X-ray imaging and microspectroscopy of plants and fungi

W. Yun,[#] S. T. Pratt,[@] R. M. Miller,[†] Z. Cai,[*] D. B. Hunter,[‡] A. G. Jarstfer,[§]
K. M. Kemner,[†] B. Lai,[*] H.-R. Lee,[*] D. G. Legnini,[*]
W. Rodrigues,[*] and C. I. Smith[†]
[#]*Advanced Light Source, Lawrence Berkeley Laboratory, Berkeley, CA 94720 USA*
[@]*Chemistry Division, Argonne National Laboratory, Argonne, IL 60439 USA*
[*]*Experimental Facilities Division, Argonne National Laboratory, Argonnne, IL 60439 USA*
[†]*Environmental Research Division, Argonne National Laboratory, Argonne, IL 60439 USA*
[‡]*Savannah River Ecology Laboratory, The University of Georgia, Aiken, SC 29802 USA*
[§]*LeTourneau University, P.O. Box 7001, Longview, TX 75607 USA*

Introduction

Approximately 90% of the world's vascular plants, including the majority of all economic crops, belong to families that commonly have symbiotic associations with mycorrhizal fungi [1, 2]. While such associations are known to increase plant viability under low nutrient conditions, in some instances mycorrhizal fungi can also moderate toxicity effects in plants growing on soils containing elevated concentrations of heavy metals [3]. Thus, an improved understanding of the plant-fungus relationship, particularly with respect to the uptake mechanisms for metals, is expected to have significant implications in both agriculture and the remediation and restoration of contaminated environments.

X-ray fluorescence microscopy and microspectroscopy can provide information on the spatial distribution, oxidation state, chemical environment, and transformations of trace elements, and offer significant advantages over other techniques. An important advantage of hard x-ray techniques is that they require essentially no sample preparation and can be applied under natural conditions. In addition, hard x-ray techniques can provide chemical-state information, which is considerably more difficult to obtain with techniques based on charged particles. While soft x-ray (< 1 keV) microscopy and microspectroscopy have considerable utility for the study of biological samples [4], the hard x-ray techniques described here provide improved fluorescence yields and access to the K edge of the third-row and heavier elements, many of which are important nutrients, micronutrients, or environmental contaminants. In the present work, we have used microimaging and microspectroscopy to study the mechanisms for the uptake of metals by mycorrhizal fungi and plants. Hard x-ray phase-contrast images, element specific x-ray fluorescence (XRF) images, and x-ray absorption near-edge spectra obtained with focused x-rays (micro-XANES) have all been performed. The new data were obtained with a minimal amount of sample preparation and clearly illustrate the potential of the new high-brilliance synchrotrons for such samples.

Methods and Materials

Hard x-ray phase contrast imaging was performed with a simplified version of a previously employed scheme [5]. Coherent, monochromatic x-ray radiation was collimated and passed through the sample and then onto a scintillation screen approximately 1.5 m downstream from the sample. The visible image on the screen was then imaged with a video camera equipped with a 20X or 40X microscope objective. Images were obtained at the video clock rate, and the spatial resolution was approximately 5 μm. Because the absorption contrast of the root-fungus sample at the x-ray energy (11.9 keV) used was very small, the phase change when the x-rays pass through the sample provided the main contrast mechanism for the image.

The high-performance zone plate [6] used in the present XRF-imaging and microspectroscopy experiments produced a focused beam of 1 μm x 3 μm cross section and 4 x 10^{10} photons/s/0.02% bandwidth. The zone plate had an effective focal length of 52.5 cm at 11.9 keV and an effective spot size of 1 μm (vertical) by 3 μm (horizontal). The x-ray beam passed through a 20 μm order-sorting aperture, and the focus was adjusted to be on the sample. Samples for the XRF images were mounted on a computer-controlled XYZ stage at 20° or 45° to the incident x-ray beam. Thus, in the latter case, the 3-μm-wide horizontal beam intercepted approximately 4.2 μm in the horizontal dimension of the sample, while the 1 μm vertical dimension of the beam was preserved. X-ray fluorescence from the sample was collected at 90° to the incident beam by using an energy-dispersive, single-element, solid-state detector. Mapping of the spatial distribution of individual elements was performed by scanning the sample in 5 μm steps through the focused monochromatic x-ray beam (11.9 keV) and integrating the selected Kα fluorescence for 3 sec/pt. The total data collection time for a 61 x 61 pixel array (300 x 300 μm^2) was approximately 4 hours, and the elemental sensitivity was approximately 500 ppb. The absolute concentrations were determined by calibrating the detector with standard films from the National Institute of Standards and Technology, but have not been corrected for effects due to attenuation of the emitted x-rays by the sample; they are expected to be accurate to within a factor of two.

The XANES spectra were recorded by aligning the focused x-ray beam to several different spatial features in the root and fungal hyphae and scanning the x-ray energy through the absorption edge while monitoring the Mn Kα fluorescence. The spatial resolution and sensitivity of the present experiments allowed the study of a single fungal hypha. Each point in the XANES spectra was averaged for ten

seconds of detector live time, and the full spectrum was recorded in approximately 45 min. The spectrum has been normalized by dividing the fluorescence signal by the intensity of the transmitted light, and energy calibrated by using an elemental Mn standard. The focal length of the zone plate depends on wavelength and in principle the distance of the zone plate to the sample should be scanned as the x-ray energy is scanned. Because the XANES spectra cover a limited energy range, however, no attempt was made to scan the zone plate distance in the present experiments. It is estimated that the effective spot size varied from approximately 1 to 2 μm in the vertical direction and from 3 to 3.6 μm in the horizontal direction. All of the experiments discussed here were performed at the 2-ID-D beamline of the Synchrotron Radiation Instrumentation Collaborative Access Team of the Advanced Photon Source.

