ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Localization and compartmentation of Al in the leaves and roots of tea plants

Localización y compartimentación de Al en hojas y raíces de plantas de té

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Abstract. Under acid soil conditions, solubility of aluminum (Al) increases leading to toxicity for plants. Al accumulator species such as tea, however, accumulate high levels of Al in tissues without toxicity symptoms. In this work, Al localization and compartmentation were studied in tea [*Camellia sinensis* (L.) O. Kuntze] grown hydroponically at 0 or 100 μM Al for eight weeks. Plant dry matter production was significantly higher in the presence of Al and accumulated up to 1.21 and 6.18 mg Al/g DW in the leaves and roots, respectively. About 40-50% of Al was partitioned into cell wall (CW)-bound fraction without any difference among leaves of different age and roots. A significant increase of the soluble phenolics fraction by Al was observed in both leaves and roots. Conventional and confocal laser scanning microscopy images of morin-stained roots indicated a high fluorescence signal in the caps and adjacent mersitematic cells. Towards basal parts, however, Al tended to accumulate mainly in the root hairs, rhizodermal and endodermal cell layers and slightly in the cortex while it was clearly excluded from the central cylinder. A high Al-morin signal was detected from the CW compared with other parts of the cells. Relatively high green fluorescence signal was emitted from the epidermal cell layer, trichomes, vascular bundle region and stomatal cells of particularly young leaves. Our study provides evidences for involvement of both avoidance and tolerance mechanisms for Al in tea plants.

Keywords: Aluminum; Morin; Fluorescence; Tea; Confocal laser scanning microscopy (CSLM).

Resumen. La solubilidad del aluminio en los suelos aumenta considerablemente bajo condiciones ácidas produciendo toxicidad en las plantas sensibles. Algunas especies adaptadas como por ejemplo el té, sin embargo pueden acumular elevados niveles de Al en sus tejidos sin mostrar síntomas de toxicidad. En este trabajo se estudió la localización y compartimentación de Al en plantas de té [*Camellia sinensis* (L.) O. Kuntze] cultivadas hidropónicamente con 0 o 100 μM de Al durante ocho semanas. La producción de peso seco fue significativamente mayor en las plantas expuestas a Al. Éstas acumularon hasta 1,21 y 6,18 mg de Al por g de peso seco en hojas y raíces, respectivamente. Tanto en raíces como en hojas, e independientemente de su edad, entre el 40 a 50% del Al se repartió en las paredes celulares. El suministro de Al incrementó de forma significativa la concentración de sustancias fenólicas solubles en raíces y hojas. Raíces teñidas con morin mostraron fuerte fluorescencia en el ápice y las células meristmáticas, visualizada tanto con microscopía de fluorescencia convencional como con microscopía confocal de láser. En las zonas más basales, sin embargo, el Al se visualizó principalmente en los pelos radiculares, en las células de la rizodermis y del a endodermis y en el córtex, mientras que quedó excluido del cilindro central. Las paredes celulares mostraron mayor señal de fluorescencia procedente del complejo Al-morin que otras partes celulares. En las hojas, especialmente las más jóvenes fluorescencia verde bastante intensa también fue emitida por la epidermis, los pelos, la zona vascular y las células estomáticas. Nuestros resultados muestran que en las plantas de té operan tanto mecanismos de exclusión como de tolerancia al Al.

Palabras clave: Aluminio; Morin; Fluorescencia; Té; Microscopía confocal de láser.

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Recibido / Received 10.III.2014. Aceptado / Accepted 28.IV.2014.

INTRODUCTION

Aluminum toxicity is a major abiotic factor limiting crop productivity on acid soils (Kochian et al., 2004; Poschenrieder et al., 2008). Nevertheless, beneficial effects of Al were reported on growth of several plant species that were well adapted to temperate or tropical acid soils (Osaki et al., 1997; Hajiboland et al., 2013b). The tea [*Camellia sinensis* (L.) O. Kuntze] plant is an Al tolerant species adapted to acid soils, and accumulates Al in leaves at concentrations as high as 30000 and 600 μg/g DW in old and young leaves, respectively (Matsumoto et al., 1976). Accumulation of high levels of Al is not accompanied by any growth impairment in tea plants. In contrast, growth stimulation has been frequently reported in this species under Al concentrations up to 400 μM in the medium (Matsumoto et al., 1976; Konishi, 1992; Hajiboland et al., 2013b). Though decline of free Al^{3+} concentrations to nanomolar orders of concentrations due to a high pH (7.0-7.5) in the cytosol, these are still potentially phytotoxic (Ma et al., 1998). Accordingly, Al accumulator species need efficient compartmentation and detoxification mechanisms.

Investigation of elemental micro-distribution in hyperaccumulator plants helps to identify the mechanisms for accumulation and detoxification of heavy metals. Such studies have been applied for evaluation of the sub-cellular distribution of Cd (Weigel & Jäger, 1980; Wang et al., 2008), Mn (Rogalla & Römheld, 2002) and As (Chen et al., 2005) in plants. Various methods have been used for investigation of Al speciation and binding form in non-accumulator (Tice et al., 1992) and accumulator (Watanabe et al., 1998) species. However, reports on the application of methods providing a quantitative estimation of Al distribution into various cell fractions are restricted to Al-accumulators other than tea (Shen et al., 2002, Klug & Horst, 2010, Campos et al., 2014).

Phenolic compounds are among the most widely distributed natural products in the plant kingdom and participate in significant ecophysiological phenomena. One of the most striking biochemical features of tea leaves is the high content of phenolic compounds. By means of ^{27}Al-NMR it was shown that Al is chelated by phenolic compounds (mainly catechin) in the leaves of tea (Nagata et al., 1992). Even more, electron microscopy allowed to know that catechin is localized mainly in the vacuole of tea leaves (Suzuki et al., 2003). In the apical parts of tea roots (0-20 mm), however, Al presents mainly as complexes with oxalate (Morita et al., 2008).

Pioneer studies on Al hyperaccumulation in tea leaves indicated a preferential accumulation in cell walls of epidermal cells (Matsumoto et al., 1976). Investigations using energy-dispersive microanalysis (EDXMA) have also shown that in old tea leaves, Al is mainly accumulated in the cell walls of epidermal cells. However, in younger tea leaves, the EDXMA was not sufficiently sensitive to obtain Al localization maps or to detect Al in symplast compartments (Carr et al., 2003). More recently, using low-energy X-ray fluorescence spectromicroscopy (LEXRF) we have shown that Al was mainly localized in the cell walls of the leaf epidermal cells, while almost no Al signal was obtained from the leaf symplast. The results suggest that the retention of Al in the epidermal leaf apoplast represents the main tolerance mechanism to Al in tea plants (Tolrà et al., 2011).

