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Micro-PIXE in plant sciences: Present status and perspectives

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Abstract

Fundamental processes of plant physiology are affected or regulated by mineral nutrients. Hence understanding the mechanisms of nutrient uptake and their functions in plant metabolism is of fundamental importance in both basic and applied plant studies. The present knowledge of ion uptake mechanisms is based mostly on techniques for bulk analysis, including analysis of small (mg-sized) samples but without spatially resolved results. On the other hand, advanced studies of elemental transport at a cellular level are conducted using techniques with high and very high spatial resolution, but with low sensitivity for elemental analysis. Thus the results obtained are usually restricted to macronutrients or elements present in high quantities. There is a high demand for studies of the functions of trace elements. In addition, it is known that, depending on their concentrations, elements can play different roles in plant life. Studies related to elemental deficiency and toxicity, as well as environmental pollution, require accurate, fully quantitative methods with good spatial resolution. Ideally, these studies should be conducted on organs and tissues as far down as the cellular level. This is where micro-PIXE has been applied until present and can in the near future play a much more important role. Progress is subject to closer collaboration between plant biologists and the PIXE community in terms of addressing problems of specimen preparation, refinement of analytical protocols such as quantitative elemental mapping and the interpretation of results. © 2002 Published by Elsevier Science B.V.

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1. Introduction

There are many aspects of plant studies in which knowledge on concentration and distribution of elements is required. Studies of the role played by particular elements in fundamental physiological processes, plant nutrition, micronutrient deficiency and elemental toxicity, eco-physiological studies documenting responses of plant tissue to environmental stress caused by pollution – all belong to this category. Depending on relative concentrations, elements present in plants can be classified as deficient, adequate and finally toxic when certain concentration levels are exceeded. The required levels of micronutrients are by no means permanently fixed, but can vary depending on many factors such as plant species, genotype and growth conditions, and between different

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organs and tissues of the same plant. From this point of view, the need for quantitative microanalytical techniques of elemental analysis cannot be over-emphasized.

Fundamental processes of plant physiology are affected or regulated by mineral nutrients (also referred to as *essential mineral elements*) [1]. Hence understanding the mechanisms of nutrient uptake and their functions in plant metabolism is of fundamental importance in both basic and applied plant studies. Currently, there are 17 elements known to be required by all higher plants [2]. Nine of them are macronutrients (C, H, O, N, K, Ca, Mg, P and S), i.e. are normally present in plant tissues at concentrations above 0.1% (dry weight). An additional eight elements are defined as micronutrients (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn) since these are required by plants in much smaller concentrations (less than 100 mg/kg dry weight). Other elements may be essential for certain plant species or for some species grown under specific environmental conditions [3]. For example, it has been reported that Si is beneficial to a wide variety of higher plants, since this element improves resistance to fungal diseases and insect damage or increases tolerance to Mn toxicity [3]. However, present knowledge does not justify adding Si to the list of essential elements for all higher plants [2].

It is interesting to note that plant scientists have added only one new micronutrient (Ni) to the list of essential elements since 1954, when chlorine was added [1,2]. At the same time animal scientists added many more trace elements to the respective list of elements essential in organisms (As, B, Br, Cd, Cr, Pb, Li, Mo, Se, Si, Sn, V and Ni) [3]. This shows the potential for further studies with practical implications in applied sciences – agriculture and horticulture – and possibilities of classifying more elements as micronutrients than known at present.

Eco-physiology is another growing research area where high demand exists for methods documenting responses of plant tissue to environmental stress, due to factors present in the natural environment and caused by anthropogenic pollution. Knowledge of localisation and quantification of toxic elements in plants indicate possible pathways of transport and mechanisms of detoxification and can assist in understanding plant adaptation in hostile environment.

Particle-induced X-ray emission with the use of focused ion beams (micro-PIXE) is an ideal tool for these types of studies. All elements from the list of macro- and micro-nutrients can be measured by either micro-PIXE or complementary techniques used simultaneously in a nuclear microprobe facility. The same applies to elements considered as toxic and constituting environmental pollution. The sensitivities are down to the part-per-million (ppm) level in areas of particular interest, along selected profiles and down to a few tens of ppm in single pixels of true elemental maps (i.e. free of artefacts related to the analytical method) that can be quantitative. Maps of elemental distribution provide information not possible to obtain using point analyses or linear scans. This makes the use of micro-PIXE, a relatively expensive facility and sometimes difficult to access by plant scientists, fully justified.

