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Phytoextraction of Au: Uptake, accumulation and cellular distribution in *Medicago sativa* and *Brassica juncea*

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ABSTRACT

The influence of metal concentration, solution pH and exposure time on the phytoextraction (i.e. separation using vascular plants) of Au was investigated for the known metallophytes *Brassica juncea* (BJ) and *Medicago sativa* (MS). Metal uptake was inferred using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and *in vivo* localisation and distribution using proton induced X-ray emission spectroscopy (μ-PIXE). MS roots accumulated a maximum of 287 mg Au g^{-1} (dry biomass) and BJ roots a maximum of 227 mg Au g^{-1} (dry biomass), both when exposed to a 10,000 ppm aqueous solution of $KAuCl_4$. MS was found to accumulate comparatively greater quantities of Au than BJ across higher substrate concentrations (40–10,000 ppm Au) whereas BJ was found to be a better accumulator of Au at lower concentrations (5–20 ppm Au). In general MS showed an increase in Au uptake with an increase in Au substrate concentration and the time exposed, whereas for BJ the maximum uptake was observed after 48 h of exposure at higher concentrations (100–10,000 ppm), and then decreased at longer exposure times. The uptake ratio (UR), defined as the ratio of Au concentration in plant tissues to the concentration in the substrate, increased with increasing concentration and exposure time, to a maximum of 995 for MS roots after 72 h exposure. Metal translocation from roots to shoots in BJ increased with increasing substrate concentration, however in the shoots, metal uptake increased from 24 to 48 h and then decreased at 72 h, indicating some threshold level had been reached and metal was then being excluded from the cells, possibly through the phloem to the Au solution. Elemental distribution maps of plant tissues measured using μ-PIXE, show Au present across the entire sample, ranging from the epidermis and cortex, with the greatest concentration occurring within the central stele. This result is suggestive of xylem loading. These results collectively suggest that the separation of Au using vascular plants for applications in mining (phytomining) and remediation (phytoremediation) are viable technologies.

1. Introduction

Gold (Au) and its chemical derivatives have been a subject of interest since ancient times [1]. The first report on the accumulation of Au in vegetation was published in 1900 [2] and was followed by research on its biogeochemistry, in particular the use of plants as an exploration tool for Au rich ore bodies [3–11] and, more recently, in the context of clean heavy metal separation, mining and remediation technologies. These studies indicated the ability of several plant species to accumulate Au in detectable (i.e. ppb) quantities, including *Pseudotsuga menziesii*, *Pinus banksiana*, *Picea mariana*, *Hordeum vulgare* and *Phacelia sericea* [3,4,6,8,10].

Phytomining is the recovery of metals using plants for commercial gain. It is a subset of a larger field of research known as phytoextraction, i.e. the process of using plants to beneficially absorb mineral species from soils, sediments and aqueous systems. Other applications of phytoextraction include phytoremediation, where non-naturally occurring contaminants are recovered for disposal or reuse and phytostabilisation, where contaminant species are immobilised in situ via plant action [12].

Au in its natural form, Au(0), is not ordinarily bioavailable and hence strategies to increase its solubility and bioaccumulation potential have been explored in the context of phytomining and phytoremediation applications. These include the use of lixiviates, e.g. cyanide, thiosulphate or thiocyanate, which aid solubilization and thus metal accumulation in plants [2]. Of these, Au complexes of cyanide are the most readily absorbed through the root membrane [3,7] and hence the sampling of cyanide secreting plants is stressed if Au exploration is the objective [7].

From the perspective of biogeochemical prospecting (i.e. concerning ppb concentrations of Au in plant tissues), Kovalevskii

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Table 1
Results of experiments conducted on *Brassica juncea* (BJ) and *Medicago sativa* (MS) showing the influence of pH, exposure time, substrate concentration and plant organ.

Plant	Plant Organ	Solution pH	Concentration (ppm)	Time (hrs)	Uptake (mg/g)	Translocation Factor
BJ	Roots	2	100	24	19.77	0.40
		3	100	24	41.44	0.09
		4	100	24	21.64	0.04
		5	100	24	14.53	0.06
		6	100	24	6.22	0.25
	Shoots	2	100	24	7.98	
		3	100	24	3.82	
		4	100	24	0.93	
		5	100	24	0.81	
		6	100	24	1.58	
BJ	Roots	2	1000	24	1220.02	0.02
		3	1000	24	129.39	0.37
		4	1000	24	174.15	0.05
		5	1000	24	149.81	0.17
		6	1000	24	72.14	0.41
	Shoots	2	1000	24	21.87	
		3	1000	24	47.57	
		4	1000	24	8.60	
		5	1000	24	25.59	
		6	1000	24	29.63	
BJ	Roots	3	5	24	0.77	0.05
		3	5	48	1.33	0.14
		3	5	72	1.49	0.15
		3	5	120	1.73	0.16
		3	5	360	2.13	0.17
	Shoots	3	5	24	0.04	
		3	5	48	0.19	
		3	5	72	0.22	
		3	5	120	0.28	
		3	5	360	0.37	
BJ	Roots	3	10	24	5.17	0.05
		3	10	48	5.92	0.13
		3	10	72	7.62	0.12
		3	10	120	8.30	0.16
		3	10	360	9.39	0.16
	Shoots	3	10	24	0.26	
		3	10	48	0.78	
		3	10	72	0.95	
		3	10	120	1.36	
		3	10	360	1.52	
BJ	Roots	3	20	24	8.82	0.04
		3	20	48	9.29	0.11
		3	20	72	10.22	0.13
		3	20	120	10.44	0.13
		3	20	360	10.77	0.12
	Shoots	3	20	24	0.38	
		3	20	48	1.05	
		3	20	72	1.28	
		3	20	120	1.31	
		3	20	360	1.33	
BJ	Roots	3	40	24	25.31	0.06
		3	40	48	28.11	0.09
		3	40	72	38.14	0.07
		3	40	120	40.57	0.08
		3	40	360	43.05	0.07
	Shoots	3	40	24	1.44	
		3	40	48	2.63	
		3	40	72	2.79	
		3	40	120	3.09	
		3	40	360	3.12	
BJ	Roots	3	80	24	38.30	0.06
		3	80	48	55.69	0.10
		3	80	72	58.16	0.12
		3	80	120	63.37	0.13
		3	80	360	68.87	0.13
	Shoots	3	80	24	2.26	
		3	80	48	5.53	
		3	80	72	6.98	
		3	80	120	7.98	
		3	80	360	8.96	

Table 1(*Continued*)

