Biophysical and physicochemical methods for analyzing plants *in vivo* and *in situ* (III):

**X-ray spectroscopy** for localising & quantifying metals and for investigating metal ligands
X-ray spectroscopy

General comments on sample preparation techniques

a) chemical fixation and resin embedding
   → Advantages: over many years best established procedure in many laboratories
   → Disadvantages: Metals will inevitably be re-distributed → ARTEFACTS

b) freeze substitution or freeze drying
   → Advantages: less element re-distribution than in (a)
   → Disadvantages: still at least intracellular (vacuole → wall) re-distribution artefacts inevitable

c) frozen-hydrated tissues
   → Advantages: hardly any element-redistribution → METHOD OF CHOICE!
   → Disadvantages: Required rapid-freeze techniques and cryostage (→ expensive)

e) non-frozen fresh tissues
   → Advantages: NO preparation necessary, “in vivo” situation
   → Disadvantages: Strong beam damage → MORE artefacts than in (c)!
(1) X-ray emission spectroscopy
(a) Energy Dispersive X-ray Analysis (EDXA)
Use of an electron microscope as an X-ray spectrometer
Signals generated in the **scanning electron microscope (SEM)**

- **characteristic x-ray photons**
- **incident electron beam**
- **backscattered electrons**
- **secondary electrons**
- **Bremsstrahlung**
- **visible light (cathode luminescence)**
- **specimen**
- **absorbed electrons**
- **elastically scattered electrons**
- **transmitted and inelastically scattered electrons**
Principle of Energy Dispersive X-ray Analysis (EDXA)

Principle of Particle Induced X-ray Emission (PIXE)
The origin of the different lines in an EDXA spectrum
Analysis of EDXA spectra

peaks of characteristic x-ray photons

spectral window

continuous background of bremsstrahlung

Analysis: 

a) recording of complete spectrum, subtraction of background --> quantification of peak areas by comparison to internal standard

b) recording of counts in spectral window --> dot maps, line scans
Detection limits of EDXA

- $E_0$
- 15 keV
- 20 keV
- 30 keV

$m_{\text{min}} \quad 10^{-12} \text{g}$

(20 keV; 3 g/cm$^3$)

$C_{\text{min}}$ %

$Z=26 \quad \text{AW}=35^\circ \quad \text{KW}=55^\circ$

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5 10 20 30 40 50 60 70 80 90

K L M
Methods of plant analysis using EDXA
Sampling of single-cells saps with micropipettes

- Micropipette filled with silicon oil, connected to air-filled syringe for controlling pressure difference
- Turgor pressure of punctured cell fills pipette with 5-20 picolitres ($10^{-12}$ l) of cell sap

Sample preparation:
1) Transfer to storage grid, addition of internal standard (e.g. RbF) and matrix (e.g. mannitol)
2) Transfer to analysis grid, drying with isopentane

Analysis:
1) Recording of EDXA spectra in SEM
2) Data processing

Typical dried sample

Methods of plant analysis using EDXRA
Quantification of elements in single-cells saps

1) net peak area is normalised by internal standard (an element not naturally present in the sample, e.g. Rb)
2) ratio obtained from 1) is quantified using calibration curve

Evaluation of the method
Advantages:
- potentially very accurate
- enables measurement of small concentrations

Disadvantages:
- only few types of cells are accessible to sampling with micropipettes
- risk of preparation artefacts
- no distinction between cytoplasm and vacuole, measurement of cell walls impossible
- very difficult to obtain information about heterogeneity of element distribution inside the analysed tissue

Methods of plant analysis using EDXA
Freeze-fracturing

Excise sample from plant, mount in/on stub or vice. The EDXA spectrum of the vice must not interfere with that of the sample!

Freeze the sample in melting nitrogen slush, transfer to cooled \((-170^\circ C)\) preparation chamber

Fracture sample with fast-moving blade (to cut rather than break the cells)

Produce conductive sample surface by evaporating carbon wire

Transfer to cooled \((-150^\circ C)\) sample stage in SEM, analyse

Methods of plant analysis using EDXSA
Analysis of bulk-frozen samples

**Effect of shading**
- Shading inside a sample leads to absorption of low-energy x-rays

**Dot -map of O Ka line (0.6)**

**Normal x-ray spectrum**

**X-ray spectrum in shadow of trichome**

**Effect of acceleration voltage**
- High acceleration voltage leads to deeper penetration into the sample!
Methods of plant analysis using EDXFA
Qualitative and semi-quantitative analysis of bulk-frozen samples

**Line scans**
Scan of the Zn K alpha line (0.6x half width) along the straight line. Amplitude represents the counts/s inside the selected spectral window.

