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Handbook of Methods used in Rhizosphere Research

2.1. Elemental Analysis of Roots and Fungi

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2.1.1. Introduction

Many aspects of studying roots and root-associated microorganisms require knowledge on the concentrations and distribution of elements, to understand the uptake and transport of ions and their roles in metabolic processes and water relationships. This chapter deals with classical and modern analytical methods to quantify elements in the roots, to localize and visualise the distribution of elements (e.g. heavy metals) in the roots and mycorrhizas. The following methods are covered in this chapter:

- plant digests
- elemental and ionic analysis of digests
- histochemical methods
- X-ray microanalytical methods

Specific contribution to rhizosphere research

None of these methods have been developed specifically for rhizosphere related question. However, elemental contents in roots and mycorrhizal fungi are often determined with the aid of these methods.

2.1.2. Plant digests

Brief description of method

Dried, ground and weighed plant tissue samples are prepared for elemental analysis through the destruction of organic matter. The two commonly used methods of organic matter destruction are dry ashing (high-temperature combustion) and wet ashing (acid digestion). Both methods are based on the oxidation of organic matter through the use of heat and/or acids. A number of procedures have been developed that utilize microwaves as source of heat. These are generally classified as closed or open vessel. Closed vessel (Parr Bomb) utilizes heat and pressure to increase reaction rate and to decrease digestion time.

Basic references

Jones, J.B.; Case, V.W. 1990. Sampling, handling, and analyzing plant tissue samples, pp. 389-427. In: R.L. Westerman (Ed.), Soil Testing and Plant Analysis. Third edition. SSSA Book Series 3. Soil Science Society of America, Madison, WI.

Kalra, Y.P. 1998. Handbook of Reference Methods for plant analysis. 1998, CRC Press, Boca Raton, Florida, USA

Watson, M.E.; Isaac, R.A. 1990. Analytical instruments for soil and plant analysis, pp. 691-740. In: R.L. Westerman (Ed.), Soil Testing and Plant Analysis. Third edition. SSSA Book Series 3. Soil Science Society of America, Madison, WI.

Application areas

Wet ashing is recommended for plant material high in Si (e.g. Graminaceae) or containing volatile elements (arsenic, mercury, or selenium) that may be lost during the dry ash procedure.

Problems, constraints, do's and don'ts

Sample material must be ground to pass through a 0.5 – 1.0 mm mesh size to ensure homogeneity. Before use, all glassware, plasticware, and Teflon digestion vessels should be thoroughly rinsed, first with a diluted acid (e.g. HCl) and then with redistilled or doubly-deionized water. It is essential that the filtrate does not contain any particles that could clog the analyzers sample introduction device (for an overview on analytical instrumentation see below). Critical factors in dry ash procedures include selection of ashing vessel, placement in furnace, ashing temperature, time, selection of acid to solubilize the ash, and final volume, whereas critical factors in wet digestion procedures include selection of the digestion vessel, temperature and control, time, the digestion mixture, and final volume. Special safety precautions are required for microwave digestion.

Related method sheets:

ID	**21_Brunner**
Parameter	**Total element concentrations in fine roots**
Soil type	forest soils
Plant species	Picea abies, Pinus montana, Pinus cembra
System	Samples from forest
Method	**CN-analyser, ICP-AES**

ID	**21_Jansa**
Parameter	**Total content of nonvolatile elements in plant shoots and roots**
Soil type	any
Plant species	any (provided roots can be washed out from the soil and cleaned)
System	both field and pot experiment samples
Method	**Atomic spectrometry, ion chromatography, and colorimetry of plant digests (dry ashing)**

ID	**21_Sainz**
Parameter	**Concentration of P, K, Ca, Mg, Na, Fe, Mn, Cu and Zn in plant material**
Plant species	Any plant species
System	Material from field, greenhouse, growth chamber
Method	**UV/VIS spectrophotometry (colorimetry), atomic emission /absorption spectrometry, ICP-OES of plant digests**

ID	**21_SasPaszt**
Parameter	**P, K, Mg, Ca, total N and microelements B, Cu, Fe, Mn, Zn in plant material**
Soil type	Mineral soil from pomological orchards
Plant species	Strawberry, apple, pear
System	Greenhouse and field experiments with mineral soils
Method	**Atomic spectrometry of plant digests (wet ashing), Kjeldahl N**

