Utilization of Laser-Assisted Analytical Methods for Monitoring of Lead and Nutrition Elements Distribution in Fresh and Dried *Capsicum annuum* L. Leaves

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KEY WORDS LIBS; LA-ICP-MS; elemental mapping

ABSTRACT Laser induced breakdown spectroscopy (LIBS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) have been applied for high-resolution mapping of accumulation and distribution of heavy metal (lead) and nutrition elements (potassium, manganese) in leaves of *Capsicum annuum* L. samples. Lead was added in a form of $Pb(NO_3)_2$ at concentration up to 10 mmol L^{-1} into the vessels that contained tap water and where the 2-months old *Capsicum annuum* L. plants were grown another seven days. Two dimensional maps of the elements are presented for both laser-assisted analytical methods. Elemental mapping performed on fresh (frozen) and dried *Capsicum annuum* L. leaves are compared. *Microsc. Res. Tech. 74:845–852, 2011.*

INTRODUCTION

In the recent years, the effort to develop and utilize methods that enable to detect contamination by toxic elements and compounds has increased, due to high mobility of the heavy elements and their ability to get into the animal and human body through the food chain. The content of heavy metals in soil, water, or biological tissues is increasing mainly in industrially developed countries (Liu et al., 2007). The mostly dangerous and for this reason also intensively tracked elements are Pb, Cd, Ag, Hg, As etc.

One of the most "natural" ways to subtract hazardous compounds or elements from soil or water is by using plants with high uptake ability. This process is called phytoremediation (Fig. 1). The aim of phytoremediation is to achieve maximum accumulation of potentially toxic compounds to hyperaccumulator plants (Krämer, 2005). Several authors deal with a different kind of remediation technologies (Chen et al., 2000; Mulligan et al., 2001). It was shown, that the concentration and form of the toxic metal in the soil solution affect the amounts of metal absorbed by a plant (Patra et al., 2004).

Finding proper plant species for phytoremediation is a complex task that includes extensive studies of selected plants in controlled environment. At laboratory condition, heavy metals are added to growth solutions in various forms. They affect the mobility of monitored nutrition elements, interaction with the other species, and try to simulate a soil solution of field scale (Adriano et al., 2004). The level of heavy metal accumulation is dependent on a lot of factors like presence of another compounds or elements [e.g., Fe, ethylenediaminetetraacetic acid (EDTA), ethylenediamine-*N*, *N*′-disuccinic acid (EDDS), tartrate, glutamate], pH, kind of plants. Phytoremediation process is affected by change of mobility of investigated elements and enables increasing of effectivity of cleaning phase. As an example, for chelated-assisted phytoremediation, increasing content of heavy metals in plant samples were observed after addition of EDTA caused by increased mobility of investigated elements (Doumett et al., 2008; January et al., 2008; Núñez-López et al., 2008; Römkens et al., 2002).

In this study as a typical heavy metal, lead was chosen for all experiments. This element is highly toxic and it is dangerous already in a small content, ~30–300 μg g^{-1} (Liu et al., 2007). In some cases, lower concentrations of lead might stimulate metabolic processes due to its similarity with calcium(II) ions, which may be replaced by lead(II) ions. It is well known that toxicity of heavy metals ions depend on their concentration, kind of salts and plant species (higher toxicity was demonstrated in the case of organic forms of heavy metals). The major processes, which may be affected, are seed germination, seedling growth, photosynthesis, plant water status, mineral nutrition, and enzymatic activities (Chen et al., 2000). Toxic elements are stored in various plants tissues and organs (root, stem, leaves, flowers, fruits, or seeds) at different concentrations. Lead is accumulated mainly in roots in comparison with its content in the other parts of plant. However, it is possible to increase the lead content in aerial parts by translocation of element from roots to these aerial parts by adding EDTA into the soil (Doumett et al.,

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Received 12 August 2010; accepted in revised form 18 October 2010

Contract grant sponsor: Ministry of Education of the Czech Republic; Contract grant numbers: ME09015, ME10061, MSM0021622412, MSM 0021622411; Contract grant sponsor: Czech Grant Agency; Contract grant number: GACR 522/07/0692

DOI 10.1002/jemt.20967

Published online 16 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

2008). This can be caused by influence of ATPases function of specialized membrane transporters (Hong et al., 2007). Increasing lead transport through xylem from roots to stem is reported in (Piechalaka et al., 2003). The same effect was monitored in *Pinus radiata* D. Don. Plants of this species were exposed to $Pb(NO_3)_2$ with and without EDTA or H-EDTA. Without addition of chelating reagent, Pb content was measured mainly in cell walls of root tissues. After application of (H) EDTA, Pb accumulation was observed inside of adjacent to cell walls and in intercellular spaces of needles (Jarvis and Leung, 2002). Similar experiment is described in the article, where authors deal with influence of EDTA to distribution of Pb, Ni, and Zn (Barona et al., 2001). Determination of the effect of NaCl irrigation on displacement of heavy metals with and without EDTA is shown in (Wahla and Kirkham, 2008).