Micro-PIXE has been used in plant science applications since its early days [4–6]. Most of the work done in this field previously has been presented in the review by Przybyłowicz et al. [7]. Here we try to emphasize some aspects of the present and future use of micro-PIXE in plant sciences.

2. Analytical techniques with no spatial resolution

Using highly sensitive techniques for bulk elemental analysis is usually the first, and often the only, step in elemental analysis related to plant science. Many techniques are available such as atomic emission, atomic absorption and atomic fluorescence spectrometry (AES, AAS and AFS), inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS) and other variants of atomic mass spectrometry as well as total reflection X-ray fluorescence (TXRF). They usually require small amounts of material (mg-sized samples). Macro-PIXE belongs to this category [8]. Some of them are able to determine the chemical species of the element analysed, which is a distinct advantage since mechanisms of micronutrient

uptake depend critically on the cationic forms of elements studied. The only possibility of tracing elemental distribution and transport is through an appropriate selection of specimens. More refined studies require the use of microanalytical techniques.

3. Specimen preparation

The aim of specimen preparation is the preservation of elemental distribution as close as possible to its native (in vivo) state. This applies to all microanalytical techniques. Proper specimen sampling and subsequent procedures/preparations are crucial parts of microanalysis, and according to the present state of knowledge, only cryotechniques are recommended. The description of preparation procedures can be found elsewhere [9–11].

Nuclear microprobe analyses are performed under vacuum conditions (of the order of 10^{-5} mbar). Therefore a specimen must be dry and immobilized in its functional state prior to analysis. The exception is analysis in air or in helium atmosphere, where an external ion beam is used. In such case, no special preparation is required.

The comparison of known schemes of specimen preparation is presented in Fig. 1. The most recommended scheme is scheme I, in which cryotechniques are used. Scheme III presents procedures involving chemical preparation. This invasive procedure is generally based on fixations with aldehydes, staining with heavy metal salts, dehydration with alcohol and embedding in plastic resin at ambient or higher temperatures (as in an oven) for a longer time. It is evident that with chemical preparation some elements are added or washed out or redistributed.

Scheme II represents a compromise between schemes I and III. It should therefore be applied with caution.

The final result will depend on the tissue sampling procedure used. Several artefacts can be produced during sampling. It is important to minimise time between sampling and cryofixation. The ideal situation would be to combine sampling and cryofixation in one step using e.g. cryopliers, a cryobiopsy needle or a cryopuncher [12–14].

Comparisons of conventional preparation and cryotechniques have already been presented in several publications.

It is important to stress that there is very limited experience with cryomicrotomy of non-meristematic plant tissues in comparison with animal and human tissues due to the structural heterogeneity of plant cells (tough cell walls compared with virtually structureless vacuoles). It is an extremely difficult task but it is possible and a few groups have achieved success in this field [15–18].

Purity at all stages of sampling and preparation is required because of the high sensitivity of the PIXE technique.

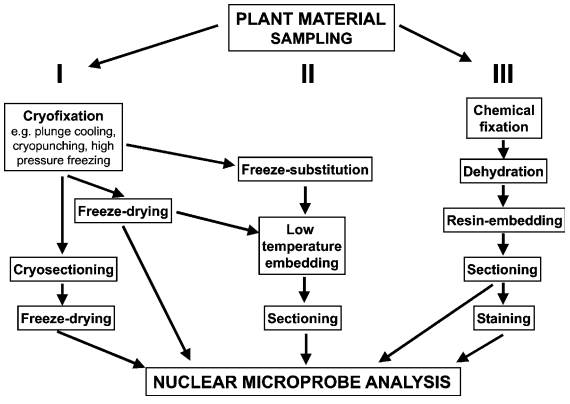

Fig. 1. Scheme of different types of preparation protocols.