2.1.3. Elemental analysis of digests

Brief method description

Element analysis of plant digests can be carried out by any method that is able to analyse total elemental or ionic contents in solutions like flame or graphite furnace atomic absorption or emission spectrometry (AAS, AES), inductively-coupled plasma atomic emission or mass spectrometry (ICP/AES, also abbreviated as ICP/OES; ICP/MS), but also colorimetry (mainly for P as phosphate) or ion chromatography (for Cl as chloride, S as sulfate, N as nitrate). Details on the principle operation and use of these analytical methods for conducting root and fungal tissue analysis have been described in the references below.

Basic references

Dulski, T. R. 1999. Trace elements analysis of metals: Methods and Techniques. Marcel Dekker, Inc. , New York

Kalra, Y. P. 1998. Handbook of Reference Methods for plant analysis. CRC Press, Boca Raton, Florida, USA

Walinga, I.; Van der Lee, J.J.; Houba, V.J.G.; Van Vark, W.; Novozamsky, I. 1995. Plant analysis manual. Kluwer Academic Publishers. Dordrecht, NL

Westerman R.L. 1990. Soil Testing and Plant Analysis. Third edition. SSSA Book Series 3. Soil Science Society of America, Madison, WI.

Application areas

The above-mentioned analytical methods were used for routine inorganic elemental analysis of plant tissue. From the point of view of rhizosphere research, macro-and micronutrients, trace elements and heavy metals can be determined in root and fungal tissues. The ICP-AES

instrument is the principal equipment in most contemporary plant analysis laboratories. It allows rapid, simultaneous multielement analysis of biological samples.

Problems, constraints, do's and don'ts

For specific problems with sensitivity, detection limits, precision, accuracy and occurrence of spectral interferences the reader should refer to basic references.

Related method sheets:

Same as for **chapter 2.1.2**.

2.1.4. Heavy metal localization using light microscopy

Brief method description

A few simple methods allowing for heavy metal detection are available to be used at the level of the light microscope or under the dissecting microscope. The most useful in the rhizosphere research are the sodium rhodizonate and dithizone techniques. Both substances form colored complexes. Sodium rhodizonate is used specifically to show Pb at pH 2.8. It reacts with several heavy metals at pH 7. Dithizone dissolved in acetone is useful in detection of Pb, Cd, Zn, Co, Ni, Cr, Fe at much lower concentrations (10^{-5} M of metal salts in aqueous solutions) than by the previous method. Both methods are very useful for pilot screening of heavy metal distribution in root and mycorrhiza samples. They give a general view of the situation and allow the selection of the most promising samples for further investigations with more specific methods including EDS, EELS, ESI or PIXE.

Basic references

Seregin, I.V.; Ivanov, V.B. 1997. Histochemical investigation of cadmium and lead distribution in plants. Rus. J. Plant Phys. 44: 791-796.

Turnau, K.; Kottke, I. 2005. Fungal activity as determined by micro-scale methods with special emphasis on interactions with heavy metals. In: The Fungal Community. J. Dighton, J.F. White, P.Oudemans (eds.), CRC Press, Boca Ration pp. 287-306.

Wierzbicka, M. 1987. Lead translocation and localization in Allium cepa roots. Can. J. Bot. 65: 1851-1860.

Wierzbicka, M.; Potocka, A. 2002. Lead tolerance in plants growing on dry and moist soils. Acta Biol. Crac. Ser. Bot. 44: 21-28.

Application areas

Both methods were successfully used to study heavy metal uptake by plants, at lethal and nonlethal concentrations, giving information on the rate of element transport. In the case of mycorhizospheric studies they were used to select mycorrhizal fungi or mycorrhizal morphotypes that accumulate heavy metals, resulting in the biofiltering effect of the extraradical and intraradical mycelium. It was used in the case of arbuscular, ericoid, orchid and ectomycorrhizas collected from various industrial wastes or cultivated in pots or rhizoboxes filled with substrata rich in heavy metals.