Plant	Plant Organ	Solution pH	Concentration (ppm)	Time (hrs)	Uptake (mg/g)	Translocation Factor
BJ	Roots	3	100	24	39.53	0.15
		3	100	48	90.62	0.25
		3	100	72	74.98	0.35
		3	1000	24	123.55	0.34
		3	1000	48	128.79	0.37
		3	1000	72	92.73	0.76
		3	10000	24	188.32	0.50
		3	10000	48	226.76	0.61
		3	10000	72	189.40	0.52
	Shoots	3	100	24	5.75	
		3	100	48	22.73	
		3	100	72	26.13	
		3	1000	24	41.75	
		3	1000	48	47.49	
		3	1000	72	70.48	
		3	10000	24	94.61	
		3	10000	48	138.16	
		3	10000	72	98.82	
MS	Roots	2	100	24	63.56	0.14
		3	100	24	75.24	0.21
		4	100	24	42.09	0.09
		5	100	24	23.76	0.21
		6	100	24	16.64	0.22
	Shoots	2	100	24	9.14	
		3	100	24	15.96	
		4	100	24	3.87	
		5	100	24	4.93	
		6	100	24	3.60	
MS	Roots	2	1000	24	51.28	0.10
		3	1000	24	354.18	0.23
		4	1000	24	67.85	0.19
		5	1000	24	169.75	0.19
		6	1000	24	155.18	0.10
	Shoots	2	1000	24	4.87	
		3	1000	24	82.83	
		4	1000	24	13.23	
		5	1000	24	32.39	
		6	1000	24	16.12	
MS	Roots	3	5	24	1.38	0.03
		3	5	48	1.59	0.09
		3	5	72	1.91	0.11
		3	5	120	2.40	0.13
		3	5	360	2.52	0.14
	Shoots	3	5	24	0.04	
		3	5	48	0.14	
		3	5	72	0.20	
		3	5	120	0.30	
		3	5	360	0.36	
MS	Roots	3	10	24	1.88	0.03
		3	10	48	2.26	0.08
		3	10	72	2.33	0.10
		3	10	120	2.40	0.12
		3	10	360	2.50	0.13
	Shoots	3	10	24	0.06	
		3	10	48	0.17	
		3	10	72	0.22	
		3	10	120	0.29	
		3	10	360	0.31	
MS	Roots	3	20	24	2.76	0.03
		3	20	48	4.41	0.07
		3	20	72	4.91	0.09
		3	20	120	5.62	0.10
		3	20	360	5.79	0.11
	Shoots	3	20	24	0.08	
		3	20	48	0.31	
		3	20	72	0.46	
		3	20	120	0.58	
		3	20	360	0.66	

Table 1(*Continued*)

Plant	Plant Organ	Solution pH	Concentration (ppm)	Time (hrs)	Uptake (mg/g)	Translocation Factor
MS	Roots	3	40	24	36.02	0.06
		3	40	48	41.33	0.10
		3	40	72	60.58	0.07
		3	40	120	68.93	0.10
		3	40	360	69.40	0.12
	Shoots	3	40	24	2.09	
		3	40	48	4.28	
		3	40	72	4.50	
		3	40	120	7.22	
		3	40	360	8.43	
MS	Roots	3	80	24	46.73	0.16
		3	80	48	65.59	0.13
		3	80	72	77.95	0.15
		3	80	120	87.14	0.15
		3	80	360	87.40	0.16
	Shoots	3	80	24	7.44	
		3	80	48	8.84	
		3	80	72	11.62	
		3	80	120	13.12	
		3	80	360	13.76	
MS	Roots	3	100	24	47.63	0.25
		3	100	48	73.27	0.11
		3	100	72	99.45	0.41
		3	1000	24	98.00	0.97
		3	1000	48	129.18	0.52
		3	1000	72	167.88	0.40
		3	10000	24	193.4	0.80
		3	10000	48	167.71	0.93
		3	10000	72	286.84	0.81
	Shoots	3	100	24	12.07	
		3	100	48	8.07	
		3	100	72	40.61	
		3	1000	24	94.59	
		3	1000	48	67.51	
		3	1000	72	66.90	
		3	10000	24	155.28	
		3	10000	48	155.96	
		3	10000	72	231.87	

and Kovalevskaya [5] classified plant species and organs into four groups:

(i) Non barrier bio-objects which give quantitative information on the Au concentration in the growth medium.
(ii) Semi-non barrier bio-objects with high concentration limits of 3–300 times the Au concentration in the growth medium.
(iii) Barriers having concentration limits of 3–30 giving only qualitative information on the concentration of Au in the growth medium.
(iv) Background barriers which provide neither quantitative nor qualitative information on Au concentration in the growth medium.

Kovalevskii and Kovalevskaya [5] recommended the inner, middle and outer bark of trees as non-barriers and confirmed them as the main organs of trees which can reflect deeply buried Au deposits based on a study of 33 separate species. Kovalevskii and Kovalevskaya's barrier concept states that every plant and plant organ offers varying degrees of resistance to metal uptake. Thus, when prospecting for an element, the focus of analysis has to be on specific plants and plant organs.

More recently, researchers have reported inordinately large concentrations (e.g. ppm to %-wt) of metals in some plants [12]. To this end, Baker and Brooks [13] classified plants into three categories: (i) excluders; plants which do not take up metals, (ii) hyperaccumulators; those which take up abnormally large quantities of metal and (iii) indicators; those which take up metal in proportion to its quantity in the soil. Hyperaccumulators of many heavy metals have already been identified; approximately 317 nickel, 24 copper and 26 cobalt hyperaccumulators to date [14], although none for the noble metals. Harris and Bali [15] previously evaluated the ability of *Medicago sativa* (MS) and *Brassica juncea* (BJ), two known metallophytes (metal loving plants), to reduce and hyperaccumulate Ag(0) from aqueous solutions of Ag(I). In this work the whole plant Ag concentration was up to 14 wt.%-dry basis and 12 wt.%-dry basis in MS and BJ respectively. To the best of our knowledge however, there are no published accounts of the hyperaccumulation of Au in the literature. Anderson [16] reported the induced accumulation of Au, up to 57 mg Au kg^{-1} dry weight in the leaves of BJ using thiocyanate as a chelating agent. Rodriguez [17] and Parsons et al. [18] showed that MS cultivated on Au enriched media accumulated 56 mg Au kg^{-1} shoot dry weight after 14 d exposure. However, neither report meets the definition for hyperaccumulation.

Despite this previous research, there is limited information on the mechanism(s) of Au absorption, its chemical behaviour, transport and localisation in plants. Relevant studies suggest that the final site of metal accumulation is the leaf [19] or leaf tip [6] however there are no published reports explaining why or how plants take up Au. Anderson et al. [20] suggested evapotranspiration (the combined effect of evaporation of water from the soil and plant surfaces and the loss of water through stomata in leaves), as a possible mechanism for induced hyperaccumulation in Au. There is a lack of information in the literature on the response of plants at higher Au concentrations and no reports of the extent of uptake of Au in BJ and MS when exposed to Au solutions for extended periods of

Table 2
Microwave digestion program used for the digestion of plant samples.

Step	Time	Temperature (°C)	Microwave Power (W)
1	8	105	1000
2	5	105	1000
3	2	25	500

time. This information is necessary to advance the development of phytomining and phytoremediation processes for Au.

In light of the above, the objectives of this work were to (i) evaluate the limits of uptake of Au in two known metallophytes, BJ and MS using hydroponic growth experiments (to exclude the complicating factors introduced when a soil matrix is present), (ii) assess the effect of critical parameters on Au uptake and (iii) determine the localisation of accumulated Au in the various plant organs.