In (Antosiewicz, 2005), regulated lead deposition in cell walls of plants was studied in four different plant species. It was observed that lead toxicity increases with decreasing calcium content. The influence of Pb content on total amount and distribution of potassium and manganese was also studied (Galiová et al., 2007).

The uptake ability of different plant species is intensively investigated. As an example, monitoring of distribution of Pb, Cd, and Zn into various parts of twelve different species and removal efficiency was studied in (Liu et al., 2007). The distribution of Pb, Ba, and Cd in different plants is investigated in (Pichtel et al., 2000). The uptake can be dependent on pH and concentration of heavy metals in contaminated soil (Komárek et al., 2007). In (Liu et al., 2000), lead was added in a form of $Pb(NO_3)_2$ at different concentrations. The influence of lead nitrate on root, hypocotyl and shoot growth and its accumulation in each part was studied. It was showed that the amount of Pb in roots increases with increasing Pb content. Lead-exposed plants showed reduction in root calcium, zinc and copper contents. The total Pb content was measured by ICP-MS (Brunet et al., 2008).

In the work (Brunet et al., 2009) the study of the lead nitrate impact on steady-state accumulation of messengers corresponding to stress responsive genes in grass pea (*L. sativus L.*) plants is presented. The plants accumulated the pollutant mostly in their roots. Direct effects of lead on these tissues included growth arrest and variations in target gene transcript accumulation.

The presence of heavy metal in plant affected by enzyme activities was studied in (Verma et al., 2003).

Regarding the analytical techniques, the total amount of toxic elements present in the plant after the phytoremediation process can be measured by several techniques, for example by atomic absorption spectrometry (AAS), inductively coupled plasma optical emission or mass spectrometry (ICP-MS/OES) (Pereira et al., 2004; Suleiman et al., 2008; Masson et al., 2010). Dissolution of samples for analysis is however a time consuming process, moreover by using this technique information on distribution of observed ions are lost. In general, there is a lack of information where monitored toxic ions are stored in various parts of hyperaccumulator plants. In order to obtain data about trafficking and storage of these ions, methods enable mapping the chemical composition of sample in solid state should be applied.

Laser-assisted analytical methods offer simple and fast tool for chemical mapping (Kaiser et al., 2009; Galiová et al., 2008; Becker et al., 2010a,b). These techniques can be applied without any or with simple and straightforward sample preparation such as ablation of impurities from the sample surface by applying "cleaning" pulses. Both, LA-ICP-MS and LIBS allow qualitative and semiquantitative direct analysis, profiling, and mapping of elements distribution. Basically, the achievable spatial resolution of these methods depends only on the diameter of ablation crater created by the focused laser beam (Kaiser et al., 2009; Galiová et al., 2008; Becker et al., 2010a,b; Novotný et al., 2008). The main disadvantage of techniques that uses laser ablation for sampling is a lack of certified reference materials for calibration. The typical detection limits (LOD) for LA-ICP-MS analyses of plant samples are approximately $\mu g\ g^{-1}$ (Becker et al., 2010; Santos et al., 2009). The detection limits of LIBS analysis are in general 10 times worse ($10\times$ $\mu g\ g^{-1}$) (Vadillo et al., 1999). It should be also noted that the LOD for LIBS can be improved by applying modified LIBS techniques as double-pulse (DP) LIBS or combining LIBS by laser-induced fluorescent spectrometry (LIFS) (Samek et al., 1998; Telle et al., 2001; Laville et al., 2009).

Here we report on a pilot study focused on monitoring of effects of lead(II) ions on important field crop—*Capsicum annuum* L. (pepper). In the case of improvement of its features, the usage of this plant in field conditions is relatively undemanding. Laser-assisted analytical methods (LIBS, LA-ICP-MS) were utilized for mapping of lead and nutrition elements distribution in pepper leaves treated by lead. The influence of water present in sample on results of LIBS and LA-ICP-MS analysis was investigated. The LIBS results are compared with outcomes obtained by LA-ICP-MS.

EXPERIMENTAL

Plants Cultivation and Sample Preparation

C. annuum L. seeds were germinated on wet filter paper in special vessels at $23^{\circ}C \pm 2^{\circ}C$ in dark. After 7 days, the pepper seedlings were placed into vessels containing Richter solution and cultivated in aeroponic system for 2 months with 14-h long daylight per day (maximal light intensity was about 5,400 lx) at a temperature 21–23°C. When the roots reached ~2 cm, the seedlings were placed into vessels contained 25 L modified aerated Richter's nutrient solution. The concentrations of macroelements per 1 L were 3 mmol $Ca(NO_3)_2$, 2 mmol KNO_3, 1.5 mmol KH_2PO_4, 1 mmol $MgSO_4.7\ H_2O$. Iron in the form of Fe-EDTA was added to the nutrient solution (0.18 mol L^{-1} Fe). Microelements were added to the nutrient solution in the form of Hoagland's AZ solution.