Conventional microscopy techniques combined with staining with fluorophores such as morin (2′,3,4′,5,7-Pentahydroxyflavone) (Vitorello & Haug, 1997) and lumogallion (Kataoka et al., 1997; Amenós et al., 2009) have been applied for Al visualization and localization in plant tissues. Several authors used morin to study distribution of Al in the roots of cereals (Tice et al., 1992; Wang et al., 2004; Arroyave et al., 2011) and *Arabidopsis* (Illéš et al., 2006). Morin is a pentaprotic acid that forms a highly fluorescent complex with Al, and is used along with fluorescence microscopy to sensitively localize Al in plants. However, all these staining procedures were applied to roots, because in both Al-sensitive and Al-tolerant species root tips are the most sensitive part of the plant to Al toxicity. For localization of Al in leaves, haematoxylin (Andrade et al., 2011; González-Santana et al., 2012) and pyrocatechol violet (Maejima et al., 2014) have been used for some Al-accumulator species other than tea. Recently, the morin staining method was applied for localizing Al in the leaves of *Andropogon virginicus* (Poaceae), an Al-accumulator species (Ezaki et al., 2013). Staining methods including morin staining have not been used for localization of Al in the leaves or roots of tea plants. Matsumoto et al. (1976) and Hajiboland et al. (2013b) showed difference in the Al accumulation between young and mature leaves on tea plants. However, comparative studies are lacking on the Al compartmentation or detoxification in the leaves of different age in this species.

The aim of this work was to study the Al localization and compartmentation in the roots and leaves of different age of tea plants using morin staining method. Two different approaches were adopted to achieve this goal, tissue fractionation and microscopic localization.

MATERIALS AND METHODS

Plant material and treatments. Seeds of tea [*Camellia sinensis* (L.) O. Kuntze] plants were collected from the garden of the Tea Research Station in Fuman (Guilan Province, Iran). Hulled seeds were surface-sterilized with 1% active hypochlorite, germinated on perlite in the dark, and moistened by distilled water and saturated with $CaSO_4$ every day. After emergence of the primary leaves, seedlings were transferred to the light. One month-old seedlings were transferred to 2 L pots containing nutrient solution (pH 4.0), and were precultured for one week. Thereafter, Al treatments were started. The composition and final concentration of nutrients were (mM) $(NH_4)_2SO_4$ (0.036); NH_4NO_3 (0.33); K_2SO_4 (0.02);

$CaCl_2$ (0.13); $MgSO_4$ 0.021; KH_2PO_4 (0.013); $MgCl_2$ (0.08); KCl (0.18), and μM: Fe-EDTA (8.0); H_3BO_3 (11.5); $CuSO_4$ (0.5); $MnSO_4$ (22.5); $ZnSO_4$ (2.27) and $(NH_4)_7Mo_6O_{24}$ (0.09) (Hajiboland et al., 2013b). Al treatments consisted of 0 and 100 μM $AlCl_3$, and activity of free Al^{3+} was 0 and 43 μM, respectively (Hajiboland et al., 2013b). Nutrient solutions were replaced every 3 days. Each treatment consisted of four replications using four pots with one plant per pot. Plants were grown under controlled environmental conditions with a temperature regime of 25/18 ˚C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and a photon flux density of about 400 μmol/m^2/s.

Plants harvest, growth and Al analysis. Eight weeks after treatment (13 weeks after sowing), plants were harvested. The shoot was separated into the 3rd oldest and the 2nd youngest, fully-expanded leaves for further analyses. Oven-dried samples were transferred to porcelain crucibles and wet-ashed with a perchloric acid-nitric acid mixture (1:3 v/v) for 4 h, resolved in 0.5 M HCl, and made up to volume by double-distilled water. Concentration of Al was determined in the samples by atomic absorption spectroscopy (AAS) (Shimadzu, AA6300).

Fractionation of tissues for Al. Leaves were cut gently into two halves along the midrib using a surgical blade, and subsamples from each half were subjected to the Al analysis either directly (total) or after one fractionation procedure. Fractionation of leaves for Al was performed according to the Tris-HCl extraction method described by Watanabe et al. (1998). Fresh samples were homogenized using a mortar and pestle in a medium containing 50 mM Tris-HCl (pH 7.4) (100 mg: 1ml buffer) at 4 ˚C. The homogenate was strained through eight layers of cheesecloth. The residue was washed twice with the homogenization buffer and liquid was expressed from the residue. The pooled washes, together with the first filtrate, were centrifuged at 3000 g for 10 min. The resulting pellet combined with the residue of the cheesecloth filtration was designated as the CW fraction. This residue (CW-bound) and supernatants or washing liquids (non-CW-bound) were both subjected to elemental analysis after drying and ashing as described above. The recovery (%) was calculated using the sum of Al content in both fractions relative to the total Al content obtained from the parallel subsamples without fractionation. Recovery (%) was mainly above 90%, thus, the amount of non-CW-bound Al fraction was reported in this work as the difference between total Al content and CW-bound Al.

Extraction and determination of soluble and CW-bound phenolics. Leaf and root samples were used for extraction of water-soluble and CW-bound phenolics according to the method described elsewhere (Hajiboland et al., 2013a) using Folin-Ciocalteau reagent for determination of phenolics in these fractions (Swain & Hillis, 1959). Total phenolics content was obtained as sum of the amounts of two fractions; data were reported as relative (% over total phenolics content) values.

Fluorescence microscopy. Free-hand sectioning without fixation and embedding was employed in all microscopy studies in this work. Lateral roots were subjected to staining and sectioning procedures. Using lateral instead of tap roots provided sufficient material for frequent sectioning and made it possible to have control roots without Al traces derived from seeds found in the tap roots. The terminal 30 mm of lateral roots were excised and washed shortly with deionized water and either directly or after free-hand sectioning subjected to staining. The same procedure was applied for the leaves. The youngest leaf at the apical region, the petiole of the young leaves and old leaves were excised and washed shortly with deionized water; after free-hand sectioning, they were subjected to staining. The sections were made with a sharp razor blade and were gently removed with a paintbrush and collected in Eppendorf microtubes containing deionized water. Root tips after excision and sectioning were kept approximately for 20 min each in deionized water before staining. Pictures shown are representative of 4-5 replicates per treatment.