2. Experimental

BJ and MS seeds were surface sterilized in a solution of 1% hydrogen peroxide for 15 min to avoid fungal contamination, washed with deionised water and then germinated on wet paper towels for 48 h in an incubator (without illumination) at 25 °C. Seedlings were transplanted into glass jars containing 250 mL Hoaglands media (Hoaglands Basal No 2, Sigma–Aldrich). All experiments were performed in the controlled environment of a plant growth chamber (Contherm Scientific Ltd) with a 12-h/12-h light/dark cycle (25 °C/18 °C). Seedlings were harvested between two and three weeks following germination and transferred to Petri plates containing 40 ml of aqueous solutions of $KAuCl_4$ (Sigma–Aldrich, 99.99%). As summarised in Table 1, the influence of Au concentration (100, 1000, 10,000 ppm), exposure time (24, 48 or 72 h), solution pH (2, 3, 4, 5 and 6) and plant organ (roots and shoots) was investigated during the course of experiments. Solution pH was adjusted by the addition of 0.1 M NaOH or 1 M HCl. The effect of longer exposure times (24, 48, 72, 120, 360 h) on Au uptake was also studied at lower Au concentrations (5, 10, 20, 40 and 80 ppm).

After exposure, plants were harvested, washed with demineralised water, dried for 48 h at 105 °C, weighed and ashed in the presence of air at 500 °C for 4 h. The ash was then digested in a mixture of 8 ml of concentrated HCl and HNO_3 acids using microwave-assisted digestion at 105 °C for 15 min (Milestone ETHOS SEL). The microwave digestion program used for metal digestion is reported in Table 2. The sample volume was raised to 10 mL with the addition of 1 M HCl prior to analysis by ICP-OES. Calibration standards were prepared from a known Au ICP standard (1000 mg L^{-1}). All calibration curves had a correlation coefficient of >0.99. Amongst the analytical techniques available to measure metal concentration in digested plant samples, ICP-OES and FAAS are most common [21]. In this work ICP-OES was chosen because of its greater sensitivity and higher detection limits (<10 ppb for Au) across the conditions investigated [22]. Each experiment had two replicates and all experiments were repeated three times. From these results, outliers were removed using a standard least-median-of-squares algorithm, followed by calculation of the mean and standard deviation of the Au concentration.

For morphological and anatomical analysis, plant samples were fixed in 2% glutaraldehyde followed by alcohol dehydration, embedded in Spurrs resin and then oven cured at 60 °C for 24 h [23]. Approximately 1 μm thick sections were cut using a microtome (Leica Microsystems). Sections were stained with 0.2% toluidine blue, heat fixed and viewed under a light microscope (Nikon Sensicam).

For preliminary μ-PIXE analysis hand sections of the roots and stem of BJ and MS were obtained. These were immediately plunged into liquid nitrogen, followed by freeze drying for 24 h. Freeze dried sections were mounted on carbon tape held within a standard ANSTO aluminium microprobe sample holder. For the remainder of the μ-PIXE experiments the modified method of Bidwell et al. [24] was used. Circular discs of root, stem and leaf tissue were cut using a hole punch and placed in Au sample holders prepared with 1- hexadecane (to exclude gas bubbles) prior to loading into a high pressure freezer (Leica EM HPF). High pressure frozen samples were transferred to a freeze substitution media (diethyl ether). The frozen leaf sections were then substituted with diethyl ether over freshly activated molecular sieve for 3 d at −90 °C. Samples were then gradually raised to −30 °C at a rate of 1 °C h^{-1} and held for 48 h at this temperature. The temperature was finally raised to room temperature at 1 °C h^{-1}. Freeze substituted samples were subsequently infiltrated with increasing ratios of Spurr's resin over 72 h (Spurr's resin: DEE, 2:8 for 24 h, 5:5 for 24 h, and 8:2 for 24 h) before finally being infiltrated with 100% Spurr's resin overnight for 12 h. Once substitution was complete samples were embedded in fresh resin and cured at 60 °C for 24 h. Approximately 6–10 μm sections were cut using a dry glass knife microtome (Leica Microsystems), and then mounted onto standard ANSTO aluminium holders ready for analysis.

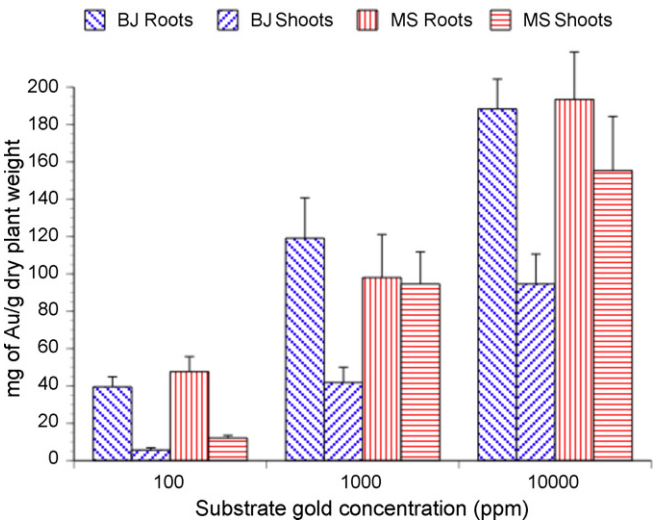

Fig. 1. Au concentration in plant tissues (roots and shoots) of BJ and MS, expressed as mg Au g^{-1} plant dry weight as a function of the Au concentration after 24 h exposure. Error bars denotes Standard deviation (SD).

3. Results and discussion

3.1. Effect of pH, metal concentration and exposure time on Au uptake

Fig. 1 shows the uptake of Au in both MS and BJ after 24 h exposure, as a function of metal concentration in the substrate. In general, the concentration of Au in both plants increased with increasing Au concentration in solution. The results in Fig. 1 clearly indicate that although all parts (roots and shoots) of the plant accumulate Au, the final concentration differs with plant type and location within the plant. BJ roots accumulated a maximum of 39 mg of Au g^{-1} dry weight when exposed to the 100 ppm Au solution after 24 h exposure, whereas BJ shoots accumulated a maximum of 6 mg Au g^{-1} dry weight. After 24 h exposure, MS roots accumulated a maximum of 48 mg Au g^{-1} dry weight when exposed to 100 ppm Au solution, and MS shoots accumulated 12 mg Au g^{-1} dry weight of Au. Roots of BJ retained more Au than roots of MS at higher concentrations (1000 and 10,000 ppm).

Metals which play no part in a plant's normal biological function (including Au) are either discarded into the leaves or rootlets This

may explain the higher retention of Au in the roots of BJ. A maximum of 188 mg of Au g^{-1} dry weight was observed in BJ roots when exposed 10,000 ppm Au solution for 24 h. MS roots accumulated 193 mg of Au g^{-1} dry weight at the same conditions. The Translocation Factor (TF), defined as the ratio of metal accumulated in the shoots to metal accumulated in the roots, was between 0.15–0.50 in BJ and 0.25–0.97 in MS. These results indicate an increase in TF with an increase in concentration and thus increase in metal transport from roots to shoots was observed with increased concentration of Au in the solution.