After that, the selected very vital, well-growing, uniform 2-month old pepper plants were placed in the vessels that contained tap water with addition of $Pb(NO_3)_2$ (0, 0.5, 1, 2, and 10 mmol L^{-1}), where they were grown for 7 days. Plants cultivated without the presence of lead served as a control.

First changes in physiology of experimental plants were well evident already after 24 h of treatment in the highest applied lead(II) concentration; these

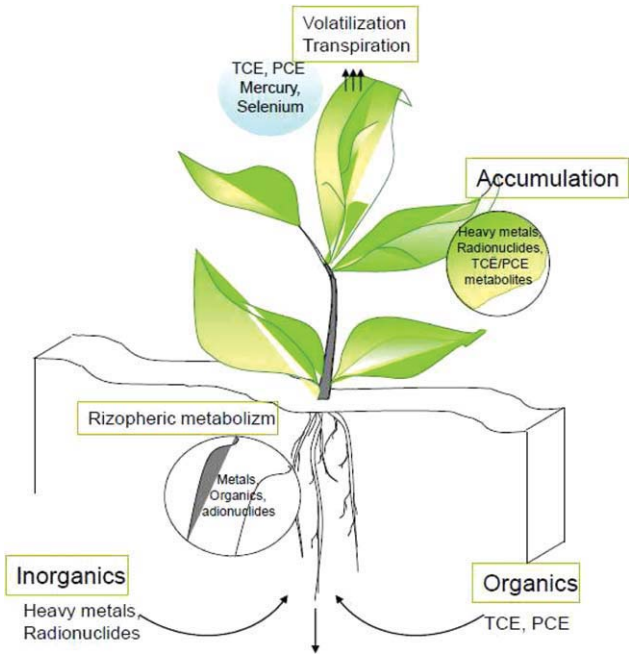

Fig. 1. Phytoremediation can occur through a series of complex intereactions between plants, microbes, and the soil, including accumulation, hyperaccumulation, exclusion, volatilization, and degradation. Plants also stabilize mobile contaminated sediments by forming dense root mats under the surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

changes included especially reduction of experimental solution uptake. After 30 h of treatment, also morphological changes resulting from water uptake reduction were well evident. After 72 h, these changes were well evident also in the case of other experimental variants—plants treated by lead(II) ions at concentrations 0.5 and 1 mmol L^{-1}. After 168 h, plants treated by highest lead(II) ions concentration (10 mmol L^{-1}) demonstrated very significant nonphysiological changes including chlorophyll degradation, necroses with leaves browning and significant reduction of water content; these changes were not so accentuated in two lower concentrations (Fig. 2). Control plants and plants treated by lead(II) ions in lowest concentration (0.5 mmol L^{-1}) did not embody growth depression (Fig. 2a).

Ontogenetically oldest leaves (n = 6) were sampled from each variant at certain time intervals (0, 48, 96, 144, and 168 h) during the experiment. After sampling, the leaves were rinsed three times in distilled water and 0.5 mol L^{-1} EDTA. In addition, each harvested leave was divided onto halves. One half was stored in deep freezer at −80°C (Sanyo, Japan) and the second one was dried for 12 h at 45°C (Memmert, Germany) (Fig. 2b).

From this relatively large set of samples for LIBS and LA-ICP-MS mapping, the representative leaves from untreated (control) sample and leaves from samples treated by 10 mmol L^{-1} $Pb(NO_3)_2$ for 2 days were used.