Vital (Fluorescein acetate-propidium iodide) staining. To visualize Al-induced membrane damage, root tips were double-stained with fluorescein acetate and propidium iodide (vital staining) according to Jones and Senft (1985).

Morin staining. After collection of sufficient root and leaf sections, distilled water was replaced by 100 μM morin (Fluka) in 10 mM MES buffer pH 5.5 for 30 min. Then, they were washed twice with MES buffer and deionized water for 5 min each (Zheng et al., 2005). A number of checks on autofluorescence were conducted including examination of control roots and leaves with and without staining, and of Al treated roots without staining.

Sections were mounted in distilled water on the slides and subjected to fluorescence microscopic observations considering excitation-emission spectra of 420-510 and 490-520 for morin and fluorescein acetate-propidium iodide stained samples, respectively. Fluorescence was visualized with a fluorescence stereomicroscope equipped with a Nikon H55OS lens, Intensilight C-HGFI and a Nikon Digital sight DS-L1.

The fluorescence emitted from the Al-morin complex was also observed under a confocal laser optic microscope operating in the reflection mode, using excitation wavelength at 420 nm and emission at 510 nm (TCS SP2 AOBS Leica microscopy Systems GmBH, Mannheim, Germany).

RESULTS

Plant dry matter production was significantly higher in the presence of 100 μM Al than in controls (Table 1). As expected, Al accumulated in both leaves and roots. The highest Al accumulation was observed in the roots, followed by the old and young leaves. A considerable Al concentration was also detected in the plants without Al treatment (Table 2).

Table 1. Shoot and root DW (g/plant) of tea plants grown hydroponically in the absence (–Al) or presence of 100 μM Al (+Al) for 8 weeks.
Tabla 1. Peso seco de partes aéreas y raíces (g/plant) de plantas de té cultivadas en solución nutritiva sin (–Al) o con 100 μM Al (+Al) durante 8 semanas.

Treatments	Shoot DW	Root DW
-Al	1.98 ± 0.25 b	0.35 ± 0.08 b
+Al	3.11 ± 0.45 a	0.51 ± 0.05 a

Data within a column followed by the same letter are not significantly different ($p<0.05$).

Table 2. Concentrations of Al (μg/g DW) in the young and old leaves and roots of tea plants grown hydroponically in the absence (–Al) or presence of 100 μM Al (+Al) for 8 weeks.
Tabla 2. Concentraciones de Al (μg/g peso seco) en hojas jóvenes y viejas de plantas de té cultivadas en solución nutritiva sin (–Al) o con 100 μM Al (+Al) durante 8 semanas.

Treatments	Young leaves	Old leaves	Roots
-Al	156 ± 39 b	759 ± 81 b	946 ± 99 b
+Al	622 ± 48 a	1211 ± 171 a	6182 ± 401 a

Data within a column followed by the same letter are not significantly different ($p<0.05$).

About 40-50% of the Al was partitioned into CW-bound fraction and the remaining was found in non-CW-bound fraction; leaves of different age and roots did not differ in this regard. Aluminum treatment slightly increased the CW-bound fraction of Al in the leaves, and reduced it in the roots (Table 3). In the leaves of control plants most phenolics (76-84%) were found in the soluble fraction. However, phenolics were partitioned evenly between bound and soluble fractions in the roots. Upon Al treatment, a significant increase of the soluble phenolics fraction was observed in both leaves and roots (Table 3).

Root microscopy studies. Roots of plants exposed for one week to Al concentrations up to 100 μM (43 μM free Al^{3+} activity) did not show signs of damage in the tips (Fig. 1).

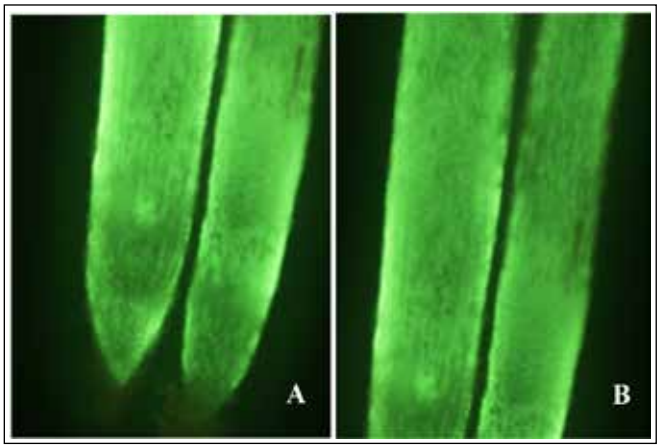

Fig. 1. Vital staining of tips (A) and basal parts (B) of the roots in control (left) and Al-treated (right) tea plants (100 μM Al for one week) (×10).
Fig. 1. Tinción vital de zona apical (A) y zona basal (B) de plantas control (izquierda) y plantas expuestas a 100 μM Al (derecha) durante 1 semana (×10).

Table 3. Relative Al and phenolics contents (% over total content) in different cell fractions of the young and old leaves and roots of tea plants grown hydroponically in the absence (–Al) or presence of 100 μM Al (+Al) for 8 weeks.
Tabla 3. Contenidos relativos de Al y sustancias fenólicas (% respecto contenido total) en diferentes fracciones celulares de raíces y hojas jóvenes y viejas de plantas de té cultivadas en solución nutritiva sin (–Al) o con 100 μM Al (+Al) durante 8 semanas.

Treatment	Young leaves		Old leaves		Roots	
	CW-bound	nonCW-bound	CW-bound	nonCW-bound	CW-bound	nonCW-bound
			Aluminum			
-Al	42 ± 9 a	58 ± 12 a	46 ± 4 a	54 ± 8 a	47 ± 5 ab	53 ± 7 ab
+Al	49 ± 5 a	51 ± 8 a	51 ± 9 a	49 ± 7 a	42 ± 3 b	58 ± 6 a
			Phenolics			
-Al	24.1 ± 3.2 c	75.9 ± 5.9 b	16.3 ± 2.1 c	83.7 ± 2.9 b	43.7 ± 8.1 b	56.3 ± 5.3 b
+Al	8.0 ± 0.98 d	92.0 ± 3.8 a	4.8 ± 0.12 d	95.2 ± 4.8 a	8.1 ± 1.7 c	91.9 ± 8.6 a

Data of each plant part followed by the same letter are not significantly different ($p<0.05$).

