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Accumulation and distribution of aluminium and other elements in tea (*Camellia sinensis*) leaves

H.P. Carr[a], E. Lombi[b]*, H. Küpper[c], S.P. McGrath[b], M.H. Wong[a]**

[a] Department of Biology and Institute for Natural Resources and Environmental Management, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China
[b] Agriculture and the Environment Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK
[c] Universität Konstanz, Mathematisch-Naturwissenschaftliche Sektion, Fachbereich Biologie, Fach M665, 78457 Konstanz, Germany

Abstract – Tea plants (*Camellia sinensis*) are well known for their ability to accumulate high concentrations of aluminium (Al). Aluminium distribution in tea leaves has been previously estimated on a qualitative basis using energy dispersive X-ray microanalysis (EDXMA). However, no comprehensive studies have been carried out to quantitatively determine the distribution of Al, especially in young leaves which are used for the manufacture of commercial tea. Cuttings of *C. sinensis* var. *sinensis* grown in acidic soil were collected and young and old leaves were used for analysis. Total Al concentrations in young and old leaves were 380 and 6866 $\mu g \cdot g^{-1}$, respectively. The leaves were freeze-fractured and the quantitative distribution of Al and other elements such as Mg, Ca and K was determined using EDXMA. In the young and old leaves Al was found to be preferentially accumulated in the upper epidermis. In particular, Al was accumulated in the cell walls, which had significantly higher levels than the vacuoles of epidermal cells. The Al concentrations in the vacuole of mesophyll cells was found not to change significantly between young and old leaves. In contrast, a large increase in Al concentration was observed in the epidermal cell walls. Calcium was present in higher levels on the cell walls and also present as crytals in the spongy mesophyll tissue. In contrast, K and Mg were evenly distributed across the leaf.

aluminium / *Camelia sinensis* / tea / accumulation / EDXMA

Résumé – **Accumulation et distribution de l'aluminium et d'autres éléments minéraux dans les feuilles de thé (*Camellia sinensis*).** Les plants de thé (*Camellia sinensis*) sont bien connus pour leur capacité à accumuler d'importantes quantités d'aluminium (Al). La distribution de l'Al dans les feuilles de thé a déjà été estimée de manière qualitative par le biais de la microanalyse par dispersion d'énergie de rayons X (EDXMA). Cependant, aucune analyse détaillée n'a été conduite pour déterminer la distribution quantitative d'Al plus particulièrement dans les jeunes feuilles qui sont celles utilisées dans l'industrie du thé commercial. Des boutures de *C. sinensis* var. *sinensis* cultivées dans des sols acides furent collectées et des feuilles jeunes et âgées furent utilisées pour l'analyse. Les concentrations en Al total dans les feuilles jeunes et âgées étaient de 380 et 6866 $\mu g \cdot g^{-1}$ respectivement. Les feuilles ont été congelées et fractionnées et la distribution quantitative d'Al et d'autres éléments tels que Mg, Ca, et K fut déterminée en utilisant une EDXMA. Dans les feuilles jeunes et âgées, on a observé que l'Al s'accumulait préférentiellement dans les épidermes supérieurs. En particulier, l'Al s'accumulait dans les parois cellulaires, qui avaient des concentrations significativement plus fortes que les vacuoles des cellules épidermiques. On a observé que des concentrations en Al dans les vacuoles des cellules du mésophylle ne changeaient pas significativement entre les feuilles jeunes et âgées. Par contre, une augmentation importante de la concentration en Al fut observée dans les parois des cellules épidermiques. Du calcium était présent en grande quantité sur les parois cellulaires ainsi que sous forme de cristaux dans le tissu du mésophylle spongieux. En revanche, le K et le Mg étaient uniformément distribués dans la feuille.

aluminium / *Camelia sinensis* / thé / accumulation / EDXMA

1. INTRODUCTION

Aluminium (Al) is the most abundant metal in the earth's crust, where it generally occurs as a component in a variety of crystalline aluminosilicates, oxyhydroxide and non-silicate-containing minerals [5, 17]. In this form, Al is usually regarded as being unavailable for chemical and biological reactions. However, under acidic conditions it is soluble and more available for various biochemical processes. Aluminium is toxic to a number of plants, primarily affecting the roots and causing