Problems, constraints, do's and don'ts

The time of incubation in solutions needs to be determined experimentally and it depends mainly on the plant material. The material for the tests should be treated with solutions of rhodizoniate or dithizone directly after harvesting. If washing of the material is needed, it should be done as fast as possible. Only material stained in rhodizonate dissolved in water was shown to be useful in further analysis for example by EDS. In other cases possible redistribution before or during further preparation steps has to be assessed.

Related method sheet

ID	**21_Turnau_c**
Parameter	**heavy metal detection at the light microscope level**
Soil type	industrial wastes or substrata enriched in heavy metals
Plant species	various plant species including those forming endo- and ectomycorrhiza
System	field material, rhizobox or pot material
Method	**rhodizonate and dithizone tests**

2.1.5. X-ray microanalytical methods

This chapter acquaints the reader with the types of principal instruments that are typically used for the localization of inorganic elements in plant tissue. X-ray microanalytical methods are a powerful technique that allows the determination of many elements of physiological interest at the subcellular level.

X-ray microanalytical methods cover various modern methods. This subchapter includes the following methods:

- Energy dispersive X-ray microanalysis (EDS)
- Electron energy loss spectroscopy (EELS) and imaging (ESI)
- Particle induced X-ray emission (PIXE)
- Synchrotron-based X-ray fluorescence and absorption-edge microtomography (F-CMT, AE-CMT)
- Synchrotron X-ray fluorescense (μ-SXRF) mapping and X-ray absorption fine structure (XAFS) spectroscopy.

2.1.5.1. Energy dispersive X-ray microanalysis (EDS)

Brief method description

The basic principle of EDS in a scanning electron microscope is that the electron beam scanned across the specimen area of interest excites atoms to emit X-ray photons which are collected by a semiconductor crystal. The energy of the X-ray photon hitting the crystal is converted into a voltage pulse proportional to the energy of the X-ray photon. The voltage pulses are sorted by a multichannel analyzer, thus forming the X-ray spectrum. EDS-detectors allow the measurement of all elements of $Z \geq 5$ (B) simultaneously. For a review consult one of the standard references below.

Basic references

Frey, B.; Scheidegger, C. 2002. Preparative techniques for LTSEM of lichens. In Methods in lichenology, Eds. I. Kranner, R.P.Beckett and A. Varma. Springer Lab Manual, Heidelberg 118-132.

Frey, B.; Zierold, K. 2003. X-ray microanalysis in botanical research. In: Science, Technology and Education of Microscopy: an overview, ed. A. Mendez-Vilas. Formatex, Extremadura, p. 313-324.

Marshall, A.T. 1988. X-ray microanalysis of frozen-hydrated biological bulk samples. Mikrochim. Acta Suppl. 15 (1998), pp. 273-282

Sigee, D.C. 1998. Environmental SEM and X-ray microanalysis of biological materials. Mikrochim. Acta Suppl. 15, pp. 283-293.

Zierold, K. 2002. Limitations and prospects of biological electron probe X-ray microanalysis. J. Trace Microprob. Tech. Vol. 20, pp. 181-196.

Application areas

EDS techniques have a broad application for various types of localizations of relevance to plant physiology, environmental pollution and root-microbe interactions. The principal field of interest is related to determining the distribution of water-soluble ions or to determining the identity and occurrence of inorganic deposits in cells such as silicon in cell walls or calcium in plant vacuoles as calcium-oxalate crystals. An interesting range of applications in physiological studies covers investigations on the uptake and transport of inorganic ions in plants using apoplastic (La) or symplastic (Rb, Cs) as tracers. A particularly successful field for EDS was the study of metal detoxification. The knowledge of the localization, distribution and quantification of toxic elements in root organs and cell compartments indicates possible pathways of transport and mechanisms of detoxification and is therefore important in reaching an understanding of tolerance mechanisms of heavy metals in plant sciences.