4. Present status of micro-PIXE

Micro-PIXE allows quantitative studies of elemental distribution with lateral resolution of the order of 1 μm for elements from Na to U and very good scanning capabilities. A proton beam is most often used and therefore a nuclear microprobe is also referred to as a proton microprobe.

4.1. Spatial resolution

The best proton beam spot size measured at a beam current of 100 pA is 400 nm [19]. However, most research related to plant science is carried out using beam spot sizes of the order of a few μm.

Although this beam spot increase is related to experimental conditions in many laboratories (e.g. older accelerators, beam line parameters not fully optimised), such lateral resolution is still perfectly adequate for studies in plant sciences including measurements of single cells. Sometimes the limits of the maximum size of scanned areas become more important than the beam spot size when larger structures are analysed. Typically, areas up to 2.5 mm × 2.5 mm can be analysed, with some systems capable of scanning larger structures, but sizes exceeding 10 mm require specimen movement. The depth resolution of PIXE analysis is poor as is for almost all techniques based on characteristic X-rays. Effective depth of analysis depends on proton energy and the energy of X-ray line(s) used to quantify an element. However, due to the porous structure of most biological specimens, it is risky to attribute any fixed value of depth of analysis for a particular element that could even exceed 100 μm.

4.2. Complementarity of techniques

Simultaneous use of at least two analytical techniques is a common practice. The most important benefit in plant science studies is that an assessment of specimen thickness and composition of major elements is possible and easily accomplished. This is a notable advantage not only over less sensitive techniques such as energy dispersive spectrometers (EDSs) coupled with scanning electron microscopes (SEMs) or scanning transmission electron microscopes (STEMs) but also over more sensitive techniques such as synchrotron radiation-induced X-ray emission (SRIXE) or ion microprobe (secondary ion mass spectrometry, SIMS). Preparation of biological standards matching composition and thickness of analysed specimens is a well-known problem, particularly in EDS analysis. The necessity of using biological standards, usually difficult to prepare and with changing composition under beam exposure, is thus eliminated.

For thick specimens, the use of backscattering spectrometry (BS) is the only choice. This technique allows quantification of light elements forming the organic matrix (C, O, N) and measurement of specimen areal density. Software used for simulation of spectra should take into account non-Rutherford cross-sections for proton scattering from C, N and O and corrections for non-homogeneous thickness distribution in the scanned area [20]. For specimens fully penetrated by the proton beam, target thickness can be determined using the energy loss contrast scanning transmission ion microscopy (STIM) method [21,22]. In such cases sample composition cannot be determined independently, and an average composition of biological tissue ($C_5H_9O_2N$) is assumed [21]. The STIM signal can also be used for sample localisation. This is especially useful for very thin, unstained specimens, difficult to view with a light microscope.

Proton-induced gamma-ray emission (PIGE) can detect some selected light elements not accessible with PIXE – F, Li, B – with limits of detection below 100 mg/kg.

4.3. Quantitative analysis

Normalization of analysis is obtained by direct measurements of accumulated charge deposited by ions or a value proportional to it. Sometimes deposited charge is obtained from BS spectra (the so-called *Q*-factor method) [23].

Matrix parameters (composition and thickness/areal density) are used by software processing PIXE spectra. All necessary procedures are well developed and weak points are known. Accurate quantitative analysis at sensitivities down to mg/kg (ppm) is possible in points, line scans and maps. Simultaneous quantitative studies of major, minor and trace elements are possible.

4.4. Radiation damage

Radiation damage is not negligible. Analysis of biological specimens should be performed using beam currents lower than those used for solid materials. For proton beams a current of ca. 100–200 pA/μm^2 seems to be a reasonable limit above which specimen damage becomes excessive. Using alpha particles may result in complete destruction of a specimen or the thin organic foil (Formvar, pioloform) on which it is mounted.

It is possible to correct for mass losses and an appropriate model of mass losses has been developed [24,25]. However, this requires additional measurements using the STIM technique. Beam currents required for studies using nuclear reactions result in excessive specimen damage and elemental losses [26,27]. Therefore elemental analysis based on nuclear reactions is seldom used in botanical applications.