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* Present address: CSIRO Land and Water, PMB2 Glen Osmond, SA5064, Australia.
** Corresponding author: mhwong@hkbu.edu.hk

major agronomic problems affecting up to 70% of potentially cultivable land [2]. Nevertheless, some plants have adapted to the high soluble Al contents in soils, especially in tropical regions, by becoming excluders or accumulators [4, 9, 11].

Tea (*Camellia sinensis*) is cultivated on soils of low pH, rich in available Al. *C. sinensis*, similarly to what is found in other related familes of the *Ericales* (+*Jansen*), accumulates large amounts of Al, especially in its shoots. Chenery [3] proposed a possible nutritive role for Al in tea. This has been explored by other authors [12, 15] but its physiological role remains unknown. The concentration of Al in the leaves is positively correlated with the age of the leaf, with older leaves accumulating about 10 times more than young leaves [24, 27]. Reported concentrations in old leaves vary from 5000–30 000 $\mu g \cdot g^{-1}$ [15].

Aluminium is reportedly neurotoxic to a number of animal species including man [1, 10], and although it is still under debate, Al has been linked to Alzheimer's disease [10, 16]. For many people tea is probably the most important single source of dietary Al [8] even though there is still an intense debate regarding the bioavailability of Al in tea infusions [for review see 7]. There are several different varieties of teas produced [27]. Most teas (green and black) use the flush or newly produced leaves. For other teas such as brick tea, old leaves and branches are used. The increased Al in the old leaves of tea could increase the Al load in the diet [24]. Therefore, it is important to enhance our understanding concerning the mechanism of accumulation of Al in tea plants.

Aluminium distribution in old tea leaves has been previously estimated on a qualitative (or semi-quantitative) basis using energy dispersive X-ray microanalysis (EDXMA). Several authors reported that Al was localised in the epidermal regions [6, 15, 18]. However, no comprehensive studies have been carried out to quantitatively determine the distribution of Al, especially in young leaves that are used for the manufacture of commercial tea.

The objective of this paper is to investigate the localisation of Al and other elements, such as Ca, K and Mg, in the leaves of *C. sinensis*. These elements were also quantified in situ using a method that we have recently developed [13].

2. MATERIALS AND METHODS

Cuttings of mature bushes of *C. sinensis* L. var *sinensis*, grown on acidic soil of pH 4.5, were obtained from the tea house of the Unilever corp. (Bedford, UK). Both young (newly produced flush leaves) and old leaves from the base of the branch were collected from different plants. The leaves were cleaned with a tissue paper and a small section of the leaves was excised, mounted in a stainless steel vice, and rapidly frozen in melting nitrogen slush. The sample was transferred to a preparation chamber, cooled to –180 °C and fractured. The sample was evaporatively coated with carbon and the specimen was transferred to a cooled stage (–170 °C) inside the Scanning Electron Microscope (model XL 40, Phillips). Energy dispersive X-ray microanalysis was performed in the scanning electron microscope (SEM), using an acceleration voltage of 30 kV. Aluminium and other elements were quantitatively analysed in situ using the method described by Küpper et al. [13]. In this method, the counts of Al and other elements were normalised on a molar basis to the oxygen counts. A semi-quantitative, two-dimensional distribution pattern was also recorded by scanning an area of the specimen repeatedly for up to 2 h and integrating the counts for Al, Ca and K within their respective spectrum windows into dot-maps.

After EDXMA analysis, the leaves were air dried and digested in a microwave with 70% nitric acid. Aluminium, Ca, Mg and K were quantified in the digest by Inductively Coupled Plasma Emission Spectrometry (Perkin Elmer Optima 3000DV). Fluoride was measured using an F selective electrode (Cole-Palmer 27502-18,19 Illinois), which was calibrated with F standard [25]. Data of element concentrations from different regions in the leaves were subjected to two-way ANOVA to evaluate the difference in elemental concentrations in the various tissues. Comparisons were made using Duncan's Multiple Range Test from SPSS 9.0.