The roots of both BJ and MS in general showed a greater UR defined as the ratio of Au concentration in plant tissues to the concentration in the solution to a maximum of 994 in MS and 906 in BJ. On average, MS exhibited a higher root and shoot UR than BJ. Fig. 2a shows a decrease in the relative uptake of Au with an increase in solution Au concentration. The greater UR of the roots suggests restricted translocation of Au to shoots once absorbed. Although the reason for this restricted translocation was not able to be established from these experiments, it is possible that significant quantities of Au were retained in the root vacuoles, and/or apoplasts. This result suggests that plant roots rapidly deplete Au ions from the solution and hence have a high affinity for Au^{+}.

To date, no biological mechanism has been elucidated for the uptake of noble metals nor has the form of accumulated Au in plants been studied. In general however, as indicated by Anderson et al. [20], plants accumulate Au because it is present in an available form in solution. Research on heavy metal uptake suggests rhizosecretion of biologically active compounds, i.e. organic acids (e.g. histidine), proteins (mainly phytochelatins and metallothionins), isoflavonoids and enzymes, occurs in response to metal exposure [13,25–28]. These compounds may be responsible for the Au accumulation observed in MS and BJ in this work. Further biochemical and physiological studies are required on metal uptake, accumulation and sequestration in plants.

The time of exposure to a metal is another critical factor influencing the uptake of metal through the root membrane [17,28]. To this end, the effect of exposure time (24, 48 or 72 h) was studied at all higher concentrations (100, 1000 and 10,000 ppm). Au uptake was found to markedly increase with increasing exposure time in MS (Fig. 2b), to a maximum of 287 mg Au g^{-1} dry weight of roots after 72 h exposure at 10,000 ppm of Au. At 1000 ppm, the roots of MS accumulated 98–168 mg Au g^{-1} dry weight at exposure times from 24-72 h. This trend of increasing metal uptake with increasing exposure time was observed across all Au concentrations studied for MS. However, for BJ, Au uptake increased from 24 to 48 h across all concentrations but then decreased from 48 to 72 h, indicating some kind of exclusion mechanism had been activated (Fig. 2c). The process of the accumulated metal elimination by the roots indicates Au to be phloem mobile.

At 1000 ppm Au in the solution, Au uptake was 124 mg Au g^{-1} dry weight after 24 h of exposure, 129 mg Au g^{-1} dry weight after 48 h reducing to 93 mg Au g^{-1} dry weight after 72 h of exposure. Cells, when starved of a metabolite, show increased influx of that metabolite. This can explain the initial high uptake of Au in both MS and BJ. Once the cells are saturated, the uptake is dependent on the external concentration and availability of free ion exchange sites at the root surface. The initial rapid uptake may be because of diffusion, metal homeostasis or an ion exchange mechanism [6] and is indicative of the fact that BJ had reached its threshold level after 48 h exposure time.

Similar results were observed at all the lower concentrations (5, 10, 20, 40 and 80 ppm) wherein the data suggest an increase in uptake with increased exposure time and concentration for both BJ and MS. ICP-OES analysis of plant tissues revealed that at each exposure concentration, the majority of Au was associated with

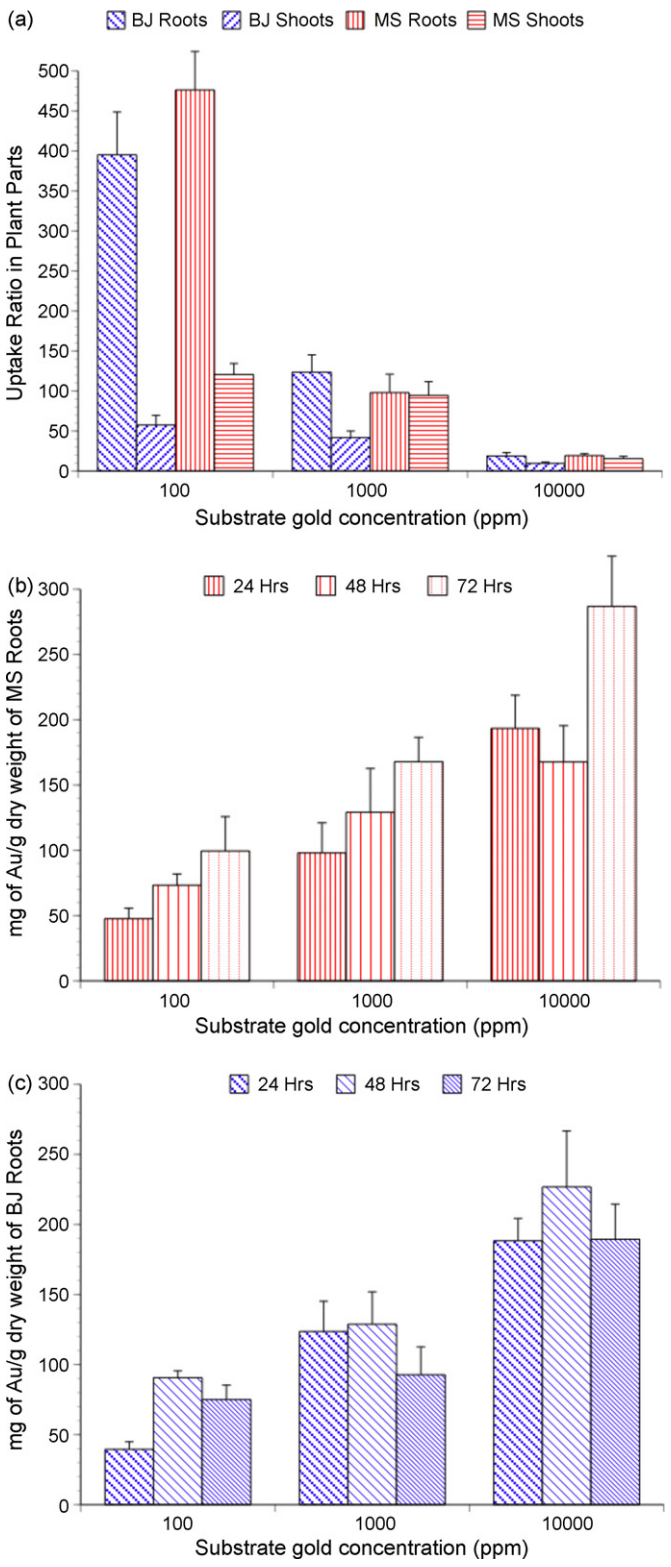

Fig. 2. (a) Uptake ratio of roots and shoots of BJ and MS expressed as a function of Au concentration after 24 h exposure time (b) Au concentration expressed as mg Au g^{-1} dry weight of MS roots, as a function of the Au concentration in the solution and the exposure time (c) Au concentration expressed as mg Au g^{-1} dry weight of BJ roots, as a function of the Au concentration in the solution and exposure time. Error bars denotes Standard deviation (SD).