4.5. Geometry of measurements

The best X-ray detection efficiency is needed in order to minimise beam damage of a specimen. More than one X-ray detector is recommended (and as close as possible to the specimen) to maximise the solid angle. A second X-ray detector may also be recommended for another reason. Crystals of typically used Si(Li) or HPGe detectors need to be shielded from protons backscattered from a specimen by using an absorbing layer of adequate thickness. Neglecting this usually perturbs the electronic system as evidenced by shifting of the signal baseline and deterioration of energy resolution. For typically used proton energies of 2.5–3 MeV, the side effect of using a layer of the required thickness is total absorption of X-rays of Na, Mg and Al. As these elements are vital in plant studies, the alternative is to either lower the proton beam energy [28,29], to use the magnetic deflector of backscattered protons [30] or to use another X-ray detector further from the specimen and with no additional absorbing layer. A possible compromise is to use an absorber with a small hole(s) to reduce the number of protons hitting the detector crystal and to allow simultaneous measurements of higher-energy X-rays and X-rays from Na, Mg and Al at a reduced count rate.

4.6. Data acquisition

Listmode (event-by-event recording) is the preferred choice of data acquisition. Time information saved simultaneously can be used for tracing possible beam damage. This allows for extraction of PIXE and RBS (or STIM) spectra from any part of the measured region. Defining borders (for example within selected plant parts) is usually very difficult or impossible before analysis.

4.7. True elemental mapping

Very easy beam steering results in unique capabilities of two-dimensional elemental mapping of major (using BS), minor and trace elements at once (using PIXE). High scanning speeds are recommended to minimise specimen damage (mostly due to heat deposited by the beam). Easy construction of elemental maps at sensitivity levels matching concentrations of micronutrients in plant parts or required in toxicity studies opens up opportunities not fully appreciated at present. However, full exploitation of this option is only possible with true elemental mapping (free of artefacts related to the analytical method) such as the *Dynamic Analysis* method or a related one [31–33].

4.8. Conditions of analysis

Analyses are typically performed in a vacuum of the order of 10^{-5} mbar. Thick specimens need to be coated with a conductive layer (carbon). Some very thin specimens do not require coating as the beam penetrates through them and there is no charge build-up on a specimen. Using an external beam allows analysis of specimens in air or in helium atmosphere. However, this option is seldom used as the beam spot size drastically deteriorates and is of the order of a few tens of μm.

5. Other microanalytical techniques

No other microanalytical technique can at present provide quantitative elemental maps at sensitivities offered by micro-PIXE. This is because data processing used by some nuclear microprobes is superior than those used by more sensitive techniques.

Micro-PIXE cannot compete with the spatial resolutions achievable by facilities based on electron microscopy. EDSs coupled with STEMs enable measurements at spatial resolutions of the order of 20–50 nm and those coupled with SEMs

usually allow for routine measurements with resolutions well below 1 μm. On the other hand, this resolution may drastically deteriorate for thicker specimens and become comparable with resolutions achievable with protons. Electron energy loss spectroscopy (EELS) is another technique coupled with SEMs or STEMs used for elemental analysis. This is sometimes the only technique available for analysis of very thin specimens, impossible to measure with techniques requiring higher electron beam currents [34]. Quantification is difficult. Information on the chemical state of a detected element is possible from energy loss near edge structures (ELNES) in the spectra [35]. Both STEMs and SEMs can be equipped with cold stages that allow analysis of plant material in a frozen state, thus preserving elements in the original in vivo locations. However, analyses are restricted to major and at best minor elements. In addition, claims that "EDAX will give much better elemental maps than PIXE when the elements are present at reasonably high concentrations" [36] are not justified. Providing the same parameters of software are used for image processing, micro-PIXE will give elemental maps of inferior spatial resolution but with higher signal-to-background ratio, which is a crucial factor in this case.

Rapid development of fluorescent dye staining techniques, coupled with confocal laser microscopes, is another type of analysis that becomes very popular in plant science studies. Techniques based on electron or confocal microscopy offer possibilities of structural observation – a natural first step in studies at a cellular level – and parallel elemental analysis. In addition, these facilities are commercially available.

The major advantage of ion microprobes (SIMS) is high sensitivity to all elements and isotopes. However, quantification for specimens composed of many elements with varying ratios of major components is difficult. It is interesting to note that some elements difficult to measure with SIMS (Cr, Zn, Cd) do not present a problem for PIXE and both techniques may to some extent become complementary [37].