**Dot maps**
Scan of the Zn K alpha line (0.6x half width) over the whole image. Each dot represents one x-ray count inside the selected spectral window.

Küpper H, Lombi E, Zhao FJ, McGrath SP (2000) Planta 212, 75-84
EDXA imaging application example:
Ni silicate accumulation in cell walls of Berkheya coddii

Dot maps (Kα lines) of the upper side of a Berkheya coddii leaf. Quantitative relation between Si and Ni in metal accumulation spots: 3.5 (± 1) Si / Ni (P = 0.0055)

EDXA
Quantitative analysis of bulk-frozen samples

Counts in spectra (A) can be normalised to either the background (B) or an internal standard. The oxygen Kα line has proven to be a reliable internal standard in bulk-frozen samples, in particular in aqueous compartments like vacuoles (C).

Küpper H, Lombi E, Zhao FJ, McGrath SP (2000) Planta 212, 75-84
EDXA quantification application example: Al accumulation in epidermal cell walls of tea (*Camellia sinensis*)

Young leaves

Old leaves

Electronoptic picture of an old *C. sinensis* leaf (upper epidermis) and dot map of the Al K α line

Evaluation of the freeze-fracturing method

Advantages:
- All types of cells and tissues can be analysed
- *In situ*-analysis with very little risk of preparation artefacts
- Easy analysis of the heterogeneity of element distribution, by use of dot-maps even in an imaging way

Disadvantages:
- Limited sensitivity (min. 1mM) and accuracy (shading)
- Elements in dead tissues with low water content cannot be reliably quantified
(1) X-ray emission spectroscopy
(b) Proton induced X-ray emission (PIXE) imaging

Imaging of potassium, calcium and nickel in a leaf of Hybanthus floribundus

→ more sensitive than EDX, but no observation of frozen-hydrated samples (samples have to be freeze-dried) → increased risk of artefacts
(1) X-ray emission spectroscopy
(c) X-ray fluorescence imaging (XRF)

Imaging of Fe, Mn, and Zn in seeds of *Arabidopsis thaliana*

→ MUCH more sensitive than EDX and PIXE, but reliability of quantification less tested, and influence of damage caused by the intense synchrotron µ-beam less known
(2) X-ray absorption and fluorescence spectroscopy
Where it is done...
How Synchrotron radiation is generated

DESY

Bending Magnet

Wiggler

Undulator

Free Electron Laser
• Electrons in atoms are arranged in shells with different atom-specific binding energies: K, L, M

• Atomic electron can absorb x-rays if:

\[ E_{\text{photon}} > E_{\text{ionization}} \]

(Pauli-principle)
X-ray absorption (II)

$\mu(E)$: linear absorption coefficient

- mainly atomic effect
- strong dependence on energy: $\propto E^{-2.78}$
- strong dependence on atomic number: $\propto Z^{2.7}$
- inner shell electrons contribute most strongly
XAS techniques
What can we learn from XAS?

Three different characteristics:

- **edge position:** oxidation state
- **near edge spectrum (XANES):**
  - local projected density of states
- **extended fine structure (EXAFS):**
  - local neighborhood of atomic species
Example of what can we learn from XANES (I)

Edge is shifted to higher energy with increasing formal valence:

- Cu
+ Cu(I)$_2$O
* Cu(II)O
# KCuO$_2$
Example of what can we learn from XANES (II)

Finger print for chemical state of element of interest:

determine concentration of chemical compounds in mixtures

- Cu
+ Cu(I)$_2$O
* Cu(II)O
# KCuO$_2$

example: inhomogeneous specimens
Principle of Extended X-ray Absorption Fine Structure (EXAFS)
Principle of single vs. multiple scattering contributions in EXAFS
Effects of single vs. multiple scattering contributions in EXAFS

\(\mu(E)\): linear absorption coefficient

Metallic Cu:
Preparation of plant material for XAS (EXAFS and XANES)

Excise sample from plant

Freeze the sample in melting nitrogen slush

grind sample in mortar cooled by dry ice

fill the still frozen-hydrated powder into an EXAFS cuvette, seal with Kapton tape

The EXAFS spectrum of the cuvette must not interfere with that of the sample!

Transfer to cooled (20 K) sample holder of beamline, analyse

Analysis of EXAFS data (I)
Analysis of EXAFS data (II)
Cd-ligands: model compounds

Cd in hyperaccumulator leaves

Application example: Speciation of cadmium and zinc hyperaccumulated by *Thlaspi caerulescens* (Ganges ecotype)
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