3. RESULTS AND DISCUSSION

Table I shows the total concentrations of metals in the leaves of *C. sinensis*. The young leaves have significantly lower metal concentrations than the older leaves. In particular, the concentration of Al increases in mature leaves by approximately 18 times. This result is in agreement with previous findings that Al is continuously accumulated in tea leaves and its concentration increases with time [3, 18]. The concentration of F in the leaves increased from 3 to 227 $mg \cdot kg^{-1}$. Yamada and Hattori [26] suggested that F could bind Al in tea leaves. However, an Al/F molar ratio of 21 in old leaves (from Tab. I) indicates that only a small part of Al could be associated with Nagata et al. [19, 20] demonstrated, using ^{27}Al and ^{19}F NMR, that most of the Al is complexed in tea with catechins and only a small portion is bound to F.

Al is preferentially accumulated in the epidermal cell walls, especially in the epidermis of old leaves (Figs. 1A and 1B). This is in agreement with observations of old leaves by Matsumoto et al. [15] and Memon et al. [18]. However, an accurate analysis of the adaxial side of the leaf showed that Al is also accumulated in the cuticle (Figs. 1C and 1D). The distribution of Al in young leaves cannot be shown by Al dot-maps due to the much lower concentration of the metal in these leaves.

Table I. Total metal ($\mu g \cdot g^{-1}$) in the leaves of *Camellia sinensis*.

	Al	Ca	F	K	Mg	Mn
Young leaves	381 ± 80	3560 ± 364	3 ± 1	2916 ± 1214	1716 ± 108	113 ± 40
Old leaves	6866 ± 645	18784 ± 2674	227 ± 21	15239 ± 2552	2596 ± 343	1340 ± 189

Values represent the mean ± standard errors. N = 4.