After 24-hour exposure to Al, intense morin staining was observed in roots (Fig. 2), especially in the root hair zone (Fig. 2B). At the same time, root tips exhibited less green fluorescence (Fig. 2A), and the older part of the roots above the hair zone was almost unstained (Fig. 2C). Such a high Al concentration in the root hairs was observed also after haematoxylin staining (images not shown).

In the root tip, conventional microscopy images of the cap zone (Fig. 3) showed a slight Al accumulation mainly in the lateral root cap. In CLSM images of morin-stained roots, however, a relatively high fluorescence signal was detected in the caps of Al-treated roots (Fig. 4B). The same was true for the adjacent meristematic cells (Fig. 4C). Any fluorescence signal could be detected in morin-stained sections prepared from the same zone, but without Al treatment (Fig. 4A).

Free-hand cross sections of the zones behind the root cap in morin-stained roots showed a significant difference in the accumulation pattern between apical and basal parts of the

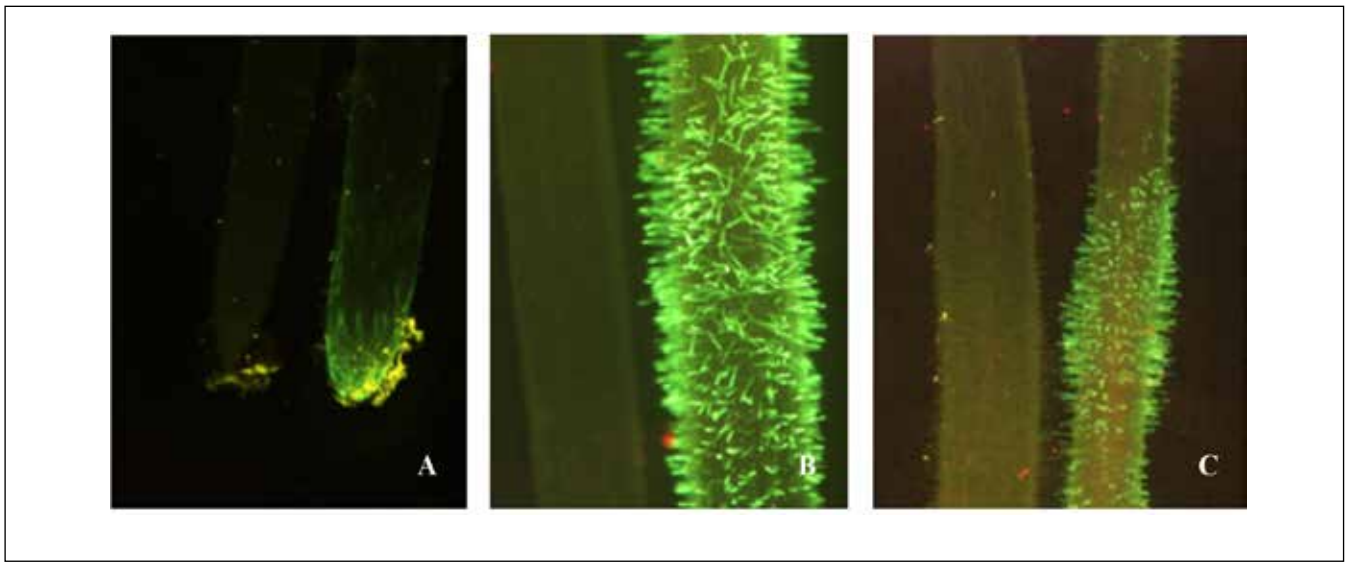

Fig. 2. Morin staining of root tips (A) and root hair zone (B and C) in control (left) and Al-treated (right) tea plants (100 μM Al for 24 h) (×10).
Fig. 2. Tinción con morin de zona apical (A), zona de pelos absorbentes (B y C) en plantas control (izquierda) y plantas expuestas a 100 μM Al (derecha) durante 24 horas (×10).

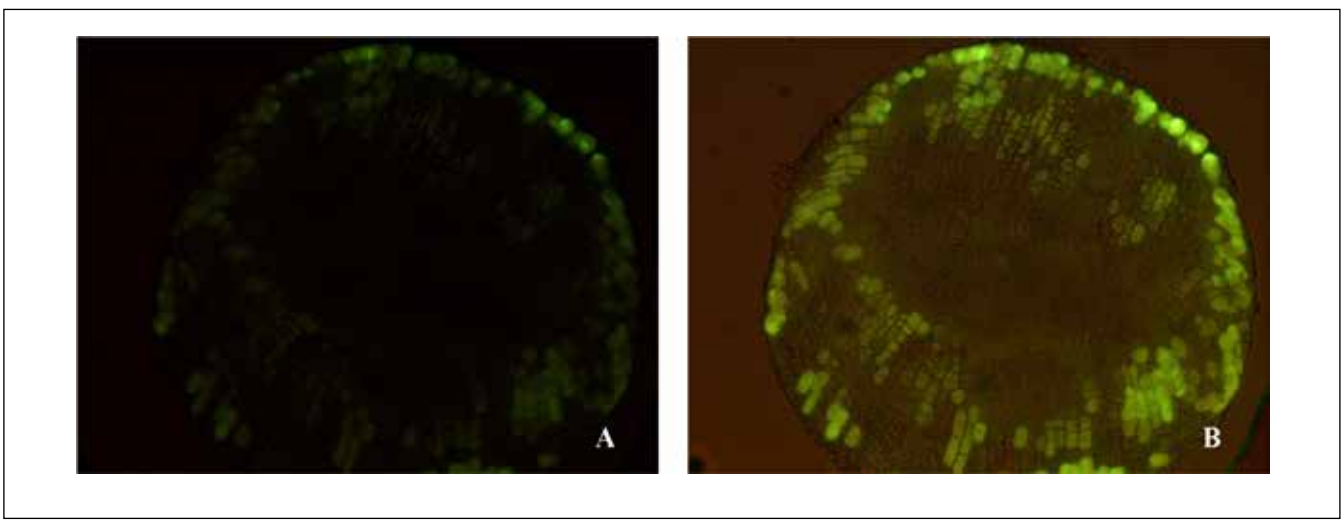

Fig. 3. Morin staining of free-hand cross sections of root caps in tea plants treated with 100 μM Al. B is the overlay of fluorescence and bright light images (×40).
Fig. 3. Tinción con morin de cortes a mano alzada de ápices de raíz de plantas de té tratadas con 100 μM Al. B muestra la superposición de imágenes de fluorescencia y de campo brillante (×40).