SRIXE (synchrotron radiation-induced X-ray emission) is a less accessible method. Synchrotron X-ray fluorescence microanalysis (SFXMA) is much less destructive and more sensitive than PIXE but lacks additional techniques able to ascertain the composition of major elements in a specimen and determine its thickness. Besides, data acquisition systems available at present do not permit true elemental imaging. Its main strength lies rather in XANES (X-ray absorption near edge structure) and EXAFS (extended X-ray absorption fine structure) analysis providing the chemical state of elements present at trace levels [38–40].

Micro-PIXE using an external beam faces competition from table-top μ-XRF systems, based on X-ray tubes and focusing of X-ray by microcapillaries, already commercially available. Some applications in botany have been reported [41,42].

6. Selected examples of applications

Since the last botanical overview [7] some more results have been published. These include plant responses to environmental stress caused by unbalanced nutrient composition in phytoplankton [43,44], salinity [29,45–51] and heavy metals (in lichens [52,53], mycorrhizas [54–58], tree rings [59], seeds [60–64], seagrasses [65] and plants accumulating heavy metals [66–68]). Physiological studies were conducted on Si [69], Al [70], Ca [71] and Fe [72]. A rather unusual application was a study of reed material used as mouthpieces of musical instruments [73].

Four selected examples of applications in physiology, agriculture and plant responses to hostile polluted environments are presented. All results were obtained using the nuclear microprobe at the iThemba LABS, Somerset West, South Africa. Quantitative elemental maps were generated using 3 MeV protons and the *Dynamic Analysis* method of imaging. All specimens were cryofixed, freeze-dried, mounted on Formvar films and carbon coated. More detailed description of analytical procedures can be found elsewhere [74].

6.1. Plant resistance to pathogen infection

Disease resistance in plants is often expressed as a hypersensitive response (HR), which is a rapid,

localised death of plant cells associated with the restriction of pathogen growth.

A resistant genotype of a wild plant (*Lagenaria sphaerica* (Sonder) Naudin, Syn. *Lagenaria mascarena* Naudin) belonging to the Cucurbitaceae was inoculated with a foliar fungal pathogen (*Sphaerotheca fuliginea* (Schlecht. ex Fries) Poll [75,76].

The accumulation of Ni, Cu, Zn, Mn, Fe, Ca, Ti, As and Sr as well as drastic depletion of macronutrient levels (P, S, Cl, K) occurred in the invaded region during the first four days.

Six days after inoculation, necrotic cells with high levels of Mn (Fig. 2) surrounded the lesions. Si deposition in concentrations of up to 23 wt.% occurred after accumulation of most metals in the fully necrotic lesions. Changes of elemental distribution with time suggest that metal accumulation to phytotoxic levels may contribute to death of infected cells, and that subsequent Si accumulation provides an impermeable, non-toxic barrier protecting the surrounding uninfected tissue. The enrichment of two selected, potentially phytotoxic, elements (Mn, Ni) as well as macronutrient (Ca) and the depletion of macronutrient (K) is shown in Fig. 2.

6.2. Quantitative elemental distribution in Lupinus angustifolius L. (Fabaceae) after treatment of seeds with molybdenum

This is an example of micro-PIXE application in agriculture [77–79]. Quantitative elemental distribution was studied in *Lupinus angustifolius* L. (Fabaceae) after treatment of seeds with molybdenum. Mo is a co-factor for a few enzymes, amongst which nitrogenase and nitrate reductase are the most important. In general, soils contain sufficient Mo to sustain normal plant growth, but this element is less readily available at low pH.

