradation in relation to the projected root area (**Figure 7**). Plants with nitrogen deficiency (either in deionised water or in nutrient

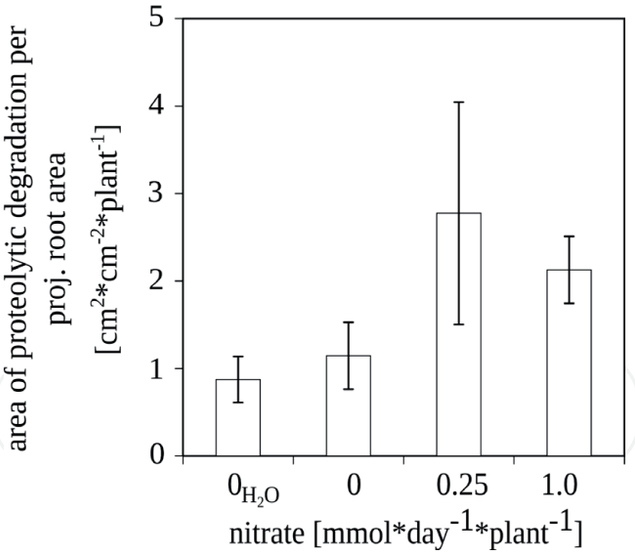

Figure 7.
Effect of plant nitrate supply on protease activity in the rhizosphere of young tomato plants. The comparison of the area of proteolytic activity along the root system to the total projected root area per plant is based on non-calibrated 8bit (greyscale) root zymographies and UV photographies of root systems analysed in WinRHIZO. Letters indicate group differences at a p-value of 0.008 (n = 4).

solution) had an on average 3.2 times lower proteolytic activity per projected root area than well-supplied plants. However, the ratio of proteolytic degradation per root area was not significantly different between optimally fed plants and plants with a surplus of nitrate, clearly indicating that the increase in proteolytic activity was not a response to nitrogen deficiency.

4. Discussion

For understanding of plant health and root adaptation to biotic and abiotic factors, three aspects of the rhizosphere are crucial: root development, biotic interactions and water and nutrient uptake [24]. This study focused on the aspect of nitrogen uptake, inquiring how protease activity in the rhizosphere may depend on nitrate availability. The results obtained using in situ rhizosphere zymography indicate that exuded proteases may serve purposes additional to nitrogen acquisition from organic compounds.

4.1 Plant growth in response to nitrate regime

Plants respond to nitrogen regimes with changes in growth and biomass partitioning as well as in root architecture [25–28], so according parameters were chosen to evaluate the deficient, optimum and excessive nitrogen supply. Plants with nitrogen deficiency showed a strongly reduced shoot biomass compared to optimally supplied plants, while the reduction in root biomass was not as grave (**Figure 4A**). This corresponds well with the general assumption that nitrogen deficiency results in overall reduced biomass due to metabolic limitation. Because nitrogen is an essential element for nucleic acids, proteins and diverse vital molecules from phytohormones to cell wall components like proteoglycans [29], its absence leads to similarity in growth of both total nutrient deficiency (deionised water) and nitrate deficiency only (**Figure 4A**). As an adaptation to nitrogen insufficiency, plants show higher root growth than shoot when nitrogen is low [30]. However, it has been proposed that reduced growth—especially of leaves and shoot—is not only a result

of metabolic limitation but an adaptive response to prevent internal starvation [28]. Nitrogen surplus can be detrimental to plants [25, 31] as indicated by reduced shoot biomass of plants with excessive nitrate supply. The root biomass and shoot/root ratio, however, did not differ significantly from optimally supplied plants (**Figure 4A**). Under nitrate overload, total plant growth would be reduced, and a constant C/N ratio has to be maintained [25].

Root architecture

In the applied rhizoboxes, the root system can only expand along the glass pane of the box, resulting in an artificially two-dimensional root system that can be easily evaluated. Changes in the root system architecture (RSA) at different external nitrate concentrations have been shown in *Arabidopsis* [27] but may be present in most plants [28].

The number of root forks was similar for plants supplied with both shortage and excess of nitrate, while plants under optimum nitrate conditions showed higher fork counts as earlier reported for *Arabidopsis* [27]. While low nitrate concentration is assumed to increase root elongation in primary and secondary roots at an overall reduced root weight, high concentrations of nitrate would reduce root elongation at a higher root weight [27], which is consistent with the presented findings. Higher root elongation at comparable root branching under nitrogen deficiency is typical for roots foraging for nitrogen [29] to achieve higher nitrogen uptake efficiency. For high nitrogen supply, on the other hand, the typically reduced branching and growth are assumed to result from nitrogen accumulation in the shoot, inhibiting auxin flux to roots and thus preventing lateral roots to pass an auxin-requiring checkpoint that is vital in lateral root development [32].