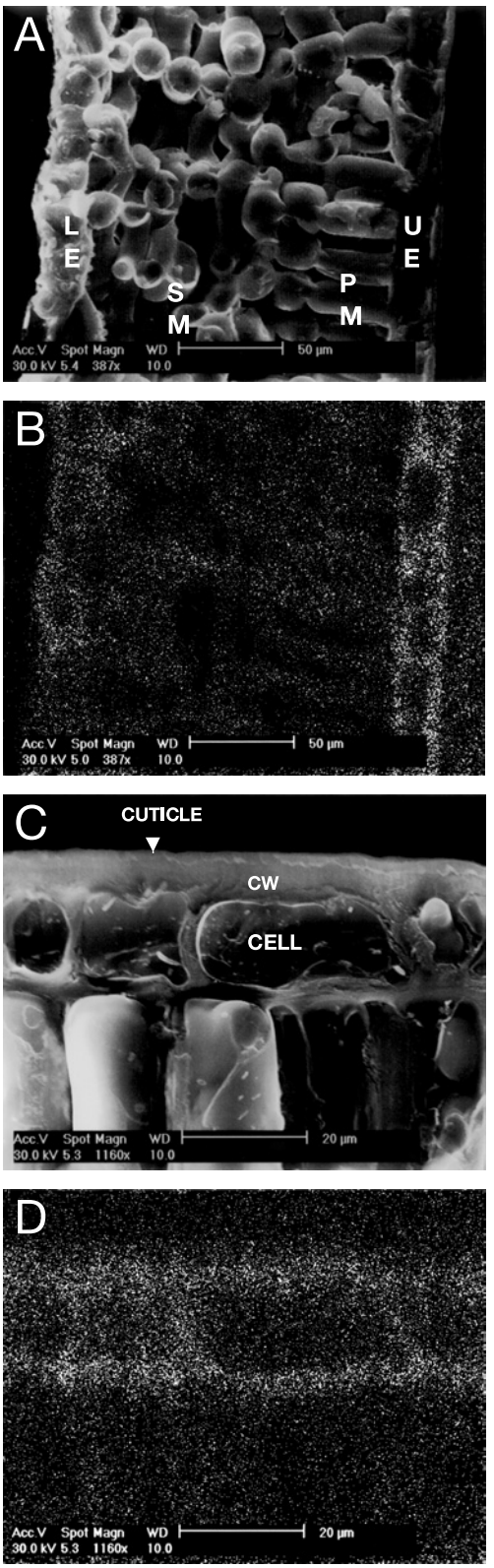

Figure 1. Al distribution in an old leaf of tea analysed using EDXMA. (A) Cross-section and (B) Al dot-map of the leaf. (C) Upper epidermal region and (D) Al distribution. LE: Lower epidermis, SM: Spongy mesophyll, PM: Palisade mesophyll, CW: cell wall.

Calcium dot-maps showed the presence of Ca crystals in the spongy mesophyll tissues (Figs. 2A and 2B).

The concentrations of the different elements in the various tissues of the leaf were determined using the method that we developed to quantify different elements in situ using EDXMA [13]. In the young leaves the cuticle and the cell wall of mesophyll cells were too thin to be analysed for their Al concentration. In both young and old leaves most of the Al is concentrated in the upper and lower epidermis (Figs. 3A and 3B). In both young and old leaves the largest accumulation of Al was observed in the outer cell walls of the upper epidermis with concentrations of 5 and 323 mM, respectively. The presence of high levels of Al in the epidermal regions is in good agreement with the results of Matsumoto et al. [15]. The palisade and spongy mesophyll regions contained significantly lower concentrations of Al, which did not significantly increase in the old leaves. In contrast, the Al concentration in the cell walls significantly increased in old leaves, especially in the upper epidermal cells, by a factor of 64 and 270 for the outer and inner parts of the cell walls, respectively. These results are in partial agreement with the data shown by Echlin [6] who did not, however, quantify Al concentrations but reported the peak/local background ratios for Al in young and

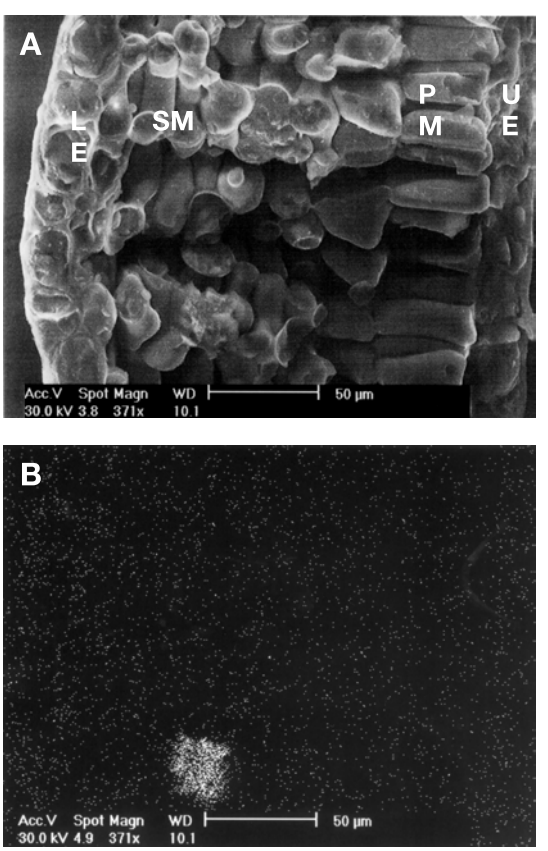

Figure 2. Cross-section of a mature leaf (A) and correspondent Ca dot-map (B) showing the presence of Ca crystals in the spongy mesophyll tissues. LE: Lower epidermis, SM: Spongy mesophyll, PM: Palisade mesophyll, UE: Upper epidermis.