Problems, constraints, do's and don'ts

Reliable quantitative EDS measurements of biological specimens require special preparation techniques. Rapid freezing methods are suitable in preserving native-state cell structure, as well as having the least detrimental effect on redistribution and translocation of elements. Such frozen botanical specimen has to be opened by fracturing or by cryosectioning in order to image and analyze the inner part. Cryofractures can

be directly analyzed in the cryo-SEM. However, the freeze-fracture method of preparing botanical specimens has not previously produced good results in studies on element distribution at the sub-cellular level. A resolution limit of about 2 µm is indicated for frozen-hydrated samples. This rather poor spatial analytical resolution is improved considerably in thin specimens such as cryosections, approximately 100 nm thick

Related method sheet

ID	**21_Frey**
Parameter	**elemental analysis of root and mycorrhiza tissue**
Soil type	forest soil
Plant species	spruce, beech
System	field and laboratory material (rhizoboxes, in vitro cultures)
Method	**energy dispersive X-ray microanalysis (EDS)**

2.1.5.2. Electron energy loss spectroscopy (EELS) and imaging (ESI) – microanalytical method

Brief method description

The distribution of elements in biological specimens on the cellular and subcellular level can be determined by electron energy loss spectroscopy (EELS) and electron spectroscopy imaging (ESI) which is a technique exploiting the interactions of the electron beam with the inner shell electrons of distinctive elements (Z = 3-92) and is an extension of the capabilities of a transmission electron microscope (TEM). Root samples collected from the field or from rhizoboxes or pots have to be fixed, dehydrated and embedded in resins. Ultra thin sections supported on grids are observed in TEM and the area for element analysis is selected. Element distribution maps can be obtained that should be supported by the spectra in the chosen areas.

Basic references

Kottke, I. 1994. Localization and identification of elements in mycorrhizas. Advantages and limits of electron energy-loss spectroscopy. Acta Bot Gallica 141:507-510.

Egerton, RF. 1996. Electron energy loss spectroscopy in the electron microscope. New York: Plenum.

Williams, D.B.; Carter, C.B. 1996. Transmission Electron Microscopy. A textbook for materials science. New York and London: Plenum Press, pp 729.

Turnau, K.; Kottke, I. 2005. Fungal activity as determined by micro-scale methods with special emphasis on interactions with heavy metals. In: The Fungal Community. J. Dighton, J.F. White, P.Oudemans (eds.), CRC Press, Boca Ration pp. 287-306.

Orlovich, D.A.; Ashford, A.E. 1995. X-ray microanalysis of ion distribution in frozen salt/dextran droplets after freeze-substitution and embedding in anhydrous conditions. J. Microsc.-Oxford 180:117-126.

Application areas

The method is primarily used to study interactions between plants, fungi and bacteria within the mycorrhizosphere. Several studies were carried out to assess the role of fungi in heavy metal detoxification using field material. The studies carried out on laboratory systems (rhizoboxes or in vitro cultures) are mostly unexplored, although there are possibilities to study even long-distance transport (using La and Ce as markers). In addition, parallel histochemical studies may help to understand the nature of substances involved in element accumulation. The method can be also used to study plant reaction to pathogenic fungi in the rhizosphere.

Problems, constraints, do's and don'ts

Sample preparation is a critical point of using micro-analytical tools on biological material. The material should be properly cleaned and washed in ice cooled water and fixed as soon as possible. Drying/rehydrating and freezing/thawing of soil samples containing fungal hyphae might result in large decrease of metal concentration in the hyphae. Artifacts resulting from faulty preparation are possible to recognize by transmission electron microscopy. The strongest changes occur in senescent and dead cells. Distinguishing between fixation-induced and natural changes usually requires experience. Methods

accompanying the microanalytical tools, such as observation with light microscope accompanied by Nomarski contrast or physiological studies, are vital to avoid misinterpretations. The most adequate protocol to study element distribution seems to be the anhydrous freeze-substitution method (Orlovich and Ashford, 1995).

Related method sheet

ID	**21_Turnau_a**
Parameter	**element localization and distribution in plant material**
Soil type	natural soils and industrial wastes
Plant species	broad range of plants including ferns, liverworts, angio and gymnosperms
System	field and laboratory material (rhizoboxes, *in vitro* cultures)
Method	**electron energy loss spectroscopy (EELS) and imaging (ESI)**

2.1.5.3. Particle induced X-ray emission (PIXE) – a microanalytical method

Brief method description

The distribution of elements in biological specimens at the cellular and subcellular level may be determined by the use of focused protons, instead of electrons, for the generation of characteristic X-rays. This technique is referred to as PIXE (particle induced X-ray emission). Detection of characteristic X-rays generated during the interaction of protons with distinctive elements (elemental range from Na to U)) in a specimen is an extension of the capabilities of a scanning electron microscope (SEM). Quantitative PIXE analysis is a sensitive technique (concentrations down to ppm can be analysed) and benefits from the possibility of simultaneous use of proton backscattering (BS) or scanning transmission ion microscopy (STIM) techniques for matrix corrections and the analysis of lighter elements (C, N, O).

