RESEARCH PAPER

Laser ablation tomography for visualization of root colonization by edaphic organisms

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Abstract

Soil biota have important effects on crop productivity, but can be difficult to study in situ. Laser ablation tomography (LAT) is a novel method that allows for rapid, three-dimensional quantitative and qualitative analysis of root anatomy, providing new opportunities to investigate interactions between roots and edaphic organisms. LAT was used for analysis of maize roots colonized by arbuscular mycorrhizal fungi, maize roots herbivorized by western corn rootworm, barley roots parasitized by cereal cyst nematode, and common bean roots damaged by Fusarium. UV excitation of root tissues affected by edaphic organisms resulted in differential autofluorescence emission, facilitating the classification of tissues and anatomical features. Samples were spatially resolved in three dimensions, enabling quantification of the volume and distribution of fungal colonization, western corn rootworm damage, nematode feeding sites, tissue compromised by Fusarium, and as well as root anatomical phenotypes. Owing to its capability for high-throughput sample imaging, LAT serves as an excellent tool to conduct large, quantitative screens to characterize genetic control of root anatomy and interactions with edaphic organisms. Additionally, this technology improves interpretation of root–organism interactions in relatively large, opaque root segments, providing opportunities for novel research investigating the effects of root anatomical phenotypes on associations with edaphic organisms.

Keywords: Cereal cyst nematode, Fusarium, mycorrhizae, root anatomy, root phenotyping, western corn rootworm.

Introduction

Root interactions with soil organisms have significant impacts on plant performance and yield. Interactions with soil biota including fungi, arthropods, and nematodes encompass a wide range of relationships including commensalism, mutualism, parasitism, and herbivory. The interface between root anatomy and these soil organisms is poorly understood, but potentially important as root anatomical features and colonizing organisms may have reciprocal effects.

Arbuscular mycorrhizal fungi (AMF) are a ubiquitous group of obligate biotrophic soil fungi that are associated with >80% of terrestrial plant species (Smith and Read, 2008; Brundrett, 2009). AMF have substantial implications for mineral nutrition...
and edaphic stress tolerance of their host, and broad ecological relevance as they provide an avenue for the exchange of resources among the root systems of co-existing plants (Kaeppler et al., 2000; Chu et al., 2013; Bárzana et al., 2014; Gerlach et al., 2015; van der Heijden et al., 2015). In both natural and agricultural ecosystems, AMF elicit a wide range of growth responses in host plant species (Klironomos, 2003; Ulibapuu et al., 2012) and, depending on the plant and fungal species combination and management practices, increased AMF colonization has been shown to improve phosphorus acquisition and yield across several species (Lebek and Koide, 2005; Sawers et al., 2010). The spatial distribution of AMF has been characterized at the cellular level through two-dimensional microscopy (Smith and Smith, 1997; Smith and Read, 2008), but these studies do not capture the spatial distribution and volume of AMF throughout the root cortex or the interaction of AMF with anatomical phenotypes in roots. The interface between root anatomical phenes and AMF may have important implications for plant performance and merits further investigation as plant species, AMF species, root anatomy, and the interactions among these factors may influence AMF colonization and consequent effects on the host (Sharda and Koide, 2008).

While AMF are generally mutualistic, nematodes occupy a parasitic role and can cause considerable yield loss, especially in areas where susceptible crops are grown in monoculture systems (Rivoal and Cook, 1993). Sedentary nematodes, including cyst and root-knot nematodes, are among the most damaging plant parasites. These parasites form feeding sites in roots, damaging root tissue and interfering with cellular metabolism, cell wall architecture, the cell cycle, and hormone homeostasis (Knydt et al., 2013). Although it is widely recognized that nematodes reduce crop productivity worldwide, determination of actual yield losses is difficult and is only possible through the use of nematicides and controlled inoculation, or annual assessment from expert plant pathologists (Brown et al., 1970; Koening and Wrath, 2010). While prospective damage to crops can be estimated by densities of root nematodes present in the soil at the time of planting (Schoomaker and Been, 2013), expeditious screening of infected root samples is also essential in the study of these root parasites (Toumi et al., 2018).

Endophytic, root-rot-causing fungi of the genus *Fusarium* degrade root tissue and are a major constraint to crop production globally (Xue et al., 2011; Niño-Sánchez et al., 2015). The impact of these pathogens is especially significant in areas of intensive monoculture (Wordell Filho et al., 2013). Beyond reducing yield, some species are also capable of producing mycotoxins such as fumonisins and trichothecenes that are transported to the shoots and can affect animal and human health (Scott, 2012). Most species infect plants by either soil-born hyphae, or germinating spores that enter through the root tip, lateral roots, or root wounds (Oren et al., 2003; Wang et al., 2019). The ability to rapidly screen crop germplasm and quantify *Fusarium* colonization and damage at the anatomical level would have relevance not only for future studies characterizing these fungi and their interaction with root phenes, but also for the screening, determination of genetic controls, and development of productive crop varieties that are less susceptible to these pathogenic fungi.