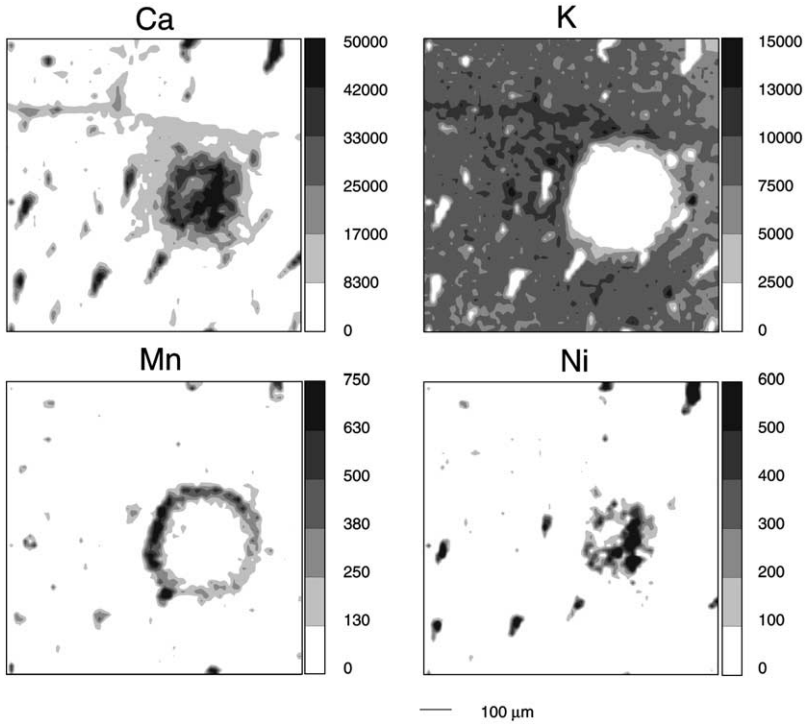

Fig. 2. Quantitative maps of Zn, Mn and Ca in 6 day lesion caused by *Sphaerotheca fuliginea* on resistant *Lagenaria sphaerica* (Cucurbitaceae) leaf. Scale of intensity in mg/kg dry mass. Scan size 920 μm × 920 μm.

There are ways to ensure its availability under such conditions, the most popular of which is lime application. However, it is costly and a much more economical method is to directly apply Mo to the seeds before sowing. In high concentrations toxic symptoms may occur.

Some specific regions of Mo and other main and trace elements enrichment were identified in the treated plants. In the leaf it was found mostly in veins, and some veins were preferred to the others. This is visible in Fig. 3, which shows Mo enrichment only in the central vein, whereas in the remaining two veins and in the other parts of the leaf, the concentration is similar. Zn and Mn are enriched in veins, but only in those which do not show Mo enrichment. Ca is depleted in all three veins. The impact of Mo on the distribution of other elements was clearly shown. The behaviour of some of the essential elements (Ca and Cl) as well as the important co-enzymes (Zn, Mn and Fe) was visibly affected. Elemental interactions are well known in plant nutrition research, but the studies of elemental distribution described here gave direct and precise information about these interactions. This helped in identification of elements on which Mo administration had the highest impact, and created a two-dimensional picture of such an impact.

6.3. Distribution patterns of the metal pollutant nickel in soybean (Glycine max (L.) Merrill (Fabaceae)) seeds

This is an example of micro-PIXE application in agriculture and environmental toxicity studies [63,64].

Seeds, especially cereals and grain legumes, form a vital component of the human diet. As a result of elevated environmental pollution, seed-bearing crop plants are grown increasingly on contaminated soils. Despite this, little research has been conducted on the effect of pollutants (specifically metals) on seed development and physiology. A research project was therefore initiated to investigate the effect of Ni on soybean seeds. An important aspect of this study was localisation of these metal pollutants within the seed tissues.