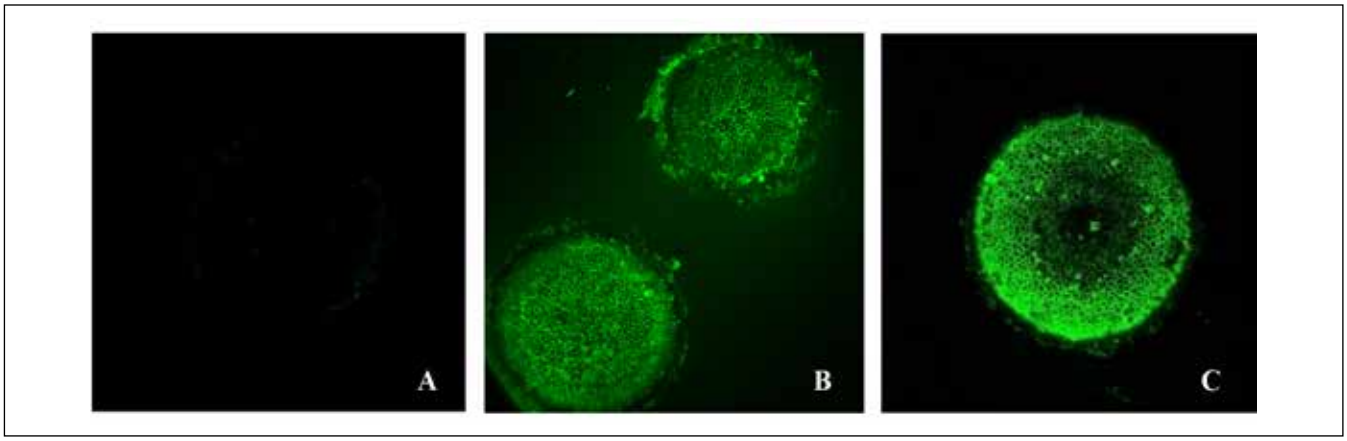

Fig. 4. Confocal laser scanning microscopy images of morin-stained sections from 0-1 mm (A and B) and 1- 2 mm (C) behind the tips of tea plants without Al treatment (A) or with 100 µM Al (B and C) (×40).
Fig. 4. Imágenes de microscopía confocal de láser de secciones de ápices radiculares teñidas con morin. (A y B) 0-1 mm del ápice, (C) 1-2 mm del ápice de plantas de té tratadas con 100 µM Al (×40).