Western corn rootworm (WCR; *Diabrotica virgifera virgifera*) is the most significant pest affecting maize across North America and Europe (Gray et al., 2009; Lombaert et al., 2018). Production losses and pest management costs attributed to WCR in the USA alone exceed US$1 billion per annum (Rice, 2004). Larval feeding of WCR on the roots of maize and other grass species reduces plant productivity by damaging the root cortex and potentially facilitating infection by fungal pathogens (Riedell and Kim, 1990). WCR tends to target nodal roots, and WCR damage in the root cortex has been demonstrated to increase the number of adventitious roots, decrease CO₂ assimilation, and decrease leaf area (Riedell and Reese, 1999). Maize genotypes vary in their responses to WCR infestation of the root cortex (Castano-Duque et al., 2017). Because larval feeding patterns on the root system of maize affect adult and larval movement (Spencer et al., 2009), observations of the larval feeding activity at the root anatomical level have great significance in developing control measures for this pest. Despite being widely studied at the molecular, chemical, and ecological scales (Spencer et al., 2009), the interaction between maize root anatomy and larva has been almost entirely neglected (Riedell and Kim, 1990; Castano-Duque et al., 2017).

Visualization of how these organisms colonize root tissue plays a significant role in understanding how they affect root health and development, as well as the development of approaches to prevent and mitigate damage caused by root pathogens, parasites, and pests. Root-colonizing organisms are notoriously challenging to identify and quantify due to the laborious nature of excavating root systems, sample preparation, and image capture and analysis. Quantitative and qualitative molecular assays have been used to detect the presence of AMF (Frey et al., 1992, 1994; Bothe et al., 1994; Olson, 1999), *Fusarium* (Nicolaisen et al., 2009; Kandel et al., 2015; Wang et al., 2015), and nematodes (Atkins et al., 2003; Berry et al., 2008) through species-specific markers including fatty acids or chitin, as well as through quantitative real-time PCR methods. While these methods enable the detection and identification of soil biota in the root, they fail to address interactions between root anatomical traits and pathogens.

The primary method for visualizing these interactions has been with microscopy. Publications exploring root colonization by AMF, *Fusarium*, nematodes, and other soil organisms utilize light microscopy (Seah et al., 2000; Nonomura et al., 2003), confocal laser scanning microscopy (Humphris et al., 2005; Olvain et al., 2006; Czymmek et al., 2007; Martino et al., 2007; Downie et al., 2014; Aditya et al., 2015; Islam et al., 2017a, b), and TEM (Rovira and Campbell, 1974; Williams and Fisher, 1993; Nurmiho-Lassila et al., 1997) to generate two- and three-dimensional images of these interactions. In addition, some of these studies utilize fluorescent reporter proteins to aid in the visualization of these structures. Although three-dimensional visualization is possible with confocal microscopy, the time required to carry out these measurements is prohibitive for screening a large number of samples, and they have limited capability to resolve features below the surface of the sample. As a result of the modest throughput and limitations in spatial visualization of traditional microscopy techniques, little is known about the spatiotemporal dynamics of root organisms.
and their interaction with root anatomy (Pivato et al., 2007). New methods for high-throughput, three-dimensional phenotyping of roots could enable large screens and genetic studies to characterize host–pathogen interactions. To gain a better understanding of the functional role and genetic control of the interaction between soil organisms and root anatomy, it would be useful to develop high-throughput methods that can spatially quantify and qualify colonization of roots by soil biota in three dimensions.

Laser ablation tomography (LAT) is an innovative imaging technology that fulfills this requirement for rapid, three-dimensional visualization of biological samples, offering significant opportunities to expand research on relationships between root anatomy and edaphic organisms. This novel, imaging technique provides high-throughput, full color scans of samples at spatial scales from 0.1 mm to 1 cm with micron-level resolution. This technology addresses a gap in sample throughput and scale that is unfulfilled by conventional microscopy techniques, yet is relevant to many applications in biology. The application of LAT for studies of root anatomy provides both volumetric quantification and tissue differentiation based on composition-specific autofluorescence.

Here we assess the utility of LAT in the analysis of root-associated organisms. LAT has been previously used to rapidly phenotype anatomical traits such as root cortical aerenchyma, cortical cell file number, cortical cell size, and secondary growth in the roots of maize and common bean (Zhu et al., 2010; Chimungu et al., 2014a, b; Saengwilai et al., 2014; Galindo-Castañeda et al., 2018; Strock et al., 2018). While these previous publications have utilized LAT for detecting statistically significant effects of treatment or genotype, the purpose of the present work is exclusively to demonstrate the qualitative and quantitative abilities of LAT as a novel methodology for anatomical analysis of root-associated organisms. This method is capable of imaging the anatomy of ~25 root segments h⁻¹ and permits the three-dimensional visualization and quantification of root interactions with AMF, nematodes, Fusarium, WCR, and presumably many other soil microorganisms.