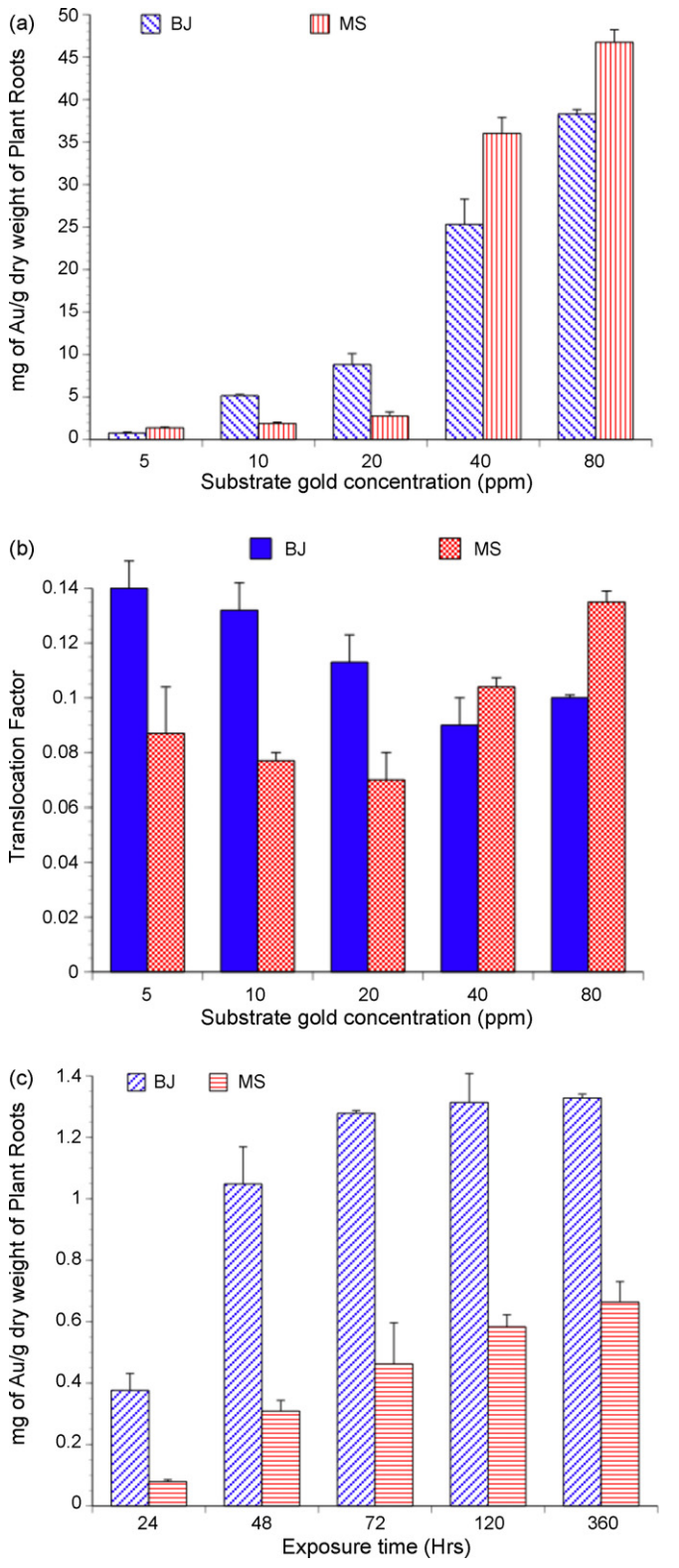

Fig. 3. (a) Au concentration in the roots of BJ and MS, expressed as mg Au g^{-1} plant dry weight as a function of the Au concentration in the solution and the exposure time, (b) Translocation Factor of BJ and MS as a function of the Au concentration in the solution and 48 h exposure time, (c) Au concentration in the shoots of BJ and MS, expressed as mg Au g^{-1} plant dry weight as a function of exposure time at a solution concentration of 20 ppm. Error bars denotes Standard deviation (SD).

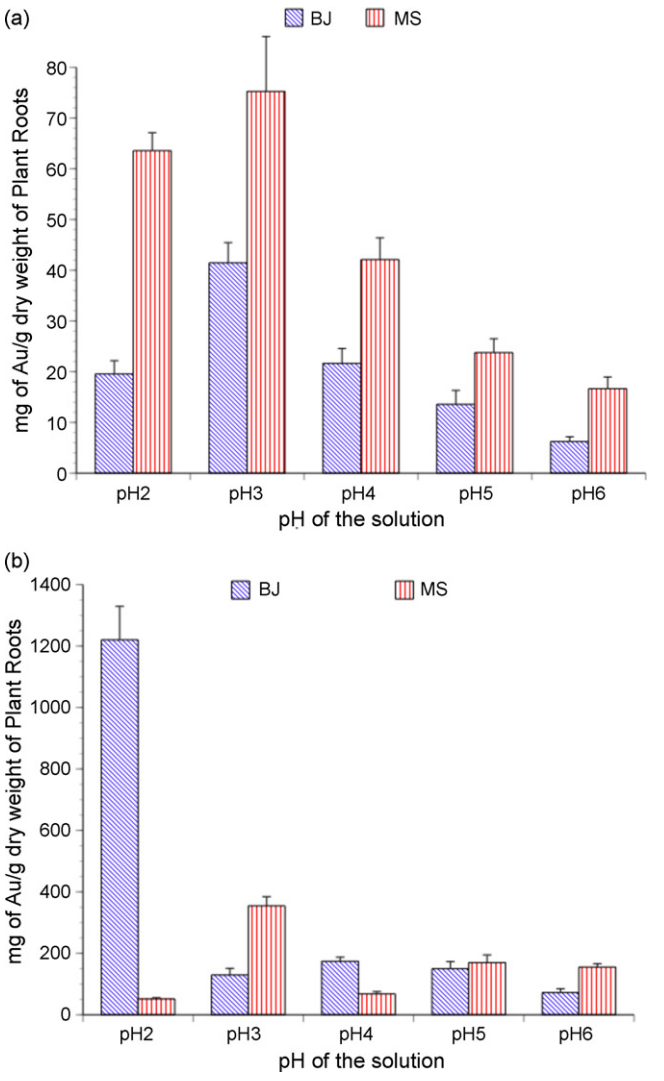

Fig. 4. (a) Au concentration in the plant tissues of BJ and MS, expressed as mg Au g^{-1} plant dry weight as a function of pH in the substrate at 100 ppm, (b) Au concentration in the plant tissues of BJ and MS, expressed as mg Au g^{-1} plant dry weight as a function of pH in the substrate at 1000 ppm. Error bars denotes Standard deviation (SD).