The samples were prepared by using standard methods. *Plantago lanceolata* seedlings were transplanted in flint sand and inoculated with approximately 40 spores of *Glomus mosseae*. The plants were watered daily, alternating each day between deionized water and 10% Hoagland's solution. After 45 days, the plants were harvested by washing the sand from the roots and fungal hyphae and rinsing with deionized water. Samples were then wet mounted between two layers of Kapton tape and mounted in the x-ray beam on the XYZ sample stage.

Results and Discussion

Phase-contrast images of the plant root infected by the mycorrhizal fungus show threadlike features coming off approximately perpendicular to the root; these correspond to the fungal hyphae. The hyphae can be distinguished from root hairs because they branch and enter the root at multiple points. The spatial resolution in our first phase-contrast images is similar to that of our optical micrographs, but the former images require far less sample preparation and can be obtained on fully hydrated samples under natural conditions. Phase-contrast images recorded near the K edges of elements of interest are expected to provide elemental selectivity.

The spatial distributions of Mn, Fe, Cu, and Zn determined in our XRF-imaging experiments show very different behavior. Interestingly, there is little evidence for Mn in the fungal hyphae, which may be related to the observation that mycorrhizal plants typically have much lower Mn concentrations than nonmycorrhizal plants [9]. The Fe tends to be most concentrated on the edge of the root, perhaps reflecting the precipitation of Fe in this location. Both Cu and Zn show up most strongly in the fungal hyphae and in the center of the root, most likely in the inner cortex where the proliferation of the fungus is greatest. This suggests the use of Cu and Zn as surrogate measures of mycorrhizal fungi in roots and additional work is underway to confirm this correlation.

The determination of both the spatial distribution and the chemical state of selected elements is important for many problems in biology and environmental science. For such studies, it is essential that the sample be in its natural hydration state because drying could modify the chemical form of the element of interest. To demonstrate the enhanced capabilities provided by a high-brilliance x-ray source and the use of high-performance zone plates, we recorded micro-XANES spectra at the Mn K edge with a 1 x 3 μm^2 spot size at selected positions within a hydrated sample. Mn is an essential micronutrient in both plants and fungi, and in natural systems it usually exists in the +2 or +4 oxidation state; the +3 oxidation state is also possible but it is generally unstable. In our sample, the Mn XANES spectrum was found to be independent of position. Comparison of our results with detailed XANES studies of a wide variety of samples and standards [7, 8] indicated that at least 90% of the Mn sampled by the x-ray beam at each of the locations is Mn^{+2}, the soluble and most useful form to plants and fungi. The lack of variation in the Mn oxidation state in the present sample limits the ability to unequivocally demonstrate the spatial resolution of the microspectroscopy unequivocally; however, the experiments clearly indicate that we now have the ability to record high-quality XANES spectra with 1 to 2 x 3 μm^2 spatial resolution and at concentrations of approximately 3 ppm. In principle, similar measurements for other third-row and heavier elements should be straightforward.

This work has been published in Reference 10.

Acknowledgments

This work was supported by the U.S. Department of Energy, Office of Energy Research, Office of Basic Energy Science and Office of Health and Environmental Research, under contract W-31-109-Eng-38, and by Financial Assistance Award Number DE-FC09-965R18546 from the U. S. Department of Energy to the University of Georgia Research Foundation. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under Contract No. W-31-109-Eng-38.

References

[1] R.M. Miller, *Ecophysiology of VA Mycorrhizal Plants*, edited by G. R. Safir (CRC Press,Boca Raton, 1987) p.135.

[2] H. Marschner, *Mineral Nutrition of Higher Plants*, Second Edition (Academic Press, New York, 1995).

[3] R. Bradley, A.J. Burt, and D.J. Read, *Nature* **292**, 335 (1981); R. Bradley, A.J. Burt, and D.J. Read, *New Phytol.* **91**, 197 (1982).

[4] J. Kirz, C. Jacobsen, and M. Howells, *Quart. Rev. Biophys.* **28**, 33 (1995).

[5] A. Snigirev, I. Snigireva, V. Kohn, S. Kushnetsov, and I. Schelokov, *Rev. Sci. Instrum.* **66**, 5486 (1995).

[6] B. Lai, W. Yun, D. Legnini, Y. Xiao, J. Chrzas, P.J. Viccaro, V. White, S. Bajikar, D. Denton, and F. Cerrina, *App. Phys. Lett.* **61**, 1877 (1992).

[7] D. G. Schulze, T. McCay-Buis, S.R. Sutton, and D. M. Huber, *Phytopathol.* **85**, 990 (1995); D.G. Schulze, S.R. Sutton, and S. Bajt, *Soil Sci. Soc. Am. J.* **59**, 1540 (1995).

[8] A. Manceau, A.I. Gorshkov, and V.A. Drits, *Am. Mineralogist* **77**, 1133 (1992); A. Manceau, A.I.

Gorshkov, and V.A. Drits, *Am. Mineralogist* **77**, 1144 (1992).
[9] S.K. Kothari, H. Marschner, and V. Römheld, *New Phytol.* **117**, 649 (1991).
[10] W. Yun, S. T. Pratt, R. M. Miller, Z. Cai, D.B. Hunter, A.G. Jarstfer, K.M. Kemner, B. Lai, H.R. Lee, D. G. Legnini, W. Rodrigues, and C.I. Smith, *J. Synchrotron Rad.* **5**, 1390-1395 (1998).