Materials and methods

Laser ablation tomography

The LAT system utilizes a Coherent (Santa Clara, CA, USA) Avia 355–7000 Q-switched Nd:YVO4 UV laser (355 nm) source with a pulsed repetition rate between 25 kHz and 40 kHz (Fig. 1). The pulse duration of the laser was <30 ns and supplied a pulse energy of ~173 µJ. A Scanlab (Puchheim, Germany) HurryScan 10 galvanometer was used to oscillate the beam over a linear distance, creating a cutting sheet. The software used to control the laser and scanner was WinLase (Acton, MA, USA). To move the root sample into the beam path, an Aerotech (Pittsburgh, PA, USA) ATS100-100 ball-screw stage with 100 mm of travel was coupled with a Zaber (Vancouver, British Columbia, Canada) low vacuum motorized linear stage with 25 mm of travel. The stage was controlled with a Zaber (Rocklin, CA, USA) motion control software coupled with laser micro-machining software (Pelham, NH, USA) which can be either incremented at pre-set intervals, or continuously traversed at a defined velocity, coupled with a set image capture frequency to achieve a desired z-step resolution. Root samples ~2.5 cm in length were affixed to a cantilever and connected to the mechanical stage along its travel axis. A Canon (Melville, NY, USA) T3i 18MP camera with a Canon 65 mm MP-E 1–5× variable magnification macro lens simultaneously images the exposed anatomy ablated by the beam.
A macro lens was used to image each subsequent laser-illuminated slice as the root sample was pushed into the beam path by the stage. This allowed a maximum theoretical resolving power of ~1 μm per pixel. The software used for image capture was the Canon EOS Utility, which transfers the data into image files. Autofluorescence from ablating root tissue by UV wavelengths was recorded in video mode at a rate of 30 frames s⁻¹ at 1080p with white balance and aperture manually adjusted to optimize visualization of features of interest. Image stacks were extracted from these videos, and root anatomical features as well as colonizing organisms were segmented from image stacks by thresholding based on red, green, and blue (RGB) spectra with the MIPART™ software (Sosa et al., 2014). RGB spectra values used for thresholding were determined in ImageJ by outlining the feature of interest and utilizing maximum and minimum spectra values in the red, green, and blue channels of pixels comprising that feature. Manual review of thresholded images was performed to verify correct segmentation. The original and segmented images were used for three-dimensional reconstruction and quantification of the root segment and organisms using Avizo 9 Lite software (VSG Inc., Burlington, MA, USA).

Dominant wavelengths of features of interest within the visible spectrum were calculated using the CIE 1931 color space from the mode RGB values determined in ImageJ. A D50 reference white and sRGB model were used for these calculations (Smith and Guild, 1931).

**Mycorrhizal colonization**

Maize (Zea mays) recombinant inbred lines and parents of the intermated B73×Mo17 population (Senior et al., 1996; Kaeppler et al., 2000) were selected for analysis based on contrasting aerenchyma formation in inoculated and non-inoculated maize root cross-sections. The minimum and maximum values for each channel were used to parameterize thresholding shown in Fig. 3, and the mode was used in calculating the dominant emission wavelength for each tissue.

![Fig. 2. RGB spectra measured in ImageJ from designated areas of non-colonized cortex, aerenchyma, and arbuscules in images of inoculated and non-inoculated maize (Zea mays) root cross-sections. The minimum and maximum values for each channel were used to parameterize thresholding shown in Fig. 3, and the mode was used in calculating the dominant emission wavelength for each tissue.](image)

**Table 1.** Dominant emission wavelength (nm) of tissues sectioned by LAT and excited by UV (355 nm)

| Root sample | Plant host   | Feature                                      | Dominant emission wavelength |
|-------------|--------------|----------------------------------------------|------------------------------|
| AMF         | Zea mays     | AMF arbuscules                               | 583.9 nm                     |
| AMF         | Zea mays     | Cortical cells                               | 477.2 nm                     |
| AMF         | Zea mays     | Stele tissue                                 | 588.6 nm                     |
| WCR         | Zea mays     | WCR (Diabrotica virgifera virgifera)          | 557.9 nm                     |
| WCR         | Zea mays     | Cortical cells                               | 473.8 nm                     |
| WCR         | Zea mays     | WCR frass                                    | 491.9 nm                     |
| Nematode    | Hordeum vulgare| Cereal cyst nematode (Heterodera avenae)      | 487.6 nm                     |
| Nematode    | Hordeum vulgare| Cortical cells                               | 473.8 nm                     |
| Fusarium    | Phaseolus vulgaris| Healthy cortical cells                      | 461.9 nm                     |
| Fusarium    | Phaseolus vulgaris| Damaged cortical cells                       | 602 nm                       |
observed in previous studies (Saengwilai et al., 2014). Seeds were planted in mesocosms (15.7 cm in diameter and 160 cm in height) filled with growth medium consisting of 50% (v/v) medium-grade sand (Quikrete Companies Inc., Harrisburg, PA, USA), 35% (v/v) vermiculite (Whittemore Companies Inc., Lawrence, MA, USA), 10% (v/v) perlite (Whittemore Companies Inc.), and 5% (v/v) autoclaved soil [Ap2 Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf), available phosphorus: 10 mg kg\(^{-1}\) as determined by Mehlich 3] (Wolf and Beegle, 1995). A complete nutrient solution was supplied through drip irrigation at a rate of 500 ml per plant d\(^{-1}\). The nutrient solution consisted of (in µM): K (3000), NO\(_3\) (7000), NH\(_4\) (1000), Ca (2000), SO\(_4\) (500), Mg (500), Cl (25), B (12.5), Mn (1), Zn (1), Cu (0.25), Mo (0.25), and EDTA-Fe (25), which had 0.2 µM phosphorus (as described by Zhu et al., 2005). Mesocosms were inoculated at planting with a liquid formulation of 400 pure aseptic spores of *Rhizophagus irregularis* (Premier Tech Biotechnologies, Quebec, Canada) by diluting aliquots of the spores in 500 ml of sterile Tween\(^*\)80 solution (0.002%, Amresco Inc., Solon, OH, USA) in water and thoroughly mixing with potting medium extracted from 5–20 cm depth (2 liters). After mixing the medium with the spores, the inoculated medium was returned to the column and topped with 5 cm depth of non-inoculated medium. Non-inoculated control plants were treated in the same manner with autoclaved spore inoculant. Control plants were used to compare the appearance of root cortical sections of non-inoculated plants with inoculated plants. Plants were grown in the greenhouse for 6 weeks (1200 µmol photons m\(^{-2}\) s\(^{-1}\) at noon, supplemented with 400 W metal halide lamps; 14:10 light:dark, 24–28 °C, 40–70% relative humidity), at which point root systems were excavated, washed, and nodal root segments 8–12 cm from the hypocotyl were collected from the second to fourth whorl. Root segments were preserved in 75% ethanol:water (v/v) for anatomical and AMF phenotyping by LAT.