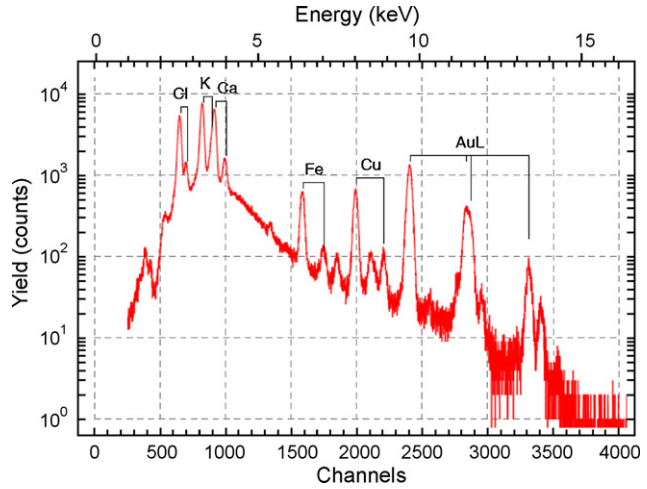

Fig. 5. Proton induced X-ray emission (PIXE) analysis spectrum of Brassica juncea root. Note the peaks Lα = 9.712 and Lβ1 = 11.440.

the roots. For all treatments, Au uptake in the roots and shoots was greater at 360 h than at lesser exposure times.

Fig. 3a shows that BJ and MS exhibited an increase in uptake with an increase in concentration. BJ had significantly higher uptake compared to MS at low concentrations (5–20 ppm) with approximately 3-fold greater uptake than MS. However, the rate of increase in uptake with an increase in concentration (5–20 ppm) slowed beyond these concentrations. This might be because of an increase in the fixed charge at the root cell surface which is believed to increase the resistance to metal uptake by slowing metal ion diffusion through the cell wall. At higher concentrations (40–80 ppm), MS exhibited higher comparative uptake than BJ.

At low solution concentrations (5–20 ppm) the roots transport greater quantities of Au to the shoots in BJ than MS (Fig. 3b). Au mobility to shoots is greater at lower concentrations indicating lower retention in the roots. At higher concentrations Au transport occurs at slower rates as indicated in Fig. 3b. A maximum TF of 0.14 was achieved at the lowest solution concentration studied (5 ppm) after 48 h exposure time. The TF for BJ decreased in general across the lower concentrations (5–20 ppm) and increased with an increase in concentration (40–80 ppm). Transport of Au to shoots in MS was different from BJ; the results show less transport compared to BJ. A maximum of 0.087 TF was achieved at 5 ppm concentration and 48 h exposure time. MS showed similar trends to BJ with a decrease in TF from 5 to 20 ppm and an increase in TF from 20 to 80 ppm.

These results suggest that a large quantity of Au is immobilised within the root tissues, e.g. in the apparent free space (AFS) associated with pectin and protein fractions. AFS is freely accessible to ions where cation exchange and complexation at the cell wall components take place. It can be inferred that the uptake of Au increases over a period of 360 h, but the rate of uptake decreases considerably after 72 h.

Experiments at low Au concentration over a period of 360 h show that although the plants were totally necrotic after 72 h, they continued to absorb Au in appreciable amounts. In general, the uptake of Au by MS and BJ shoots increased with time exposure. The data clearly suggest that at shorter exposure times, the absolute quantity of Au accumulated by BJ shoots was 3–4 orders of magnitude greater than for MS shoots (Fig. 3c). At longer exposure times the accumulation of Au by BJ was greater still, however it was only twice the uptake by MS shoots. Thus BJ is a better accumulator of Au at low concentrations and longer exposure times.

The effect of the solution pH was studied at Au concentrations of 100 and 1000 ppm. Fig. 4a shows the pH dependent uptake of both plants. The highest Au concentration in the plant was observed at pH 3. This is consistent with Girling and Peterson [29] who observed increased uptake of Au at acidic pH. Au uptake in MS was always higher than in BJ, although BJ showed a 6-fold increase in uptake at pH 3 compared to pH 6. The effect at 1000 ppm was inconclusive for both MS and BJ (Fig. 4b). Maximum uptake of 75 mg Au g^{-1} dry weight in MS and 41 mg Au g^{-1} dry weight in BJ was observed. The Au concentration in the shoots of both BJ and MS was not significantly influenced by a reduction in pH, hence translocation of Au from roots to shoots was pH independent. This result is consistent with the findings of Rodriguez [17].

In general, uptake of a metal involves three phases: (i) metal transport across the root cell plasma membrane, (ii) root to shoot translocation, and (iii) metal detoxification and sequestration [30]. Of these, the most critical is the transport of metal across the plant cell membrane. Plant cell walls have different chemical functional groups that act as binding sites for metal uptake, mainly carboxyl, amine and hydroxyl groups. Although considerable research has been undertaken on elucidating the mechanism of uptake of essential and non-essential metals, to date the mechanism for Au uptake in plants remains unclear. To understand the uptake of Au and its intracellular transport, understanding the chemistry of Au in solution is crucial. Au is a soft acid in its cationic form, will bind with soft bases (S and N containing functional groups) and hence it is expected to form covalent bonds [31]. However, initial studies indicate that the uptake of Au is via a complex mechanism and not ion exchange, because ion exchange reactions are rapid and Au uptake and reduction reactions are time dependent [32]. In solution, the tetrachlorate ion may easily be reduced to Au(I) and finally to Au(0) [33,34] by many processes, most likely hydrolysis or reduction involving the release of protons, organic acids or amino acids using nitrogen or sulphur donor ligands [1,34]. For enhanced uptake these hydrolysed ions bind with the functional groups present on the cell walls. Au(I) is most likely stabilised by CN- and S containing ligands, whereas N containing functional groups stabilise Au(III). In addition, pH dependent and independent Au uptake has been observed, indicating the possibility of different mechanisms of reduction and uptake in different plant species. pH independent results indicate covalent bonding (expected considering Au is a soft acid), however pH dependent results are attributed to electrostatic interactions between positively charged amino groups present on the cell walls and negatively charged $AuCl_4^-$ species in the hydrolysed aqueous solution. It is worth mentioning here that the few FT-EXAFS studies reported in the literature indicate binding through N-containing ligands rather than S-containing ones, confirming that Au(III) accumulation involves multiple binding sites [35]. The other question which arises is whether the reduction is extracellular or intracellular, i.e. in the aqueous growth media solution or within the cell membranes of the plant. The mechanism of uptake and reduction of Au is still unknown, in particular whether the metal is first reduced and then absorbed or the hydrolysed species is first adsorbed by the root surface and then reduced. Once the metal has passed the root membrane, metals move acropetally through the xylem, however the process of xylem loading also remains unclear. Some initial studies suggest the use of transport ATPases in xylem loading, by creating negative electrochemical gradient in parenchymal cells [36]. ATPases are responsible for carrying numerous ions besides protons (K^+, Na^+, Ca^{2+}, Cu^{2+}) and might therefore also be responsible for Au transport. Various amino (histidine, cysteine, methionine) and organic acids (malic acids, citric, malonic acids) are also believed to aid in xylem loading [37,38]. Different metals have been found to be transported using different metal complex reactions, e.g. Ni is transported mainly thorough histidine binding and Zn uptake is enhanced in the presence of malic acids [36,39]. However, it is important to note that the chemical analysis of xylem fluids indicates the presence of different amino or organic acids for different metals and plant species. Once transported the metals has to be stored, where they are rendered non toxic and no longer interfere in normal plant metabolism. The two major heavy metal binding compounds in plants are phytochelatins (PCs) and metallothionins. The presence of a Cd–phytochelatin complex in the vacuoles is one such example of metal tolerance [40]. At the tissue level, metals are stored in epidermal and subepidermal cells, e.g. Ni in *Senecio coronatus*, apoplasts and vacuoles are the cellular locations for the metal once sequestered [41,42]. Girling and Peterson [6], confirmed the presence of AuCN in the leaf vacuoles and insoluble AuCl in cell walls. Recently however, the presence of Au as discrete metallic particles in the shoots of *Medicago sativa* have been observed [43]. At the molecular level, a number of genes have been discovered for ion transport in plants, e.g. ZIP gene family (Zn uptake), Nramp proteins (Mn uptake) and ATPases [42]. However, genes responsible for noble metal transport across plant cell membranes have not been elucidated. Further research is needed at the physiological and biochemical level to thoroughly understand the underlying processes involved in Au uptake.