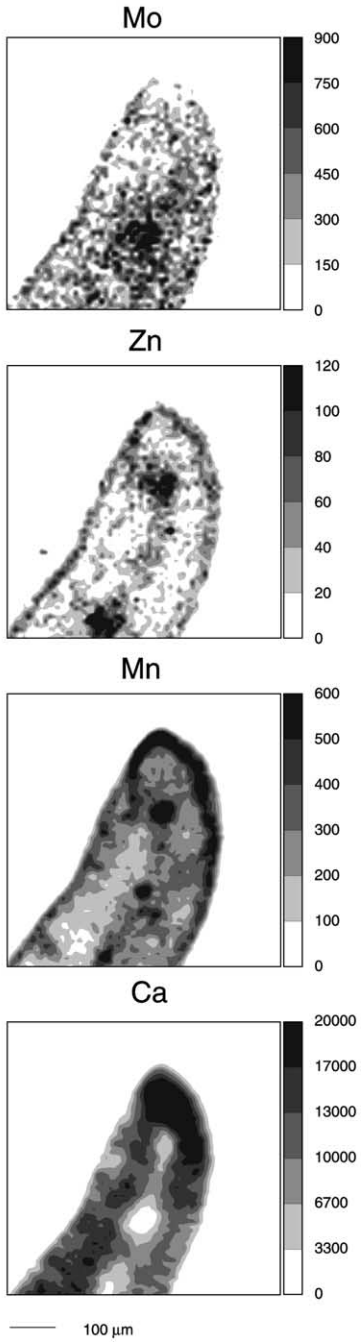

Fig. 3. Quantitative maps of Mo, Zn, Mn and Ca from the leaf tip cross-sections of *Lupinus angustifolius* L. Mo-treated plant. Scale of intensity in mg/kg dry mass. Scan size 600 μm × 600 μm.

Soybean seeds were grown in nutrient solution. In the case of metal-treated plants, the nutrient solution was amended with either 0.05 ppm Cd^{2+} or 1 ppm Ni^{2+}. Seeds from these plants were harvested and the distribution of Ni within the tissues was investigated. Average concentrations for separated seed parts were obtained by using ICP-AES, and the nuclear microprobe was used for elemental mapping. Results were compared with those of control seeds harvested from plants that had not been subjected to metal pollutant stress.

Results using PIXE were found to agree with those obtained with ICP. Nickel was located primarily in the radicle of the embryo axis with levels of up to 100 ppm being recorded. Fig. 4 shows Ni mapping of the radicle tip and from this it can be seen that this metal is concentrated specifically in the tissues of the cortex. It is largely absent from the root cap and central vascular area. The control seeds did not show a comparable metal distribution pattern for Ni. The reason for the distinctive accumulation of Ni in the radicle cortex is not known. Despite the accumulation of metal pollutants, no damage was detectable at the ultrastructural level; nor was the germination potential of the seeds compromised.

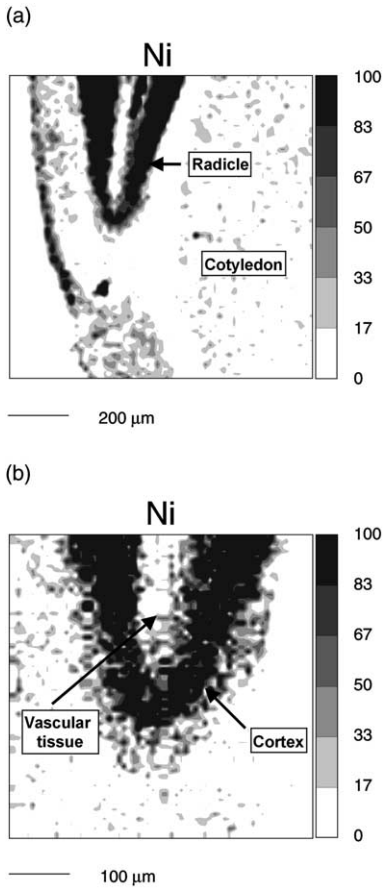

Fig. 4. Quantitative maps of Ni in the seed of *Glycine max* (L.) Merr. Scan size 1 mm × 1 mm (a). Radicle tip enlarged. Scan size 0.5 mm × 0.5 mm (b). Scale of intensity in mg/kg dry mass.

6.4. Comparison between Zn distribution in seeds from a zinc dump in Olkusz, Southern Poland

Silene vulgaris (Moench.) Garcke and *Gypsophila fastigiata* L. (Caryophyllaceae) are the common species of zinc dumps flora in the Olkusz area in the southern part of Poland [60,61]. The concentration of Zn in the soil is about 40 000 mg/kg and around 6000 mg/kg is in a dissolved form. Plants growing on such a polluted substrate have different adaptation mechanisms to this chemically hostile environment. Zn allocation in selected seeds was therefore investigated for a better understanding of the physiology of adaptation.