Basic references

Johansson, S.A.E.; Campbell, J.L.; Malmqvist, K.G. 1995. Particle Induced X-ray Emission spectrometry (PIXE). New York: John Wiley & Sons.

Mesjasz-Przybylowicz, J. 2001. The nuclear microprobe – a challenging tool in plant sciences. Acta Phys Pol A 100 (5):659-668.

Mesjasz-Przybylowicz, J; Przybylowicz, W.J. 2002. Micro-PIXE in plant sciences: Present status and perspectives. Nucl. Instr. Meth. in Physics Res. B 189: 470-481.

Turnau, K.; Kottke, I. 2005. Fungal activity as determined by micro-scale methods with special emphasis on interactions with heavy metals. In: The Fungal Community. J. Dighton, J.F. White, P.Oudemans (eds.), CRC Press, Boca Ration pp. 287-306.

Application areas

The method was used to study heavy metal distribution in the mycorhizosphere and differences in metal sequestration within mycorrhizas of different morphotypes, to determine the localization of a broad range of elements including Cl, As, Pb, S, P, Cd, Zn in arbuscular mycorrhizas (AM) and in AM mycelium collected from substratum enriched with Cd or from industrial wastes. It elegantly demonstrates element precipitation at the surface of soil mycelium and in the rhizophere, being also very useful to study the transformation of minerals by fungi, bacteria and plants. It can potentially be applied in studies on nutrition, interactions between elements in the rhizosphere, transport, sequestration and functions of minor and trace elements. Scanned areas are usually 2.5 mm x 2.5 mm in the case of general maps of the whole sections. They might be complemented by analyses of smaller regions of particular interest of any sizes down to the beam spot size (1 um). The possibility of quantitative, precise maps of comparatively large area is the main advantage of the PIXE method over EDX and EELS; however, the higher magnifications obtained with the last two techniques are not available with PIXE, making studies at the ultrastructural level impossible.

Problems, constraints, do's and don'ts

Specimen sampling and preparation are the critical steps in elemental microanalysis. Cryotechnique is the best option among available preparation techniques. Good results in analysis of various types of mycorrhiza were obtained

while the samples were rapidly frozen by plunging them into liquid cryogen (propane, isopentane) cooled by liquid nitrogen and subsequently freeze-dried. Cryosectioning is the most difficult part of the specimen preparation protocol, because of sample heterogeneity. Chemical fixation and embedding of samples should be avoided due to element redistribution and washing out. Quantitative, two dimensional PIXE (particle induced x-ray emission) maps of elemental distribution are obtained.

Related method sheet:

ID	**21_Turnau_b**
Parameter	**distribution and quantitative analysis of elements in plant material**
Soil type	natural soils and industrial wastes
Plant species	broad range of plants including ferns, liverworts, angio- and gymnosperms
System	field and laboratory material (rhizoboxes, in vitro cultures)
Method	**Particle induced X-ray emission (PIXE)**

2.1.5.4. Synchrotron-based X-ray methods

Brief method description

Synchrotron X-ray fluorescence (SXRF) spectroscopy provides *in situ*, highly sensitive, and well-resolved 2D elemental maps and, when coupled with X-ray absorption fine structure spectroscopy (XAFS) is able to determine the elemental speciation. Synchrotron-based X-ray absorption-edge and fluorescence CMT are imaging techniques that utilize a high-intensity, tunable X-ray beam to nondestructively interrogate a sample as it is translated and/or rotated within the beam. The result, after computational reconstruction, is the cross-sectional two- and three-dimensional distributions of specific elements within the sample. Unlike conventional X-ray CMT instruments, synchrotron-based fluorescence CMT can provide a highly resolved picture of the multi-elemental distribution through a virtual slice of the sample at concentrations down to approximately 100 μg g^{-1} (element dependent). Absorption-edge CMT provides a fully 3D image of the metal distribution, albeit, with some loss of sensitivity.