3.2. Au cellular localisation and distribution

Proton induced x-ray emission spectroscopy (μ-PIXE) was performed at the Australian Nuclear Science and Technology Organisation (ANSTO) using the 10MV tandem accelerator [44]. Samples were analysed using a 3 MeV proton beam with a typical spot size between 3 and 5 μm. At this spot size, beam currents between 0.1 and 0.5 nA can be achieved, which is sufficient for μ-PIXE analyses. A high-purity Ge detector was used with a 100 mm^2 active area, located 33 mm from the sample. A 100 μm Mylar foil was used to reduce low energy X-rays and thus pile-up in the μ-PIXE spectrum. This set-up allowed the detection of the accumulated trace metals such as Ni and Cu with as a high sensitivity as for Au.

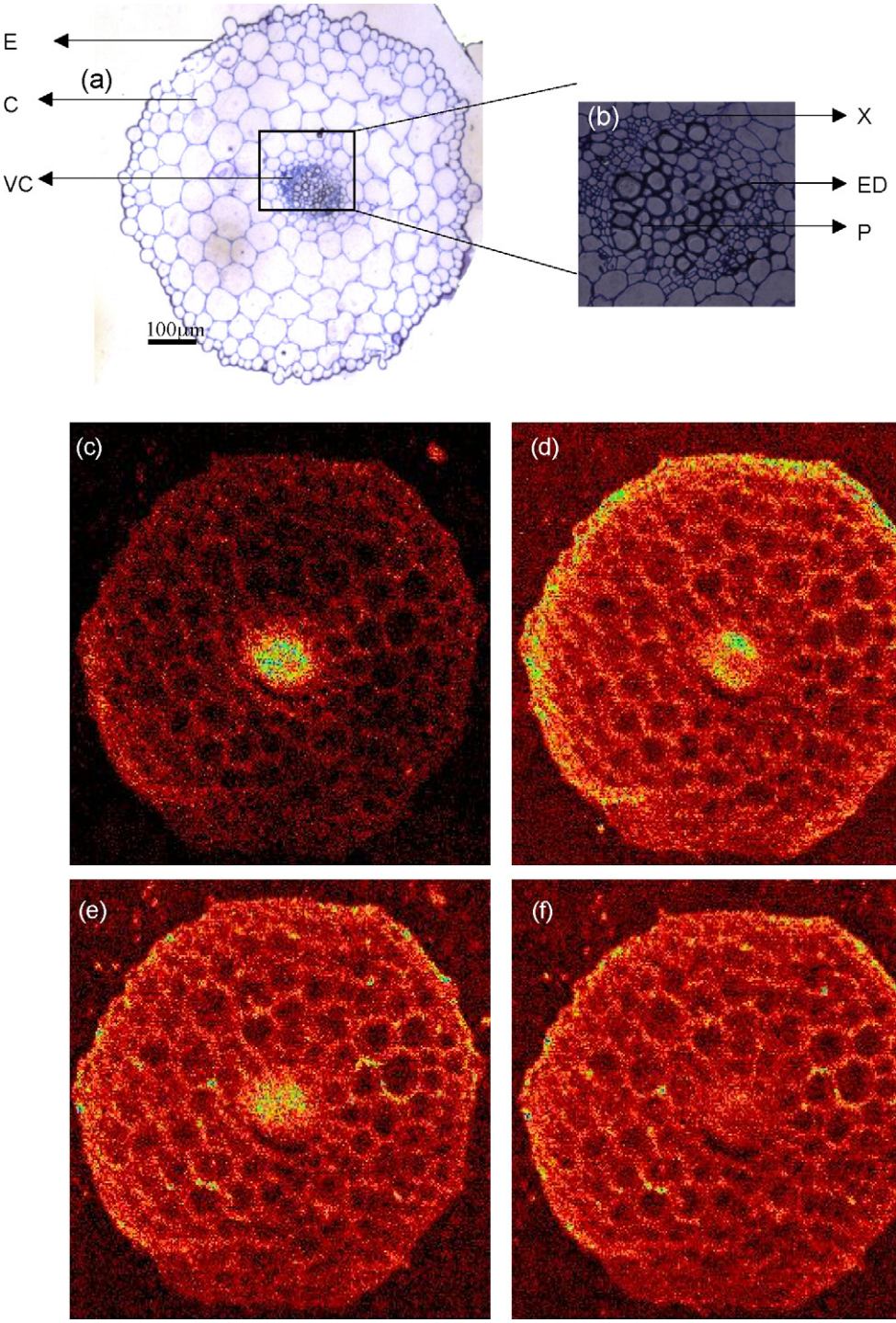

Fig. 6. Typical light micrograph (a) of BJ root cross section showing E, epidermal cells, C cortex cells and VC vascular cylinder. Central stele (b) showing xylem (X) phloem (P) and Endodermis (ED). Elemental maps of BJ root after 24 h of exposure to Au solution Gold (c), Calcium (d), Chlorine (e) Potassium (f), elemental maps of Gold (g) and Potassium (h) of MS stem after 24 h of exposure to 40 ppm Au solution, elemental maps of Gold of BJ root after 24 h of exposure to 80 ppm (i), elemental maps of Gold of BJ root after 48 h of exposure to 80 ppm (j), elemental maps of Gold of MS root after 24 h of exposure to 1000 ppm (k), elemental maps of Gold of MS root after 48 h of exposure to 1000 ppm (l). The concentration legend shows the qualitative measure of the relative concentration of the mapped element.