Root samples were scanned with LAT at a speed of 100 µm s\(^{-1}\). Image stacks extracted from videos of these scans were subsampled into stacks of ~450 images, with a longitudinal resolution of one image per 3 µm root, and the stack representing a root segment 1.5 mm in length (transverse resolution 1173 pixels mm\(^{-1}\)).

To differentiate AMF aerenchyma, and non-inoculated cortical tissue, transverse cross-sectional images from non-inoculated control roots and AMF-inoculated roots were analyzed for RGB colorspace in ImageJ (Fig. 2). Dominant emission wavelengths of regions colonized by AMF and non-inoculated tissue were determined from the RGB colorspace. AMF were defined as having a dominant emission wavelength of 583.9 nm (Table 1), with red values ranging from 200 to 255, green values from 159 to 220, and blue values from 115 to 163 (Fig. 2). Non-colonized cortical tissue had a dominant emission wavelength of 477.2 nm (Table 1), with red values ranging from 24 to 218, green values from 22 to 218, and blue values from 23 to 216 (Fig. 2). Stele tissue was defined as having a dominant emission wavelength of 588.6 nm (Table 1), with red values ranging from 99 to 176, green from 62 to 131, and blue from 44 to 110 (Fig. 2). Root cortical aerenchyma was defined in ImageJ as having red values from 15 to
Image stacks were segmented (Fig. 3), three-dimensionally resolved (Fig. 4), and quantified using MIPAR™ and Avizo 9 Lite software as described above.

Western corn rootworm infection

B73 maize seeds were surface sterilized with 10% NaOCl:water (v/v) and placed between moist germination paper in an incubator at 28 °C to germinate. After 7 d, seedlings were transplanted in steam-sterilized field soil in 500 ml plastic pots. Plants were grown in a greenhouse with supplemental lighting (metal halide and high-pressure sodium lights; 16:8 light:dark; ~24 °C; ~60% relative humidity) and watered daily. At the V3 stage, 20 freshly hatched (within 24 h) WCR neonates were applied with a fine paint brush to the roots of each plant near the crown. WCR were left to feed for 4 d, after which roots were excavated, washed, and sampled for LAT.

Samples were dehydrated in a graded ethanol series of 75, 85, 95, and 100% ethanol:water (v/v) for 30 min at 25 °C. Once samples were in 100% ethanol, samples were dried in a critical point dryer (Leica EM CPD300) to preserve their cellular structure. For each sample, 2 mm of damaged root was sectioned at a longitudinal resolution of one frame per 4 µm of root, with white balance and aperture being manually adjusted to optimize visualization of the WCR.

To differentiate the WCR, and damaged and intact root tissue, transverse cross-sectional images from WCR-infested roots were analyzed for RGB colorspace in ImageJ (Fig. 5). The dominant emission wavelength of WCR tissue was determined from the RGB colorspace. WCR were defined as having a dominant emission wavelength of 557.9 nm (Table 1), with red values ranging from 73 to 181, green values from 107 to 255, and blue values from 68 to 238 (Fig. 5). Healthy cortical tissue was defined as having a dominant emission wavelength of 473.8 nm (Table 1), with red values ranging from 6 to 153, green from 92 to 255, and blue from 133 to 255 (Fig. 5). Damaged tissue was defined in ImageJ as having red values from 17 to 28, green values from 19 to 31, and blue values from 19 to 30 (Fig. 5). Root segments were segmented (Fig. 6), three-dimensionally resolved (Fig. 7), and quantified using MIPAR™ and Avizo 9 Lite software as described above.

Fig. 4. Three-dimensional reconstruction showing the distribution of arbuscules in the cortex of maize (Zea mays) roots. Raw images were obtained with laser ablation tomography (a), processed (b), and aerenchyma lacunae (c) and mycorrhiza (d) were differentiated in green and yellow, respectively, and reconstructed in a three-dimensional model (e); a side view shows how arbuscular networks are spatially distinct from aerenchyma channels (f).

Fig. 5. RGB spectra measured in ImageJ from designated areas in cross-sectional images of maize (Zea mays) roots containing the western corn rootworm (Diabrotica virgifera virgifera) and damaged tissue. The minimum and maximum values for each channel were used to parameterize thresholding shown in Fig. 6 and the mode was used in calculating the dominant emission wavelength.
Avizo 9 Lite as described for AMF samples. Some overlap in segmentation of different features existed between spectra of WCR and root tissue, but these overlapping regions were manually removed in MIPAR™ prior to 3D reconstruction of the segment.