Basic references

Hansel, C. M.; Fendorf, S. 2001. Characterization of Fe plaque and associated metals on the roots of mine-waste impacted aquatic plants. Environ. Sci. Technol. 35: 3863-3868.

Hansel, C. M.; LaForce, M. J.; Fendorf, S.; Sutton, S. 2002. Spatial and temporal association of As and Fe species on aquatic plant roots. Environ. Sci. Technol. 36: 1988-1994.

Howe, J. A.; Loeppert, R. H.; Derose, V. J.; Hunter, D. B.; Bertsch, P. M. 2003. Localization and speciation of chromium in subterranean clover using XRF, XANES, and EPR spectroscopy. Environ. Sci. Technol. 37: 4091-4097.

Keon-Blute, N.; Brabander, D. J.; Hemond, H. F.; Sutton, S. R.; Newville, M.; Rivers, M. 2004. Arsenic sequestration by ferric iron plaque on cattail roots. Environ. Sci. Technol. 38: 6074-6077.

McNear, D.; Peltier, E; Everhart, J.; Chaney, R.L.; Sutton, S.; Newville, M.; Rivers, M.; Sparks, D.L. 2005. Application of quantitative fluorescence and absorption-edge computed microtomography to image metal compartmentalization in *Alyssum murale*. Environ. Sci. Technol. 39: 2210 - 2218;

Pickering, I. J.; Prince, R. C.; Salt, D. E.; George, G. N. 2000. Quantitative, chemically specific imaging of selenium transformation in plants. Proc. Natl. Acad. Sci. U.S.A. 97: 10717-10722.

Scheckel, K. G.; Lombi, E.; Rock, S. A.; McLaughlin, N. J. 2004. In vivo synchrotron study of thallium speciation and compartmentation in *Iberis intermedia*. Environ. Sci. Technol. 38: 5095-5100.

Application areas

The XAFS microprobe (SXRF, XAFS, XRD) provides a nondestructive suite of spectromicroscopic techniques useful for the investigation of molecular speciation, complexation, oxidation state, as well as spatial distribution and associations of elements.

Application of these techniques has included imaging of various phenomena in earth and material sciences. For example, absorption-edge CMT was used to explore the association between cation sorption sites in soils, and Fe and pore-space distributions. There are some applications of synchrotron tomographic techniques to biological systems (e.g. roots), where fluorescence CMT was used to help characterize Fe plaques and associated

metals on the surface of roots from the aquatic plants *Phalaris arundinacea* and *Typha latifolia*. It was shown that Pb and Fe accumulated on the surface of the root in a juxaposed pattern, forming a rind on the root surface while As was isolated to distinct regions on the exterior and interior of the root. Similarly, fluorescence CMT was used to reveal that As was sequestered by Fe(III) oxyhydroxides within cattail root plaques from a contaminated wetland.

Problems, constraints, do's and don'ts

However, because SXRF is 2D and the beam penetrates into or through the sample, the resulting SXRF image is actually a projection showing all of the entrained elements from one specific direction. Therefore, it may be difficult to tell exactly which compartment or specific tissue contains an observed element-specific-rich region. For instance, a face-on view of a root will show elements on both surfaces as well as those in the interior. Thus, a metal could be entrained on one side as well as on the other side of a root tissue, which would appear associated in the SXRF image but are instead separated by the thickness of the root. Therefore, because of these "thickness" effects, determining the compartmentalization of metals using SXRF should be done with caution and if possible, verified using another technique.

Related method sheets

ID	**21_McNear**
Parameter	**Quantitative Elemental Compartmentalization in Plant tissues**
Plant species	Any plant tissue with elemental concentrations exceeding ~100µg g^{-1} DW
System	material from field or laboratory systems
Method	**Synchrotron based X-ray fluorescence and absorption edge computed microtomography (F-CMT and AE-CMT)**

ID	**21_Tappero**
Parameter	***(In-situ)* Elemental distributions, associations, and molecular speciation in plant material**
Plant species	Techniques applicable to plant tissue with element concentration exceeding ~100 mg/g D.W.
System	Material from field and laboratory systems
Method	**Synchrotron X-ray fluorescense (µ-SXRF) mapping and X-ray absorption fine structure (XAFS) spectroscopy**