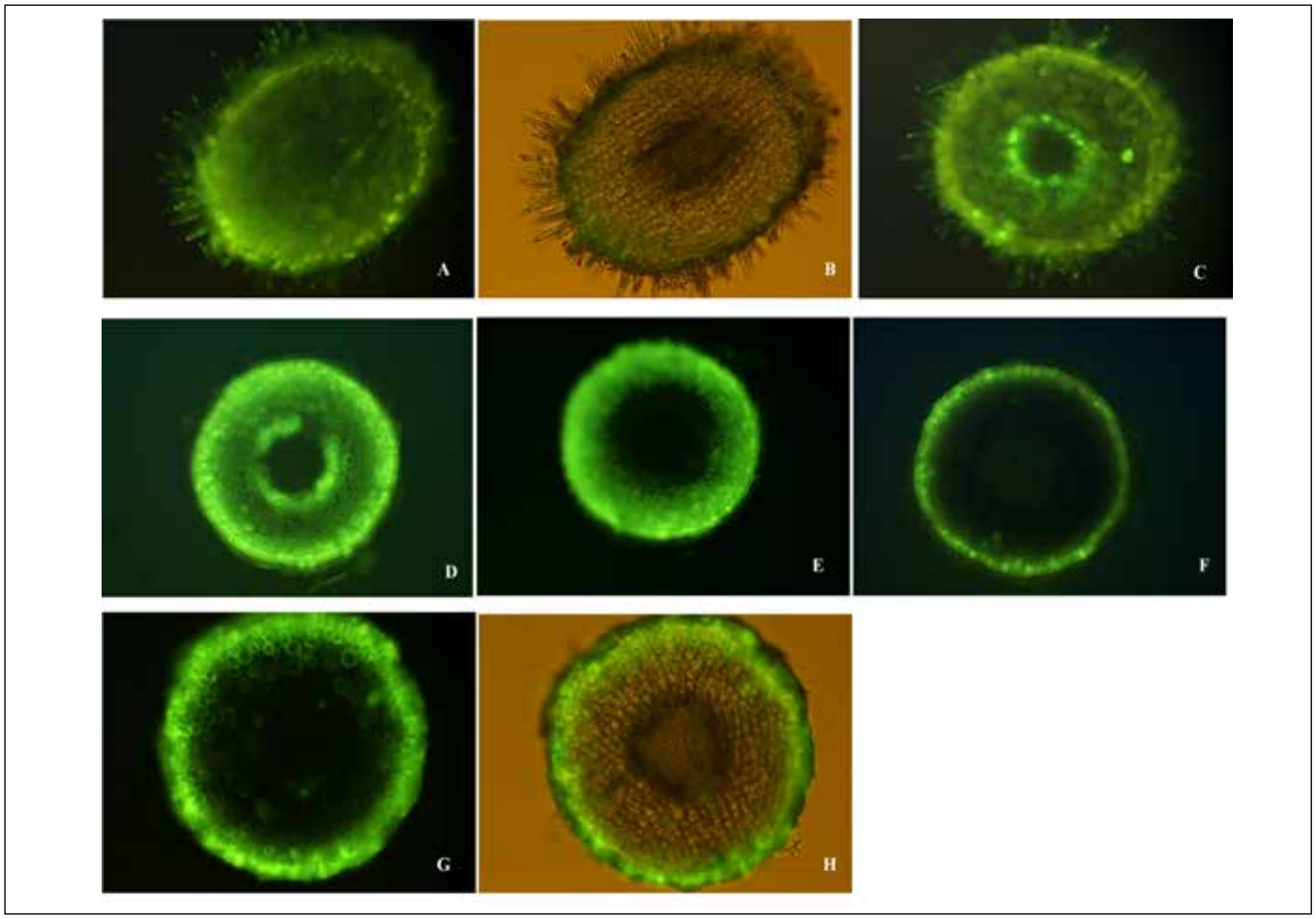

Fig. 5. Morin staining of free-hand cross sections of root tips from 2.5-5.0 mm (A and B), 5-10 mm (C), 10-15 mm (D) and 15-20 mm (E) and 20-25 mm (F, G and H) behind the root tips in tea plants treated with 100 µM Al. B and H are overlay of fluorescence and bright light images corresponded to the A and G, respectively (×40).
Fig. 5. Cortes transversales a mano alzada de raíces de plantas de té tratadas con 100 µM A. Se muestran zonas a diferentes distancias del extremo apical (A y B) 2.5-5 mm; (C) 5-10 mm; (D) 10-15 mm y (E) 15-20 mm; (F,G,H) 20-25 mm. B y H muestran superposiciones de imágenes de fluorescencia y de campo brillante correspondientes a A y G (×40).

root axis (Fig. 5). From the 2.5-5.0 mm of the root tip towards the basal parts (<15 mm), Al tended to accumulate mainly in the rhizodermal and endodermal cell layers and slightly in the cortex of the root hairs. However, it was clearly excluded from the central cylinder (Fig. 5A-D). This tendency was continued for rhizodermal zones in the following sections towards the more basal areas (Fig. 5E-H). In the 15-20 mm behind the root tips, Al was mainly restricted to the epidermis and outer cortex (Fig. 5F-H).

In the longitudinal free-hand sections of the root tips, CLSM images showed a high Al-morin signal from the CW compared with other parts of the cells (Fig. 6). Such clear accumulation of Al in the CW of root tips could be observed also in the CLSM images of cross sections (Fig. 4).

Al accumulation was detected in the free-hand sections of roots in the lateral root zone; a high Al signal could be easily detected in the zone of second-order lateral root initiation, and in the apical parts of these new emerging roots (Fig. 7). In the CLSM images of longitudinal free-hand sections, Al accumulation could be observed in the tips of new emerging second-order lateral roots (Fig. 8). The same could be observed in the CLSM images of longitudinal sections: in addition to the clear Al accumulation in the epidermal and endodermal zones, the tip of emerging leaf branches could be characterized by a high Al concentration (Fig. 8).

Microscopy studies on leaves. Free hand sections of the apical regions of shoots with young and developing leaves showed relatively high green fluorescence signal from the epidermal cell layer and vascular bundle region of the leaves (Fig. 9A). Under the same exposure time (1/15 sec), old leaves did not show green fluorescence signal. However, under a longer exposure time (1/2 sec), they showed a weak green fluorescence corresponding to morin excitation wavelength. It was coming from the epidermal cell layer and also from the vascular bundle region corresponding mainly to the phloem (Fig. 9B, 3C, 3D). The fluorescence signal from the epidermal cell layer was restricted mainly to the external and radial walls (Fig. 9C). An intense red fluorescence signal related to chlorophyll was detected in the old leaves. A similar signal from the young leaves was much weaker, particularly considering the lower exposure time applied for obtaining the image of the latter leaves.

From the same leaf parts we prepared thin sections from the epidermis. An intense fluorescence signal could be detected from the stomatal cells of the young (the inlet of Fig. 9A) and mature leaves (the inlet of Fig. 9C). In the petioles, the fluorescence signal came not only from the epidermal cell layer, but also from the trichomes (Fig. 10).

Images from CLSM also showed an intense fluorescence signal from the epidermal cell layer and vascular bundles in the apical region of young and old leaves, as well as the petiole of young leaves (Fig. 11). The intensity of the fluorescence emission from the epidermal cell layer of old leaves (Fig. 11C) was considerably lower than that from the apical region (Fig. 11A), expanding young leaves (Fig. 11B) and the petiole of young leaves (Fig. 11D). Sections were thick in some places. Therefore, it was not possible to distinguish the florescence light emitted from the outer epidermal CW from that of the radial and inner epidermal CW. However, in some locations

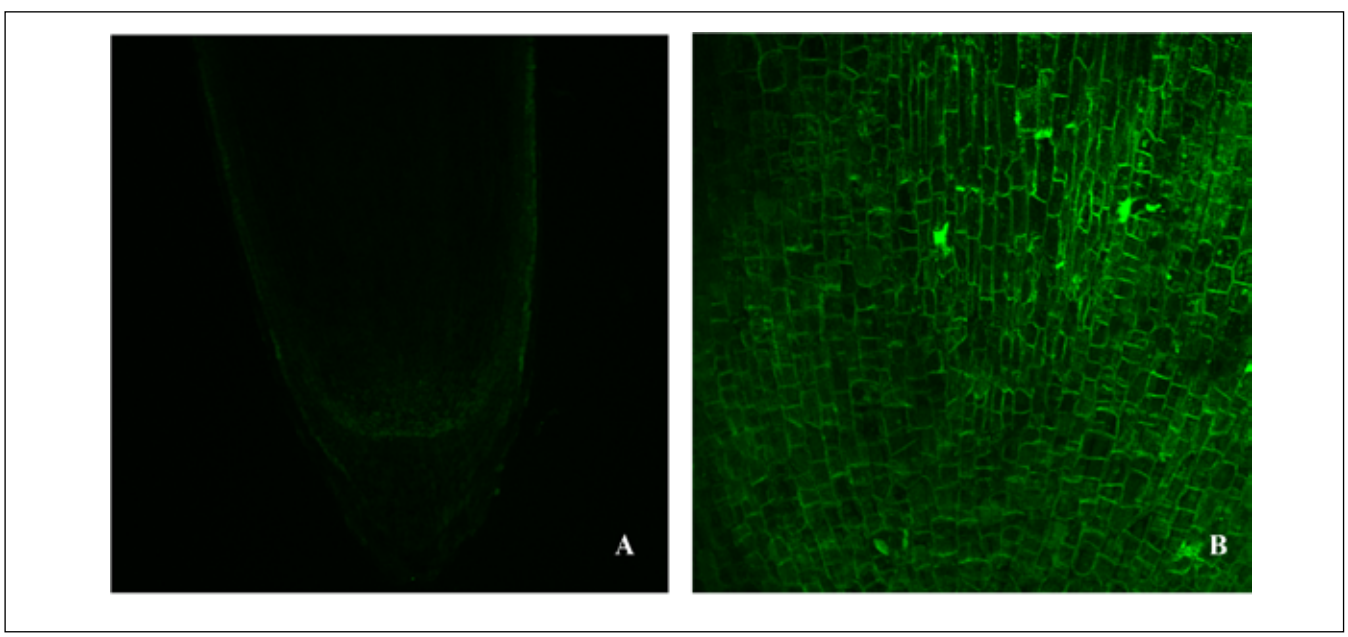

Fig. 6. Confocal laser scanning microscopy images of morin stained longitudinal sections of control (A) and 100 μM Al-treated (B) plants (×100).
Fig. 6. Imágenes de microscopía confocal de láser de secciones longitudinales de plantas control (A) y tratadas con 100 μM Al (B) (×100).