Cereal cyst nematode infection

Seeds of barley (Hordeum vulgare) cv. Sloop (developed by the University of Adelaide), which is known to be susceptible to cereal cyst nematode (CCN) (Heterodera avenae), were obtained from the University of Adelaide barley breeding program, surface sterilized in a 10% NaOCl:water (v/v) for 10 min, rinsed in deionized water, placed on moist filter paper, and incubated overnight at 4 °C. Seeds were then transferred to sterile 2% agar plates and placed in a growth chamber at 15 °C with a 12:12 (light:dark) photoperiod. CCN (H. avenae) inoculum was prepared as described by Aditya et al. (2015). Three days after plating the seeds, when barley roots were ~2–3 cm long, root tips of barley seedlings were inoculated with one drop of freshly hatched nematodes.

Ten days after inoculation, 5 cm segments of root, each including a swollen region that was considered to be indicative of the formation of one or more feeding sites, were excised and preserved in 75% ethanol:water (v/v). Root segments were dehydrated and critically point dried as described for the WCR sample. For each sample, 1.5 mm of the swollen region containing the nematode and feeding site was sectioned at an axial resolution of one frame per 1 μm of root.

To differentiate the nematodes and their feeding sites (syncytia), transverse cross-sectional images were analyzed for RGB colorspace in ImageJ (Fig. 8). The dominant emission wavelength of nematode tissue was determined from the RGB colorspace. Nematodes were defined as having a dominant emission wavelength of 487.6 nm (Table 1), with red values ranging from 64 to 102, green values from 131 to 167, and blue values from 158 to 193 (Fig. 8). Cortical tissue was defined as having a dominant emission wavelength of 473.8 nm (Table 1), with red values ranging from 14 to 53, green from 75 to 123, and blue from 162 to 215 (Fig. 8). Feeding sites were defined as having red values from 19 to 33, green values from 46 to 64, and blue values from 46 to 71 (Fig. 8). Root segments were segmented (Fig. 9), three-dimensionally resolved (Fig. 10), and quantified using MIPAR™ and Avizo 9 Lite as described above.

Fusarium colonization

Fusarium inoculum was prepared on sorghum grains that were soaked in water overnight, transferred into mushroom spawn bags (Fungi Perfecti, Olympia, WA, USA) containing 1.8 kg of soaked sorghum grains, autoclaved for 8 h, and allowed to cool. One plate of potato dextrose agar containing a 14-day-old culture of Fusarium virguliforme isolate Mont-1 was added to a sterile blender with 100 ml of sterile deionized water and blended for 30 s. The inoculum slurry was aseptically added to one bag of cooled, autoclaved sorghum grain and sealed with a heat sealer. The inoculum was incubated at room temperature and gently mixed every other day until the grains were visually well colonized, ~3 weeks. A 12 g aliquot of inoculum was added to 300 ml of medium vermiculite, homogenized, and transferred to a 350 ml pot. Seeds of common bean (Phaseolus vulgaris) cv. Red Hawk (developed by Dr...
James Kelly, Michigan State University) were obtained from Michigan State University and surface sterilized by soaking in 6% NaOCl:water (v/v) for 10 min, rinsed with sterile deionized water, and dried on sterilized paper towels. The seeds were sown on top of the homogenized inoculum and vermiculite, and covered with 70 ml of non-inoculated medium vermiculite. The plants were grown in a growth chamber at 25 °C with 14:10 (light:dark) photoperiod and irrigated every other day. Two weeks after sowing, a 3 cm segment of taproot was collected from directly below the hypocotyl and stored in 75% ethanol:water (v/v). Samples were dehydrated and critically point dried as described for the WCR sample. For each sample, 0.5 mm of infected root was sectioned at a longitudinal resolution of one frame per 1 µm of root, with white balance and aperture being manually adjusted to optimize visualization of damaged root tissue.

To differentiate between healthy root tissue and tissue damaged by *F. virguliforme*, cross-sectional images from non-inoculated control roots and inoculated roots were segmented in ImageJ using the modified Isodata thresholding method based on RGB colorspace (Figs 11, 12). The dominant emission wavelength of healthy root tissue and damaged tissue was determined from the RGB colorspace. Damaged cortical tissue was first defined in ImageJ from inoculated roots as having a dominant emission wavelength of 602 nm (Table 1), with red values ranging from 34 to 159, green values from 15 to 100, and blue values from 11 to 111 (Fig. 11). Healthy tissue was defined from non-inoculated control roots as having a dominant emission wavelength of 461.9 nm (Table 1), with red values ranging from 69 to 213, green values from 44 to 198, and blue values from 21 to 255 (Fig. 11). There was some overlap in segmentation between healthy and damaged tissue, but, on the scale of a root cross-section, thresholding on these values was an effective method for defining these two groups of tissue in inoculated root samples. Additionally, because the autofluorescence from the metaxylem vessel lumen was similar to that of damaged tissue, these misidentifications were removed prior to three-dimensional reconstruction of the inoculated segment (Figs 11, 12). Root segments were segmented (Fig. 12), three-dimensionally resolved (Fig. 13), and quantified using MIPAR™ and Avizo 9 Lite as described above.