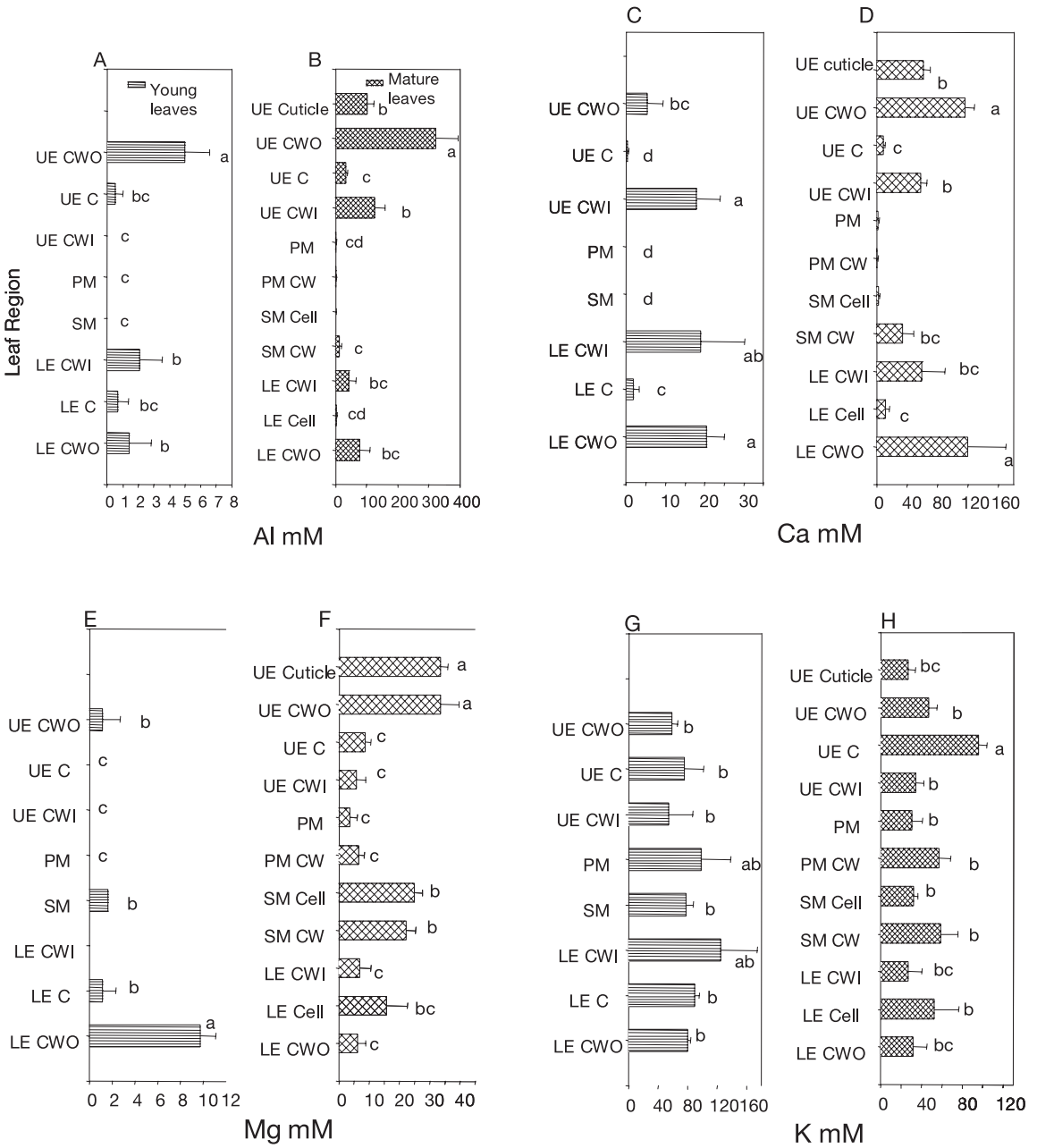

Figure 3. Distribution of Al (A, B), Ca (C, D), Mg (E, F) and K (G, H) in different regions of young and mature leaves of *C. sinensis*. Error bars represent standard errors of 3–12 replicates. Different lower case letters indicate significant differences according to Duncan's multiple range test ($P < 0.05$). UE: upper epdermis, CWO: outer cell wall, C: cell, CWI: inner cell wall, PM: palisade mesophyll, SM: spongy mesophyll, LE: lower epidermis.

old leaves. For example, in the case of epidermal cells we quantified concentrations of Al in the cell walls to be about 10–20 times larger than in the vacuoles of the same cells, whereas Echlin [6] reported a concentration factor of less then 2. However, the latter is based on peak/local background ratios that can be inaccurate, due to saturation of the signal at high metal concentrations.