PIXE microanalysis shows that Zn is accumulated in certain regions, implying that selective exclusion of metals occurs during seed development (Figs. 5 and 6). These results indicate a different adaptation strategy in the anatomically similar seeds from the same area. Zn is relatively homogeneously distributed throughout the *Silene vulgaris* seeds, with slightly higher amounts in the hilum, testa and endosperm. *Gypsophila fastigiata* seeds accumulated higher amounts of Zn and the distribution pattern shows significant differences. The highest concentration is found in the hilum area and in the radicle. The distribution of other heavy metals does not follow the Zn pattern – it is specific for Zn only. Results suggest that the adaptation strategy is specific for a heavy metal and also for a taxon or a genotype. It is possible that plants strictly and actively select elements and amounts, which are absorbed in different tissues, and this mechanism allows survival. In future investigations genotypes from different locations outside Zn dumps will be compared.

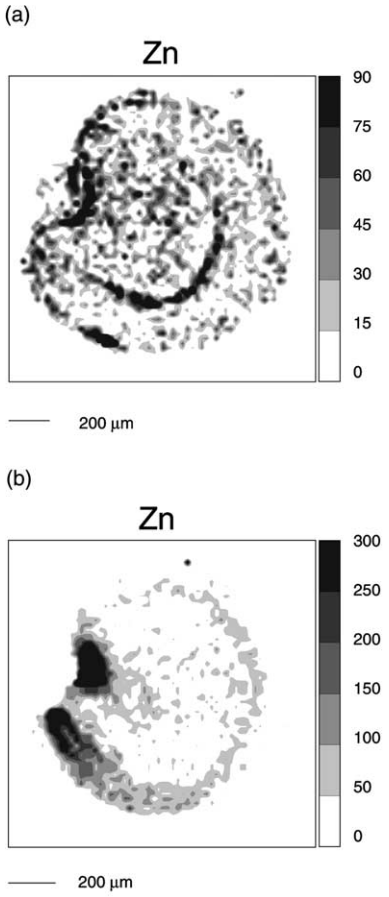

Fig. 5. Quantitative maps showing distribution of Zn in the seed of *Silene vulgaris* (Moench.) Garcke (a) and *Gypsophila fastigiata* L. (b) from a zinc dump. Scale of intensity in mg/kg dry mass.

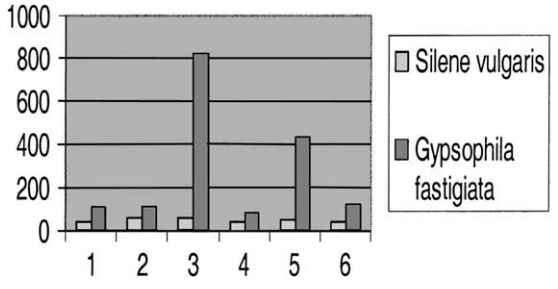

Fig. 6. Average Zn concentrations (mg/kg dry mass) in different parts of seeds analysed by micro-PIXE. 1: Whole seed; 2: testa; 3: hillum area; 4: perisperm; 5: radicle; 6: cotyledon.

7. Conclusions

Micro-PIXE is an important tool for quantitative investigations of trace elements and their interactions with other elements. Imaging capabilities are particularly attractive. However, the number of applications in plant sciences is not high. The possible reasons are:

1. In comparison with bulk techniques and electron microprobes this is a more expensive and less readily available technique. Technically, the nuclear microprobe is not an easy instrument to operate by a novice. Usually systems using micro-PIXE are developed individually at every laboratory and only a few of them have been developed with biological applications as the main aim. Therefore part of these facilities cannot be utilized for plant science studies.
2. Preparation of plant specimens is difficult. There are many related technical problems. In particular, cryosectioning of plant material is very difficult due to the heterogeneity of plant cells. On the other hand, sampling of human or animal tissues like brain is also very complicated and challenging.

When planning research in this field, an improvement in both areas is advisable. In particular, the new generation of accelerators will probably enable investigations at the ultrastructural level thanks to reduction of beam size. Construction of a cold stage should be contemplated to allow analyses in the frozen hydrated state. Close collaboration between physicists and specialists from plant sciences is the key to success in this multidisciplinary research.

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