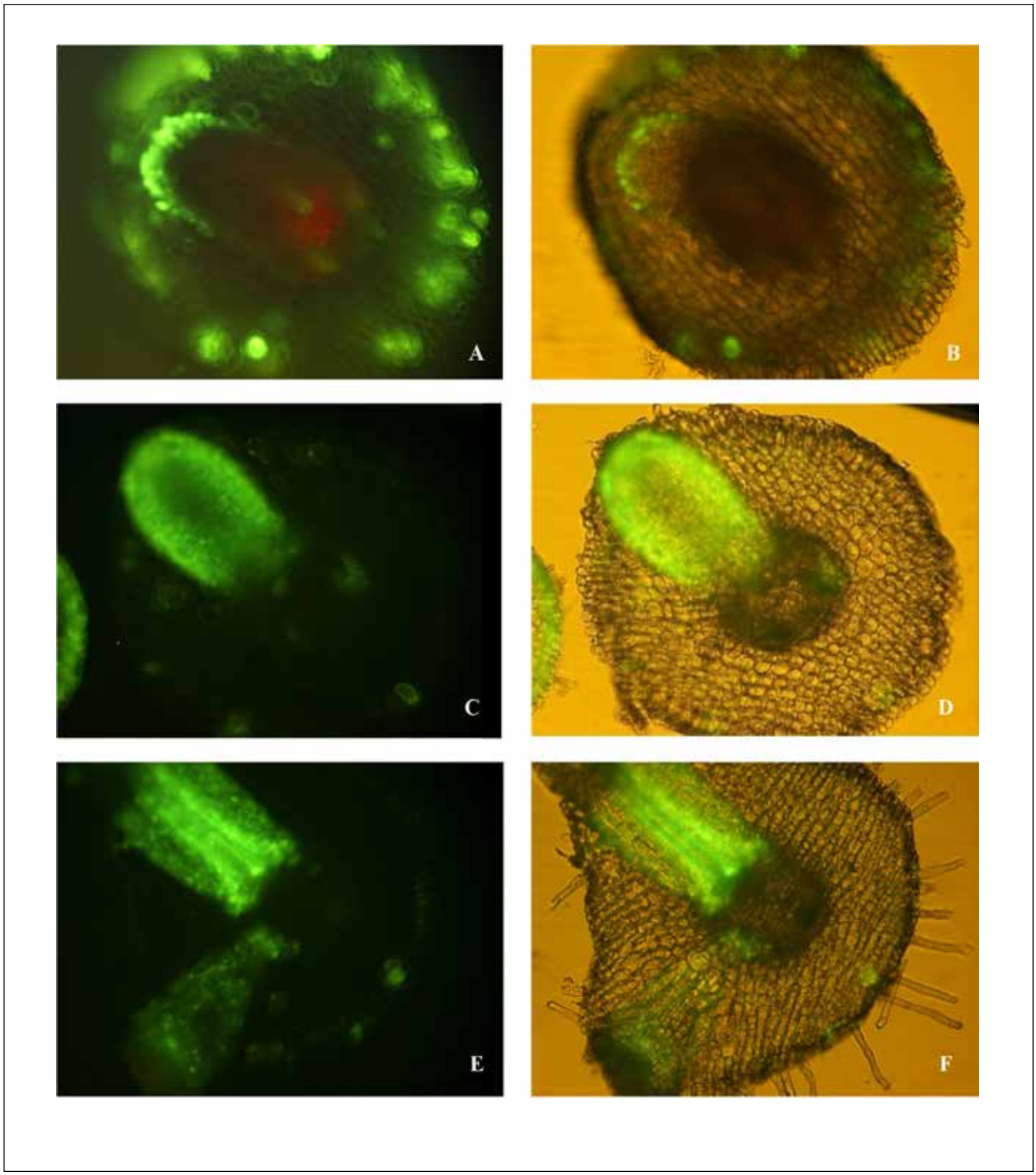

Fig. 7. Morin staining of free-hand cross sections of 25-30 mm behind the tips of tea plants treated with 100 μM Al. A, C and E: fluorescence image and B, D and F: overlay of fluorescence and bright light images. Initiation of new branching could be observed at first (A and B), intermediate (C and D) and final (E and F) developmental stages (×40).

Fig. 7. Secciones a mano alzada y teñidas con morin de zona 25-30 mm del ápice de raíces de plantas de té tratadas con 100 μM Al. (A, C y E) imagen de fluorescencia y (B, D y F) superposición de imágenes de fluorescencia y campo brillante. Se observa formación de nueva raíz lateral en fase inicial (A y B), fase intermedia (C y D) y final (E y F) (×40).