**Results**

**Mycorrhizae colonization**

AMF colonization was successfully segmented from maize root tissue due to differences in autofluorescence between root and fungal cell walls. Distinct autofluorescence emission spectra
were observed for arbuscules, cortical tissue, and stele tissue [Table 1; Fig. 3; Supplementary Video S1 available at the Zenodo repository (http://doi.org/10.5281/zenodo.1479847)]. AMF are characterized by their yellow fluorescence with a dominant wavelength of 583.9 nm (Table 1; Fig. 2), which was not present in non-colonized cortical tissues and allowed for the rapid segmentation of fungal structures (Fig. 3). Cortical tissue was characterized by a blue fluorescence with a dominant wavelength of 477.2 nm, while stele tissue fluoresced orange with a dominant wavelength of 588.6 nm (Table 1). The spatial distribution of AMF colonization in the cortex was characterized in relation to root anatomy, and volumes of these structures could be quantified in a semi-automatic manner (Fig. 4; Supplementary Video S2).

To define spatial localization of AMF colonization and aerenchyma formation, we quantified the volume in three cortical bands: the outer, middle, and inner cortex, as previous research has demonstrated that cortical cell size and anatomical traits can vary by cortical region (Chimungu et al., 2015). The average root segmented was 0.17 mm$^3$ in volume, the average volume of aerenchyma was 0.032 mm$^3$ (18.8% of total root volume), and the average volume of fungal colonization was 0.0081 mm$^3$ (4.7% of total root volume) (Fig. 4; Supplementary Table S1 at JXB online). In the inner, middle, and outer cortical bands, the ratio of aerenchyma to fungal colonization was ~4:1. Visualization of AMF colonization with light microscopy shows how LAT compares with conventional imaging techniques (Supplementary Fig. S1).

Western corn rootworm infection

The first instar larva was ~2 mm in length, had a diameter of 0.42 mm, and was observable entirely within the cortical tissue of the maize root (Figs 6A, B; 7; Supplementary Videos S3, S4). The fluorescence from the WCR larva was yellow/green in color and had a dominant emission wavelength of 557.9 nm, while cortical tissue fluoresced blue with a dominant emission wavelength of 473.6 nm (Table 1; Fig. 6). Root anatomical features were clearly observable including the epidermis, cortex, stele, and metaxylem vessels. WCR damage comprised 19% of the total volume of a 0.75 mm maize root segment and was visible as longitudinal channels running parallel to the root axis through the root cortex. WCR frass deposited within herbivorized channels fluoresced blue/green similar to intact cortical tissue and had a dominant emission wavelength of 491.9 nm (Table 1; Fig. 6). WCR damage affected both the cortical and vascular tissue, with damage affecting 28% of the total cortex volume and 8% of the total stele tissue volume. Six of the 15 (40%) metaxylem vessels in the root segment were compromised by WCR damage (Fig. 6C, D; Supplementary Table S2). Within the WCR larva, anatomical features including the midgut, fat bodies, and muscle fibers were clearly visible (Fig. 7; Supplementary Video S4). Visualization of WCR damage with light microscopy shows how LAT compares with conventional imaging techniques (Supplementary Fig. S2).

Nematode colonization

In the barley root segments, nematodes were easily identified as they emitted a distinctive green/blue fluorescence with a dominant emission wavelength of 487.6 nm. In contrast, intact cortical tissue fluoresced a darker blue at 473.8 nm, reflecting compositional differences between tissue of the parasite and cell wall of the root (Table 1; Figs 8, 9). In contrast to both the nematode and intact cortical tissue, the feeding sites appeared as dark voids directly adjacent to the central metaxylem vessel wall (Figs 8, 9; Supplementary Videos S5, S6). Internal cell walls were not clearly visible within the feeding sites. Cortical cells near the feeding site were asymmetrically shaped and enlarged, and portions of the central metaxylem near the feeding sites appeared enlarged with thickened cell walls (Figs 8, 9; Supplementary Videos S5, S6). LAT enabled the rapid identification, quantification, and spatial localization of nematodes, and the feeding sites and their spatial location in the root.

The root segment shown in Fig. 10 was 1.337 mm in length, had a maximum diameter at the feeding sites of 0.896 mm, had a total volume of 0.706 mm$^3$, and contained two nematodes and two feeding sites (Supplementary Table S3). The nematodes in this root segment had lengths of 0.396 mm and 0.313 mm, maximum diameters of 0.021 mm and 0.024 mm, and total volumes of 0.000145 mm$^3$ and 0.000146 mm$^3$ (Fig. 10;
These dimensions are consistent with the J2 stage of *H. avenae*. The feeding sites had maximum axial lengths of 0.232 mm and 0.255 mm, maximum diameters of 0.115 mm and 0.129 mm, and total volumes of 0.000662 mm$^3$ and 0.00152 mm$^3$, together occupying 0.003% of the total volume of the 1.337 mm root segment and intersecting with much of the phloem tissue and several metaxylem vessels (Fig. 10; Supplementary Table S3). Visualization of nematode colonization with light microscopy shows how LAT compares with conventional imaging techniques (Supplementary Fig. S3).

**Fusarium colonization**

As a result of the high-contrast differentiation of tissue through autofluorescence on the LAT (Supplementary Video S7), damaged epidermis and cortex from *F. virguliforme* inoculation in *P. vulgaris* was readily segmented from healthy tissue in a three-dimensional model and quantified. Autofluorescence from damaged tissue appeared as red/orange with a dominant emission wavelength of 602 nm, while intact cortical tissue appeared blue with a dominant emission wavelength of 461.9 nm (Table 1).