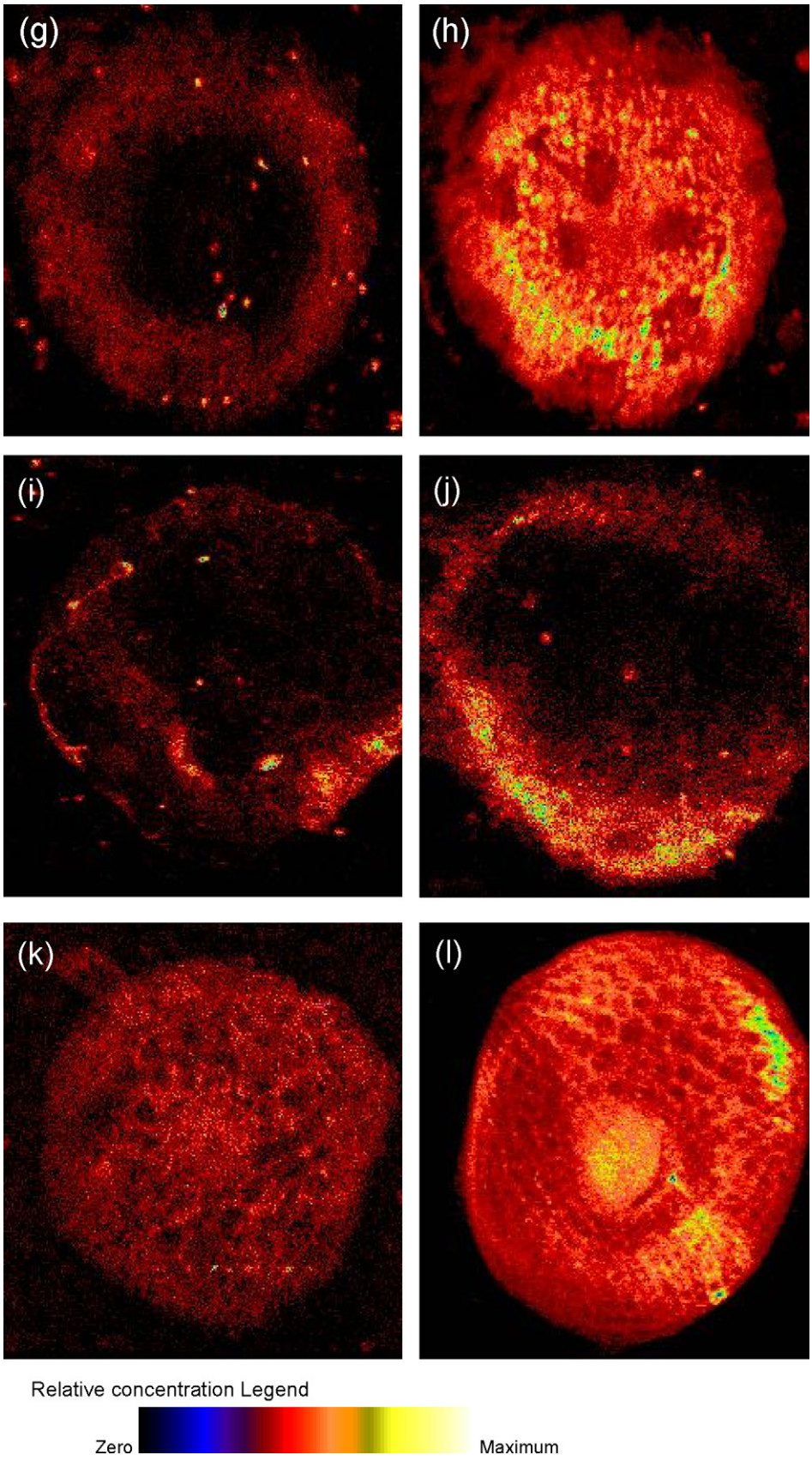

Fig. 6. (*Continued*)

Listmode data files were collected using the MICRODAS data acquisition system supplied by the University Of Melbourne. Data analysis was performed using the GeoPIXE II software package [45]. Real time quantitative elemental maps were generated using a dynamic analysis matrix transform method built into GeoPIXE II [46]. These are overlap resolved and include background subtraction.

A typical micro-PIXE spectrum from BJ root tissues is shown in Fig. 5. The peaks in the spectra clearly indicate the presence of Au, as well as the essential trace elements, Calcium (Ca), Potassium (K), Chlorine (Cl), Iron (Fe) and Copper (Cu).

For morphological and anatomical analysis, light micrographs of stem and roots cross sections were analysed. The transverse section of BJ root (Fig. 6 a) clearly shows the three tissue systems, i.e. dermal, ground and vascular. A single epidermal layer, followed by multi-layers of parenchymatous ground tissue and intercellular spaces, characteristic of the root system are visible. In the centre is the vascular bundle; strands of phloem are observed to alternate with the xylem ridges. Four xylem ridges (a tetrarch) is clearly indicated in the cross-section.

μ-PIXE analysis of BJ and MS stem and root show the presence of Au in all three tissue systems. A series of typical in situ, Au elemental maps for BJ root after 24 h exposure at 1000 ppm and MS stem after 24 h of exposure at 40 ppm are given in Fig. 6. Fig. 6 also indicates the elemental maps achieved by varying the exposure time for MS root at 80 ppm and BJ root at 1000 ppm.

These maps show the highest accumulation of Au occurs in the centre, indicating xylem loading, followed by the epidermis and cortex cells. BJ root sections show that the highest metal localisation is in the vascular bundles (VB) and xylem cells mainly because they are the means of metal translocation. The presence of Au was most notable in the vascular bundles (xylem cells), followed by epidermal cells in all the root and stem sections mapped for both BJ and MS. The results from μ-PIXE analysis of MS stems after 24 h exposure at lower Au concentration of 40 ppm (Fig. 6) indicate the accumulation of gold on the outer part of the stem section which constitutes epidermal cells and outer cortical region, whereas at higher concentrations of 1000 ppm the metal was present everywhere in the stem section, but highest in the vascular bundles.

Elemental maps for BJ root at 80 ppm after 24 and 48 h exposure clearly indicate the presence of Au in the outer epidermal cells and outer cortical cells (Fig. 6). The maps also suggests the presence of higher concentrations of Au in the epidermal and cortical cells with longer exposure time of 48 h as compared to 24 h. Similar results were observed at higher concentrations (1000 ppm) in MS root, although the metal was present everywhere in the section, higher concentrations were observed after longer exposure time. The most striking difference between BJ and MS root at 1000 ppm after 24 h is the high concentration of metal in the central stele in BJ root, where as in MS root the metal is dispersed equally throughout the section. However, at 48 h the metal seem to be concentrated in the central stele (Fig. 6).

4. Conclusions

Concentration data from ICP-OES and μ-PIXE analyses show that MS and BJ have the ability to accumulate large quantities of Au within plant tissues under idealised conditions (i.e. in the absence of a soil matrix and under high concentration driving forces) and can transport and concentrate Au to the stem at higher concentrations and longer exposure times. The study also shows that plants accumulate comparatively more Au in acidic media. Metal localisation studies with μ-PIXE indicate epidermal cells and vascular bundles to be the sites where Au accumulates in the greatest quantities.

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