The physiological role of Al accumulation in tea is not known. Matsumoto et al. [15] reported that Al promoted the growth of seedlings and hypothesised that Al could accelerate the formation of new roots and might be translocated to the leaves and accumulated as a waste material. Rengel [23] suggested that the extent of Al accumulation may be related to the capacity of a plant to detoxify and/or store Al rather than the

uptake rate. Our results clearly showed that Al is mainly stored in the cell walls and that the concentration of Al in this compartment increases with time. Detoxification of toxic metals by means of compartmentation at subcellular level is a common feature of hyperaccumulator plants. However, the pattern of compartmentation seems to be species- and metal-specific. For instance, heavy metals such as Ni, Cd and Zn are generally stored in the vacuoles of epidermis cells [14, 22]. In the case of Al in *C. sinensis*, the results of our work suggest that Al compartmentation in cell walls may be the major mechanism responsible for Al detoxification.

The concentration of Ca increased significantly in the old leaves. It appears that in the tea plants the Ca uptake systems are very efficient even when the plants are exposed to large concentrations of available Al, that would reduce the uptake of Ca in other plants [21]. The molar Ca/Al ratio decreases from 6.7 in the young leaves to 1.8 in the old leaves.

A large proportion of the Ca was localised in the cell walls of the epidermis (Figs. 3B and 3C) (116 ± 11 and 120 ± 49 mM, respectively). Crystals containing large amounts of calcium (up to 2 M) were observed in the spongy mesophyll (Fig. 2). The nature of these crystals is not known but EDXMA analyses did not reveal the presence of phosphate or other inorganic anions. Apart from this, the largest concentrations of Ca were observed in the outer walls of the upper and lower epidermis of old leaves (116 ± 11 and 120 ± 49 mM, respectively). Significantly ($P < 0.05$) less Ca was found in the vacuoles (1.9–11.9 mM in the old leaves).

Young leaves contained more K than the older leaves, whereas the concentration of Mg increased with the age of the leaves (Figs. 3E, 3F, 3G, and 3H). The distribution of K and Mg in the old leaves was similar. Both elements were more evenly distributed across different cell types than Al and Ca. The mean concentration of Mg in the cell walls and vacuoles was 13.6 (±11) mM and 13.2 (±9) mM, respectively. In the case of K the mean concentration in the cell walls was 42.8 (±13.5) mM and vacuolar concentration 53.0 (±30) mM.

4. CONCLUSIONS

The method used to quantify the concentration of Al in situ proved to be suitable for analysing the distribution of Al in young leaves of tea that are used for commercial purposes. The stimulatory effect of Al on the growth of tea plants has been shown by various authors [e.g. 12]. However, its physiological role is still poorly understood. In the present study we have demonstrated that Al in both young and old leaves is preferentially accumulated in the epidermis. This distribution resembles the localisation of Zn in the leaves of the hyperaccumulator *Thlaspi caerulescens* [14]. However, in *Thlaspi* Zn is preferentially accumulated in the vacuoles, whereas Al in tea is mainly accumulated in the cell walls. This pattern of Al compartmentation, restricted to areas outside the cytoplasm, suggests that Al may not play a large role in metabolic processes in leaves of *C. sinensis*.

The Zn accumulated in the vacuoles of *Thlaspi* leaves is largely soluble (approximately 80%) whereas Al accumulated in the cell walls is probably much more tightly bound and this may help in reducing the concentration of Al in tea infusions.

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