The inoculated three-dimensional taproot segment was 0.5 mm in length (Fig. 13; Supplementary Video S8; Supplementary Table S4). The total volume of the entire segment was 0.57 mm$^3$, undamaged tissue was 0.13 mm$^3$, and damaged tissue was 0.44 mm$^3$, consuming 77% of the segment's total volume (Fig. 13; Supplementary Table S4). Destruction by *F. virguliforme* was almost entirely confined to epidermal and cortical tissue, while vasculature and cortical tissue immediately around the stele were left intact. Visualization of *Fusarium* damage with light microscopy shows how LAT compares with conventional imaging techniques (Supplementary Fig. S4).
Discussion

LAT enabled observations of root anatomy and root organisms across a diversity of plant species and edaphic organisms as a result of the contrasting autofluorescence spectra emitted from different tissues. This spectral differentiation between tissues in roots and the colonizing organisms highlighted variation in chemical composition not visible under bright field conditions. Comparably with our observations of intact cortical tissue in maize, barley, and common bean roots, other studies have also reported blue autofluorescence from plant cell walls under UV excitation. Cellulose (420–430 nm), suberin (465 nm), lignin (455 nm), and ferulate (480 nm) have all been found to emit maxima close to our observations of cortical cell walls (461.9–477.2 nm) (Table 1) (Donaldson and Williams, 2018). Additionally, plant-derived terpenes, alkaloids, and flavonoids are also known to autofluoresce within the blue/green spectra (470–525 nm). The orange fluorescence of stele tissue in maize (588.6 nm) may be a product of anthocyanins, azulenes, polycyclics, isoquinoline, and acridone alkaloids, which are also known to emit yellow/orange/red (Table 1) (Roshchina, 2003). Other cell wall compounds such as cutin are not known to be autofluorescent under UV (Donaldson and Williams, 2018).

Root cell walls are primarily composed of lignin, cellulose, hemicellulose, suberin, and tannins (White et al., 2011), while chitin is a principal component of cell walls in fungi, and is also produced by nematodes and arthropods (Cohen, 1987; Foster et al., 2005). Additionally, the epidermis of nematodes is made up of a thick cuticle made of collagen (Anya, 1966). Overlapping with our spectral observations of cereal cyst nematode (487.6 nm) and WCR (557.9 nm) from the LAT (Table 1), under UV excitation, aphids display maxima of 475, 540, and 675–680 nm (Roshchina, 2012). With LAT, AMF arbuscules in the cortex fluoresced yellow (583.9 nm), similar to observations by Jabaji-Hare et al. (1984) where hyphae, vesicles, and arbuscular branches of Glomus species fluoresced bright yellow under UV (Table 1). Red fluorescence of Fusarium-damaged tissue (602 nm) may be the product of secondary metabolites produced in response to fungal invasion such as phenols, which have been observed to fluoresce under UV excitation (Table 1) (Wolfbeis, 1985). Specifically, plant tissue damaged by reactive oxygen species forming lipofuscins has been observed to fluoresce red (>600 nm) (Roshchina, 2012). Unlike with AMF, fungal structures of Fusarium were not visible with the LAT. This absence of autofluorescence under UV excitation has been previously reported (Ames et al., 1982). Gange et al. (1999) indicate that AMF are unique among a broad range of fungal taxa in their capacity to autofluoresce under UV. Variance in the dominant spectra of a given tissue may reflect differences in the host and root symbiont species, sampling location within the root system, and plant growth conditions.
arbuscules occupied ~5% of the total root volume, and a 4:1

phenes and root-associated organisms were rapidly qualified

teral qualities, physical interactions between root anatomical

tification and spatial resolution of the chemical composition of

2010). Spectral imaging is a promising technique for the quan-

tion by capturing and quantifying autofluorescent emission

able light (400–700 nm), hyper- or multispectral imaging may

edaphic organisms were measurable across the spectrum of vis-

structural features in root samples without the need for dyes as

In addition to the discrimination of tissues based on spec-

Although differences in autofluorescence of root tissues and

Despite this variation in autofluorescence, UV excitation of

Fig. 11. RGB spectra measured in ImageJ from designated areas

ratio of aerenchyma to arbuscules was maintained throughout

inoculation (Fig. 4; Supplementary Video S2). In contrast to pre-

first instar larva were found to feed on both cortical and vascular tissue along the length of the root (Figs 6, 7; Supplementary Video S3). As expected, cereal cyst nematodes were positioned entirely within the cortex of barley roots; their feeding sites extended into the stele, reaching metaxylem vessels (Figs 9, 10; Supplementary Video S3). In common bean roots, destruction of tissue by F. virguliforme was primarily confined to the epidermal tissue and cortex 2 weeks after inoculation (Figs 12, 13; Supplementary Video S8), although colonization of the vascular tissue has been reported by 20 d after inoculation (Islam et al., 2017a, b).