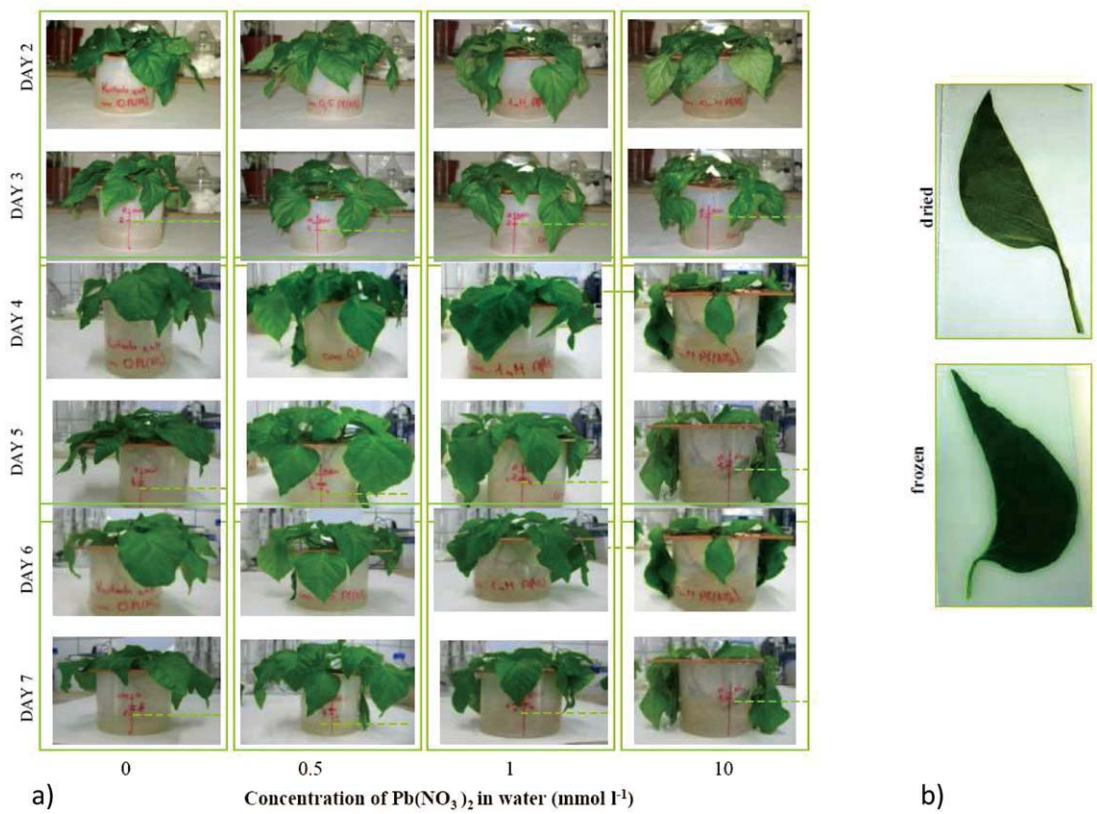

Fig. 2. (**a**) Set of *C. annuum* L. samples used for monitoring of effects of lead(II) ions. (**b**) Example of the investigated *C. annuum* L. leaves. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

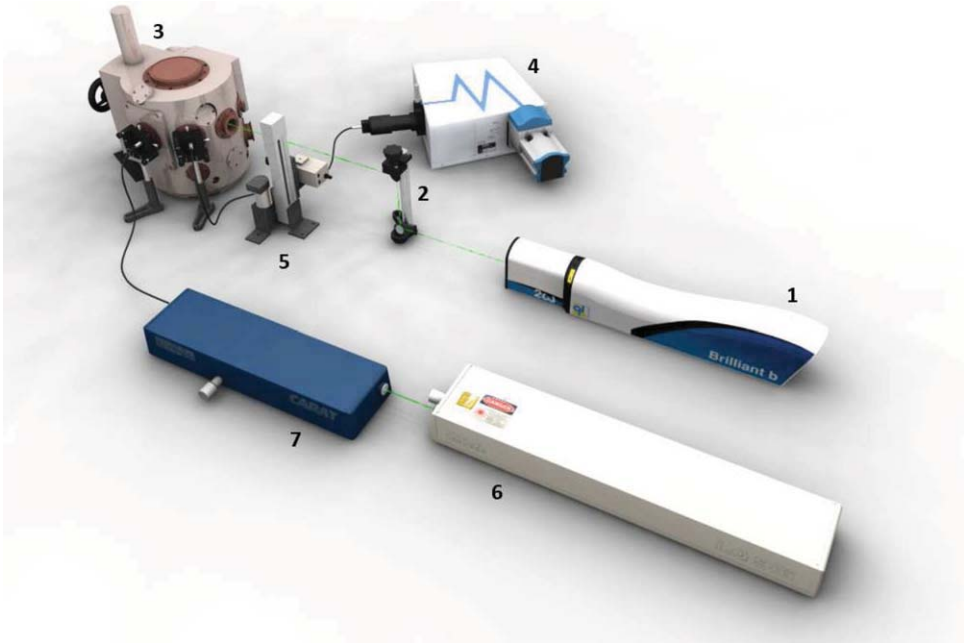

Fig. 3. Computer rendering of the LIBS experimental setup: 1. Nd:YAG ablation laser with a module for second harmonic (532 nm) generation (Quantel Brilliant B, France), 2, Periscope, 3. Interaction chamber (TESCAN s.r.o., Czech Republic), 4. Spectrograph (LOT Oriel 260i, USA) equipped with ICCD camera (Andor IStar 734i, UK), 5. CCD camera for sample alignment, 6. Nd: YAG laser (Solar LS LQ529A, Belarus), and 7, Ti: Sa laser (Solar LS LX325, Belarus) for further developments (LIBS+LIFS). [Color figure can be viewed in the online issue, which is available at wiley onlinelibrary.com.]