4.2 Proteolytic activity in the rhizosphere

While inorganic nitrogen forms such as ammonium and nitrate are usually in focus for plant nitrogen nutrition, plants are also able to take up organic nitrogen in the form of amino acids [28, 33]. Because of root-derived protease activity, even the uptake of small proteins was suggested for root hairs [34]. Plant roots can exude both endo- and exopeptidases [16]. Endopeptidases cleave within peptide chains, while exopeptidases only cleave amino acids at the termini of the substrate [10]. Additionally, proteases can be of different specificity for a substrate, ranging from unspecific to highly regulated hydrolysis, the latter depending on a certain amino acid sequence or pattern [35].

Gelatin as protease substrate is processed mainly by endopeptidases due to the molecule structure. Gelatin is a complex biopolymer made of solubilised collagen, long polypeptide-chains composed of the amino acid triplet gly-x-y with x often being proline and y hydroxyproline [36]. While a total of 16–20 amino acids can occur in gelatin, it is approximately made of 33% glycine and 15–25% proline plus hydroxyproline [37, 38]. Chain lengths of roughly 1000 amino acids [37] hugely increase the probability of endopeptidase activity, while the monotony of the triplet pattern suggests higher rates of unspecific proteolytic activity. The addition of an organic nitrogen source (gelatin) several hours previous to the zymography did not result in any priming effect (data not shown).

The zymographically observed increase in proteolytic activity in the rhizosphere with increasing nitrate availability suggests an additional function of the exuded proteases. Nitrogen acquisition as the main protease function would result in an opposed pattern with high proteolytic activity under nitrate limitation. The controlled growth conditions should also limit pathogen occurrence, hence limiting the number of proteases exuded for pathogen defence. Thus, developmental

functions of exuded proteases might be worth investigating. Recently, a subtilase TREXS has been identified in *Nicotiana tabacum* root exudates and is assumed to fulfil a role in root development according to its relative SDD1 in *Arabidopsis* which is involved in stomata development [39]. For the intracellular subtilases XSP1 and AIR1 in *Arabidopsis*, a function in lateral root formation has been proposed due to the specific expression in the respective tissues [40, 41]. Papain-type cysteine endopeptidases are expressed in root epidermis cells that are separated for lateral root emergence. Loss of papain-type endopeptidases AtCEP1 or AtCEP2 in maize caused delayed emergence of lateral root primordia [42].

4.3 Localization of proteolytic activity

The activity detected in the in situ zymographies results from extracellular proteases. Like other extracellular enzymes, extracellular proteases may diffuse from the cell wall of their mother cell to the rhizosphere soil unless they are held back, e.g. by root or microbe mucus [12].

In contrast to other studies [9] reporting high protease activities at root zones of high exudation rates, especially at root tips, the distribution of protease activity was observed roughly along the whole root system in this study. Protease activity was documented for 70–90% of the root length for all treatments. This corresponds with the exudation zone proposed by [2] to surround the whole root system. In grasses, this zone forms the rhizosheath where microbial activity has been suggested to be especially high due to carbon deposits [43]. This phenomenon may also occur in dicots [2], suggesting microbial activity as part of the observed proteolysis in the rhizosphere. However, proteolytic degradation along the root system was also visible with plants grown from surface-sterilised seeds under aseptic conditions (data not shown, plants grew untypically/were deformed). The cultivation of plants on sand instead of microbe-rich soil, the short time span and the greenhouse conditions may all put a limit to microbial colonisation in comparison to natural conditions. Additionally, rhizosphere bacteria usually absorb and assimilate nitrate only in the absence of either organic N—e.g. plant exudates—or ammonium [44], while it is a major N source for most higher plants [45]. Nitrate might even decrease microbial growth in comparison to ammonium or anorganic nitrogen compounds [46, 47]. Hence, the participation of bacteria in proteolytic activity in rhizobox experiments can be assumed to be of limited importance.

Since the root system was always clearly outlined in the zymographies, the spatial definition for the rhizosphere observed in this context is straightforward and restricted to those areas of proteolytic degradation that were linked to the root system. Apart from this, however, minor to moderate proteolytic degradation could also be observed at small, random spots across the substrate. These might coincide with colonies of microorganisms. Interestingly, the intensity of the proteolytic degradation occurring in these spots increased with increasing nitrogen supply, suggesting higher growth rates at higher nitrogen supply. In comparison to proteolytic activity in the rhizosphere, however, both size and number of these spots were very low.

5. Conclusion

Differences in protease activity between the nitrogen treatments could be a result of two different regulative possibilities: firstly, the differences in protease amounts—based on expression and exudation of the proteases—and, secondly, the differences in activity of the available proteases depending on abiotic factors and protease processing [48]. Since proteolytic activity in the rhizosphere of

young tomato plants increased with increasing nitrate availability, a function of the observed proteases for nitrogen acquisition seems unlikely. The supplication of nitrogen as inorganic nitrate alone probably limited the microbial growth in the rhizosphere and additionally avoided any priming for organic N hydrolysis.

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