Although three-dimensional quantification of root-colonizing fungi has been accomplished non-destructively via confocal microscopy of plants in agar gels (Czymmek et al., 2007), the creation of a z-stack in confocal microscopy is achieved at much lower throughput than with LAT, which can scan through samples at a rate of 30–100 µm s⁻¹. Furthermore, sample size is more constrained with confocal microscopy as the z-stack dimension is typically restricted to 1.5 mm, and is limited even further due to the opacity of root tissue. In contrast, the length of a root segment for three-dimensional reconstruction using LAT is limited only by the maximum linear distance of travel of the stage. In addition to the reduced z limit of conventional microscopy, the field of view for light and confocal microscopes is generally too restricted for the scale required for observations of field-grown roots of crop species. While confocal microscopy has benefits for cellular-level visualization on small samples with transparent tissue, LAT enables tissue-level visualization on large samples of opaque tissue. With LAT, the limitation of root volume analyzed is determined by the camera sensor size, camera lens, and the laser galvanometer. In the current LAT configuration, the sample width is constrained to 7 mm due to galvanometer limitations. With alteration of this component, the capability of LAT could be expanded to ablate samples with a width >5 cm. Similar to confocal microscopy, X-ray microscopy also fills the role of a competing technology for three-dimensional analysis. Although it is a non-destructive imaging method, X-ray microscopy has a low spatial resolution which only allows for the identification of large anatomical structures and suffers from low throughput by requiring a long imaging time and intensive data analysis (Karunakaran et al., 2015). Additionally, X-ray microscopy is very costly and lacks the ability to provide compositional information of tissues that LAT or confocal work is capable of.

While LAT has capabilities that exceed those of traditional microscopy, it does have several limitations. Obviously, LAT is destructive, although it is not unique in this regard; many other microscopy techniques necessitate destructive tissue preparation and sampling. Another shortcoming of LAT is that it only allows visualization of plant cell walls, but not cellular contents. While cell walls remain rigid and contain dense networks of structural polymers that emit a bright fluorescence when ablated, the contents of the cell lumen
are vaporized without significant production of visible light. Along with this inability to visualize the intricacies of cell contents, in contrast to the capabilities of other microscopy techniques (Navi and Yang, 2008; Islam et al., 2017a, b), the resolution of the present camera system did not permit the visualization of the fine and spatially diffuse fungal hyphae in the apoplast of AMF- and *Fusarium*-inoculated samples. Similarly, fine cell wall fragments were not visible within the syncytial feeding sites of a cyst nematode. Alternative LAT configurations could overcome these limitations by improved camera and lens technologies affording increased magnification and resolution.

LAT improves interpretation of root–organism interactions in relatively large, opaque root segments through rapid, three-dimensional visualization. This provides opportunities for novel research investigating the effects of root anatomical phenes on associations with edaphic organisms. Owing to its capability for high-throughput imaging of root anatomy, LAT serves as an excellent tool for breeders and pathologists to conduct quantitative screens characterizing resistance to root pathogens. Additionally, many aspects of the interactions among root anatomy, architecture, and edaphic organisms remain unexplored and would be well suited for investigation with LAT. Differences in autofluorescence emission of root tissues, fungi, arthropods, bacteria, and nematodes in three dimensions would greatly facilitate the study of these interactions. Deeper understanding of the relationships between root-inhabiting biota can provide important insights in designing integrated systems of pest management and agricultural productivity. With significant opportunities to improve upon the present capabilities of LAT through modification of laser wavelengths, increased resolution, and hyperspectral imaging technologies, many research applications remain for the employment of LAT.

Fig. 12. Comparison of healthy common bean (*Phaseolus vulgaris*) root (a, b, c), with a *Fusarium*-inoculated common bean root (d, e, f). Healthy tissue (b, e) was separated from compromised tissue (c, f) using RGB spectra measured from images (Fig. 11).
Supplementary data

Supplementary data are available at JXB online.

Table S1. Dimensions of features measured in maize (Zea mays) root segment colonized with arbuscular mycorrhizal fungi shown in Fig. 4.

Table S2. Dimensions of features measured in maize (Zea mays) root segment colonized with western corn rootworm (Diabrotica virgifera virgifera) shown in Fig. 7.

Table S3. Dimensions of features measured in barley (Hordeum vulgare) root segment colonized with cereal cyst nematode (Heterodera avenae) shown in Fig. 10.

Table S4. Dimensions of features measured in common bean (Phaseolus vulgaris) root segment colonized with Fusarium (Fusarium virguliforme) shown in Fig. 13.

Fig. S1. Comparison of images of maize (Zea mays) roots colonized with arbuscular mycorrhizae (Rhizophagus irregularis) taken with a stereo-microscope and LAT.

Fig. S2. Comparison of images of maize (Zea mays) roots damaged by western corn rootworm (Diabrotica virgifera virgifera) taken with a stereo-microscope and LAT.

Fig. S3. Images of barley (Hordeum vulgare) roots colonized with cereal cyst nematode (Heterodera avenae) taken using a stereo-microscope.

Fig. S4. Comparison of images of common bean (Phaseolus vulgaris) roots damaged by Fusarium (Fusarium virguliforme) taken with a stereo-microscope and LAT.

Video S1. LAT scan of maize (Zea mays) root segment colonized with AMF.

Video S2. Three-dimensional reconstruction of AMF colonization in a maize (Zea mays) root segment, highlighting the spatial relationship between AMF (yellow) and aerenchyma (green).

Video S3. LAT scan of maize (Zea mays) root segment colonized with WCR.

Video S4. Three-dimensional reconstruction of WCR colonization and damage in a maize (Zea mays) root segment.

Video S5. LAT scan of barley (Hordeum vulgare) root segment colonized with cereal cyst nematode.

Video S6. Three-dimensional reconstruction of nematodes (red) and associated feeding sites (black) within a barley (Hordeum vulgare) root segment.

Video S7. LAT scan of common bean (Phaseolus vulgaris) root segment with damage from Fusarium.

Video S8. Three-dimensional reconstruction of a common bean (Phaseolus vulgaris) root segment with damage from Fusarium.

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