LIBS Setup

The LIBS setup utilized in our experiments is shown in Figure 3. For creating the microplasma, Nd:YAG laser system (Brilliant B, Quantel, France) working on its second harmonics frequency (532 nm) was used. The laser pulse width was ~5 ns and beam diameter 8 mm. LIBS signals were obtained using the energy of the laser pulse 10 mJ at the sample that was set and controlled by an energy meter (Field Master LM-P10, Coherent, USA). The samples with sample holder were placed inside the ablation chamber (TESCAN s.r.o., Czech Republic) to the stage with precision movements (2 μm in x, y, and z direction). The LIBS measurements were realized in air at atmospherics pressure. CCD camera placed outside of the chamber was used for targeting and controlling of each ablation spot (Novotný et al., 2009). Laser beam was focused with 16 mm focal-length glass doublet (Sill Optics, Germany) and emitted radiation was collected by objective and transported with a 3-m fiber optics system onto the entrance slit of the 1/4 m monochromator (LOT Oriel 260i, USA). For LIBS measurements, the grating 2,400 g mm^{-1} of the monochromator and 10 μm entrance slit were used. As a detector an ICCD camera (Andor IStar 734i, UK) was employed. The camera was triggered by the Q-switch signal of the laser. The ICCD detector was gated 1 μs after the Q-switch signal and the integration time was 10 μs.

Analytical lines of Pb (I) 405.78 nm, Mn (I) 403.07 nm, 403.31 nm, and K (I) 404.41 nm, 404.72 nm were observed in the selected spectral interval. The LIBS ablation patterns had a spacing of 500 μm and the diameter of ablation craters was ~200 μm. To obtain two-dimensional maps, background was subtracted for each shot and the area under the selected peak (for appropriate chemical element) was calculated.

LA-ICP-MS Device

Instrumentation for LA-ICP-MS consists of a laser ablation system UP 213 (New Wave, USA) and an ICP-MS spectrometer Agilent 7500 CE (Agilent, Japan). A Q-switched Nd:YAG laser ablation device works at the 5th harmonic frequency (213 nm). The ablation device is equipped with programmable xy-stages to move the sample along a programmed trajectory during ablation. Target visual inspection is accomplished by means of built-in microscope/CCD-camera system. A sample was enclosed in the SuperCell (New Wave, USA) and was ablated by the laser beam, which was focused onto the sample surface through a quartz window. The ablation cell (volume 20 cm^3) was flushed with helium (carrier gas), which transported the laser-induced aerosol to the inductively coupled plasma. A sample gas flow of argon was admixed to the helium carrier gas flow after the laser ablation cell. Therefore, the total gas flow was 1.6 L min^{-1}. Optimization of LA-ICP-MS conditions (gas flow rates, sampling depth, electrostatic lenses voltages of the MS) was performed with the glass reference material NIST SRM 612 with respect to maximum S/N ratio and minimum oxide formation (ThO+/Th+ counts ratio 0.2%, U+/Th+ counts ratio 1.1%). The optimized ICP-MS parameters are summarized in Table 1.

To analyze specific locations in the sample, for LA-ICP-MS line scanning and 2D mapping the ablation laser was used in hole drilling mode (fixed sample

position during laser ablation), for the duration of 1 s for each spot. The LA-ICP-MS ablation pattern consisted of ablation craters of ~100 μm in diameter, placed in distances of ~200 μm. Both ablation patterns (LIBS and LA-ICP-MS) were placed near to each other. Time delay between end of laser ablation of one spot and initiation of laser ablation of following spot was 1 s. Laser ablation was performed with laser spot diameter 100 μm, laser fluency 3 J cm^{-2} and repetition rate 10 Hz. The isotopes ^{208}Pb, ^{55}Mn, and ^{39}K were measured with integration time 0.1 s/isotope.

RESULTS AND DISCUSSION

Laser Ablation Measurements

In this study, the elemental mapping using the comparison of potassium (404.41 and 404.72 nm), manganese (403.07 and 403.31 nm), and lead (405.78 nm) LIBS signals measured on fresh (frozen) sample and on dried samples was realized. All results were compared with outcomes obtained by LA-ICP-MS. Typical single-shot LIBS spectra taken at two different positions on the pepper leaf samples and temporal LA-ICP-MS signals for Pb and K of *C. annuum* L. leaf samples are shown in Figure 4. Potassium was mapped as a nutrition element present in the LIBS spectral window suitable for the lead detection.

For elemental mapping, LIBS signals were recorded for each shot on the area 7.5 × 9.5 mm^2 for fresh (frozen) and 7 × 14 mm^2 for dried control leaf sample of *C. annuum* L. Size of LA-ICP-MS ablation pattern was 2 × 6 mm^2 for all samples. In Figure 5, maps of potassium obtained from the studied area of the control (untreated) leaf measured by both techniques Figure 5a LIBS and Figure 5b LA-ICP-MS are shown. There is a comparison of potassium distribution in fresh (frozen) and dried samples. Potassium as a nutrition element is spread homogenously within area of the control sample similar to manganese distribution (not shown). The distribution of potassium (and manganese) is not affected by presence of water in the case of fresh sample.

In Figure 6b, results of leaf sample analysis obtained after 2 days of lead (10 mmol L^{-1}) treatment, are demonstrated. The area investigated by LIBS was 6.5 × 7.5 mm^2 for fresh (frozen), 6 × 12.5 mm^2 for dried leaf and 2 × 6 mm^2 was measured by LA-ICP-MS. Maps of potassium distribution in the studied area by laser ablation based methods are shown in Figure 6b. As it is evident from obtained experimental data, there are nonsignificant differences between control and treated plants.

We should note that also in the case of lower applied concentrations (0.5 and 1 mmol L^{-1}), there were no changes detected in potassium accumulation in comparison with potassium distribution in untreated leaf sample, in both cases of fresh and dried samples. The same results were obtained for manganese distribution in leaf samples.

The important difference was observed for lead distribution in the 2 days 10 mmol L^{-1} lead treated *C. annuum* L. leaf sample. The maps of lead obtained by LIBS and LA-ICP-MS analyses (Fig. 7) show that lead is accumulated mainly in the central vein and in surrounding area. Distribution of potassium is not affected by accumulation of the toxic element. No influence of frozen water present in the sample for analytical outcomes was detected. The results obtained by LIBS are the same for fresh and dried samples. Moreover, LA-ICP-MS analyses confirmed LIBS outcomes.

On the base of this study, we can conclude that both, fresh and dried samples can be used for monitoring of nutrition and toxic elements distribution in plant tissue. The distribution of investigated elements is not affected by the drying process.

TABLE 1. ICP-MS optimized parameters

RF power	1,350 W
Carrier gas flow	1.00 L min^{-1} He
Makeup gas	0.6 L min^{-1} Ar
Plasma gas	15.0 L min^{-1} Ar
Sampling depth	8.0 mm

Statistical Analysis

Peak area of potassium and lead from each investigated leaf derived from LIBS measurement and intensity of selected elements recorded by LA-ICP-MS were analyzed by statistical software. It was obtained that

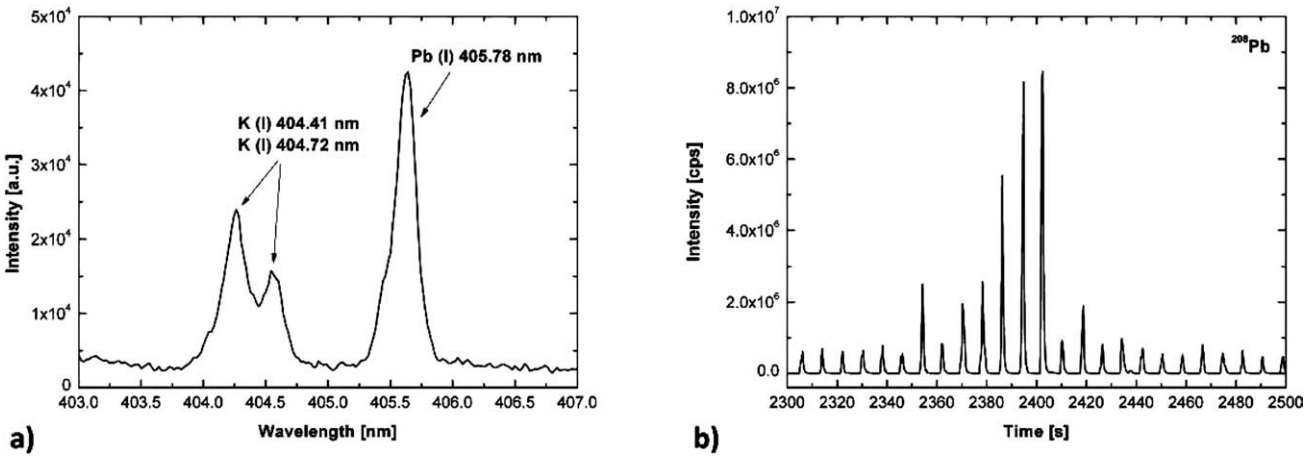

Fig. 4. (**a**) Typical single shot LIBS spectra taken at two different position and (**b**) temporal LA-ICP-MS signal for Pb and K of the *C. annum* L. leaf sample.

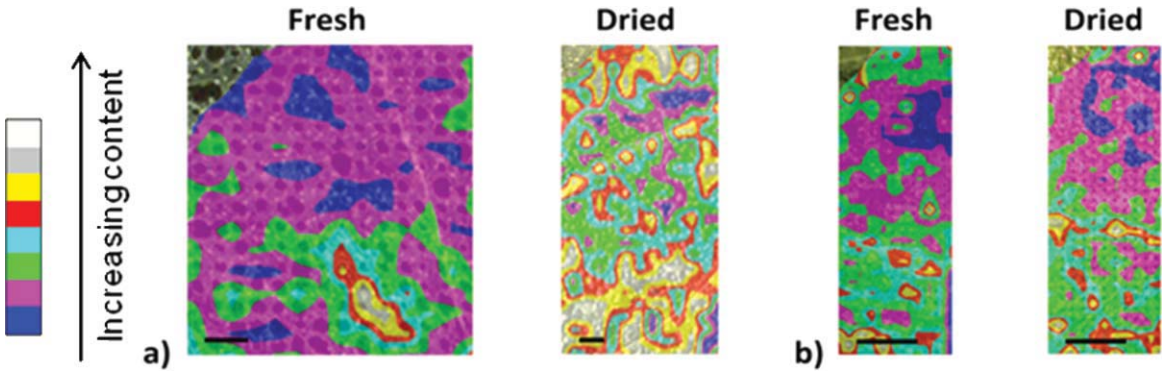

Fig. 5. The maps of K obtained from the studied area of the control (untreated) *C. annuum* L. leaf. The K distribution in fresh (frozen) and dried leaf measured by (**a**) LIBS and (**b**) LA-ICP-MS. The maps are shown together with the photos of the samples. In the upper left corners, the part of the photograph is uncovered. The length of bar is 1 mm.

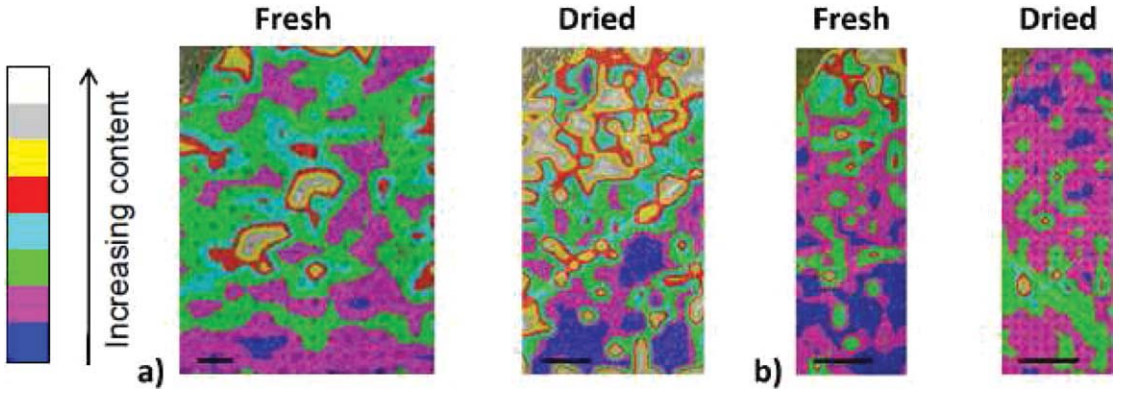

Fig. 6. The maps of K obtained from the studied area of the 2 days 10 mmol L^{-1} $Pb(NO_3)_2$ treated *C. annuum* L. leaf. The K distribution in fresh (frozen) and dried samples measured by (**a**) LIBS and (**b**) LA-ICP-MS. The maps are shown together with the photos of the samples. In the upper left corners, the part of the photograph is uncovered. The length of bar is 1 mm.

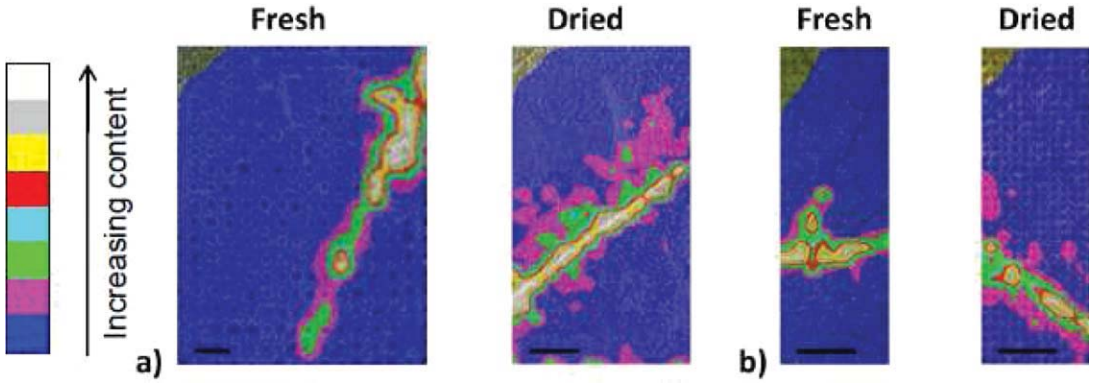

Fig. 7. The maps of Pb obtained from the studied area of the 2 days 10 mmol L^{-1} $Pb(NO_3)_2$ treated *C. annuum* L. leaf. The K distribution in fresh (frozen) and dried leaf measured by (**a**) LIBS and (**b**) LA-ICP-MS. The maps are shown together with the photos of the samples. In the upper left corners, the part of the photograph is uncovered. The length of bar is 1 mm.

data of these values had the Log-normal distribution (Reimann et al., 1999) of these values was revealed. The average of the logarithm of intensity values or peak area was calculated (Limpert et al., 2001). The comparison of the lead signal obtained by LIBS and LA-ICP-MS analyses of 2 days 10 mmol L^{-1} lead

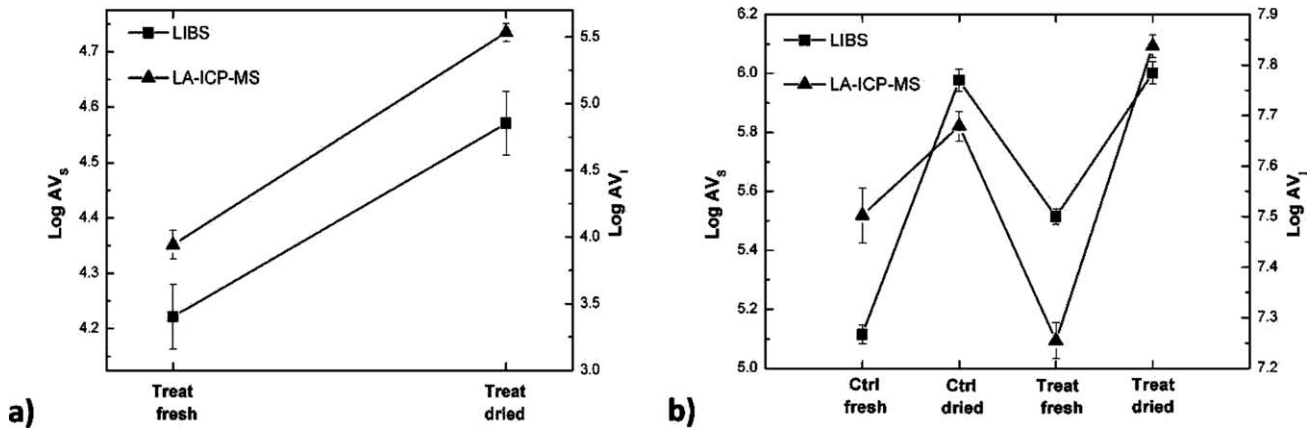

Fig. 8. The logarithm of average value of the peak areas ($LogAV_S$) and the logarithm of average value of the intensities ($LogAV_I$) for (**a**) K and (**b**) Pb on untreated (Ctrl) and 2 days 10 mmol L^{-1} $Pb(NO_3)_2$ treated *Capsicum annuum* L. samples measured by LIBS and LA-ICP-MS.

treated *C. annuum* L. fresh and dried leaf is shown in Figure 8a). The results of this simple statistical analysis shows the logarithm of average value of the peak areas (Log AV_S) and the logarithm of average value of the intensities (Log AV_I) for the appropriate element (potassium manganese and lead) in the case of LIBS and LA-ICP-MS, respectively.

The increase in the intensity of the MS signal and on the peak areas for LIBS in the case of dried sample is evident for both elements. It should be noted, that however the presence of the water decreases the LIBS/LA-ICP-MS signal intensity; the frozen samples are much less fragile than the dried one, thus denser ablation patterns can be created on them. It means that frozen samples are more suitable for analysis with high spatial resolution.

Figure 8b demonstrates the capability of LIBS and LA-ICP-MS to record the changes in accumulation of nutrition element (K) in different areas of untreated (control) and 10 mmol L^{-1} lead treated leaf sample. For LIBS and LA-ICP-MS measurement, higher signal of investigated element was observed for dried leaves on contrary to the fresh (frozen) samples. In comparison of logarithm of average value of the intensities and logarithm of average value of the peak areas obtained by LIBS with values measured by LA-ICP-MS, lower value of Log AV_I was calculated for fresh 10 mmol L^{-1} lead treated leaf sample that for untreated sample measured by LA-ICP-MS. In the case of LIBS measurement, decreased amount of potassium was determined for untreated leaf sample. These differences can be explained by different position of LIBS and LA-ICP-MS ablation patterns on the sample. The Log AV_I and Log AV_S for dried samples are similar. The same behavior was observed for manganese.

CONCLUSION

In summary, by utilizing two laser-ablation based diagnostic methods, namely LIBS and LA-ICP-MS, the accumulation mechanism of lead pollutant in *C. annuum* L. plant species was revealed. The lead is accumulated preferably in the vein structure of leaves. Moreover, the LIBS and LA-ICP-MS signal on dried and fresh samples were compared, and the possibility to achieve lower detection limits on dried samples demonstrated. It should be noted that despite the number of advantages of laser-ablation based methods, the major drawback of these techniques in the case of analysis of environmental samples in their natural form is the difficult standardization. The measurements presented in this work gave the map of the intensity (or area) of a spectral line, which may be proportional to the local concentration, but there was not attempt to calibration or a demonstration of linearity of response. In the ongoing work complimentary techniques, mainly X-ray microradiography and microtomography (Kaiser et al., 2005, 2007) will be applied for validation of LIBS and LA-ICP-MS outcomes.

ACKNOWLEDGMENTS

The authors acknowledge the help of T. Vaculovič in realization of LA-ICP-MS measurements and Violetta Shevstiska for technical assistance.

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