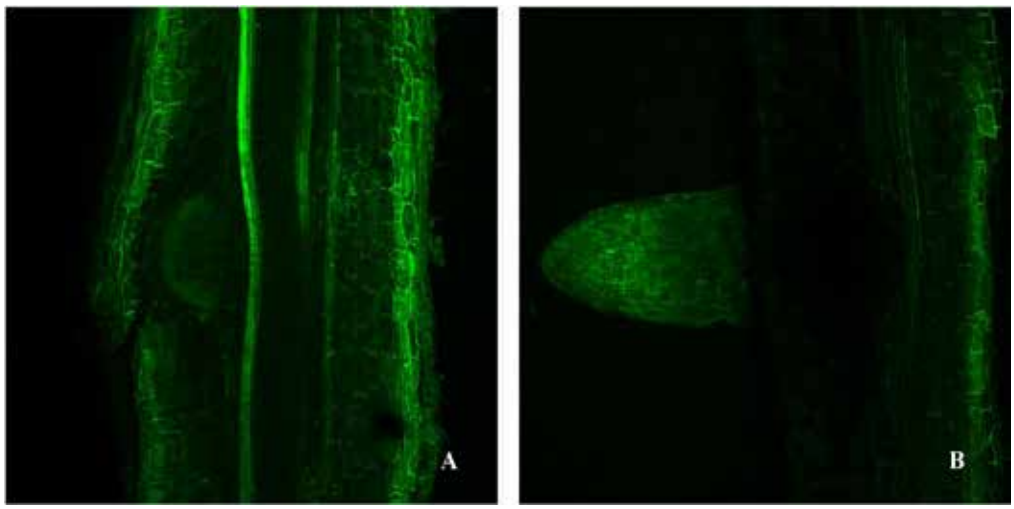

Fig. 8. Confocal laser scanning microscopy images of free-hand longitudinal sections of 25-30 mm of morin-stained roots of tea plants treated with 100 μM Al with an emerging (A) and developed (B) new branching (×100).
Fig. 8. Secciones a mano alzada y teñidas con morin de zona 25-30 mm del ápice de raíces de plantas de té tratadas con 100 μM Al con ramificación emergente (A) y ya desarrollada (B) (×100).

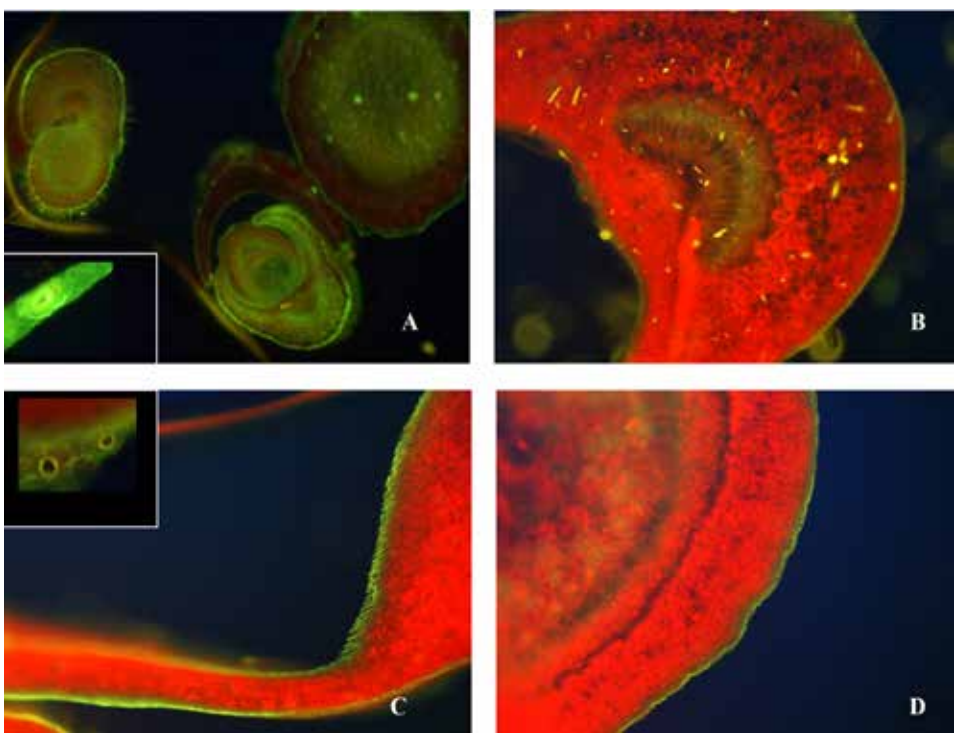

Fig. 9. Fluorescence images of morin stained free-hand sections of young leaf (A) and old leaf (B, C and D) in tea plants with Al treatment. The exposure time was 1/15 second for A and 1/2 second for B, C and D. The inlets are the stomatal cells selected from the high-magnification images obtained from the corresponding sections (×40).
Fig. 9. Imágenes de fluorescencia de cortes a mano alzada teñidas con morin de hoja joven (A) y vieja (B, C, D) de plantas de té tratadas con Al. Tiempos de exposición de la toma de foto fueron 1/15 s (A) y ½ s B, C y D. Las imágenes insertadas representan células estomáticas tomadas con mayor aumento en las secciones correspondientes (×40).

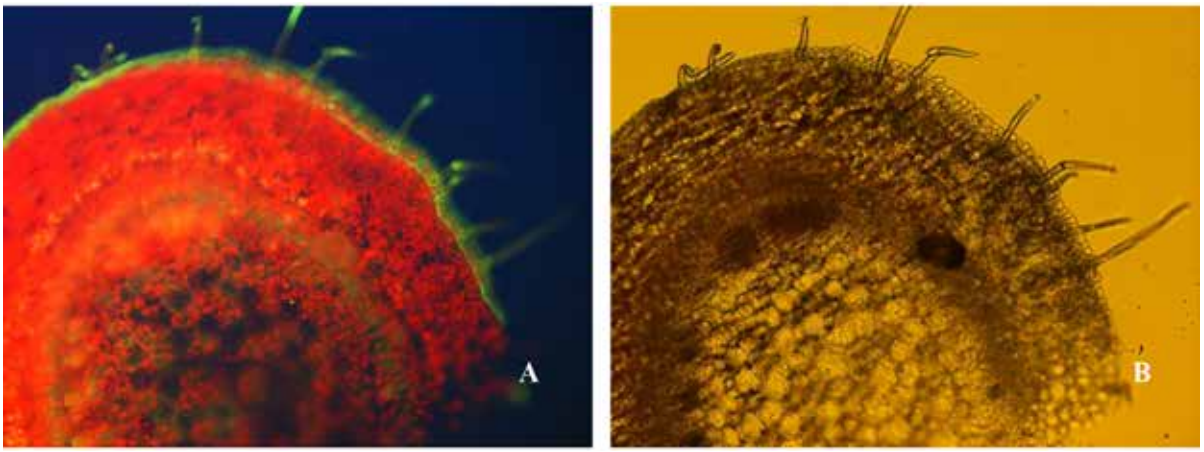

Fig. 10. Fluorescence images of morin stained free-hand sections of the leaf petioles in tea plants with Al treatment. B is the bright-filed image of the A (×40).
Fig. 10. Imagen de fluorescencia de cortes a mano alzada de peciolos foliares de plantas de té tratadas con Al (A). (B) Imagen de campo brillante de A (×40).

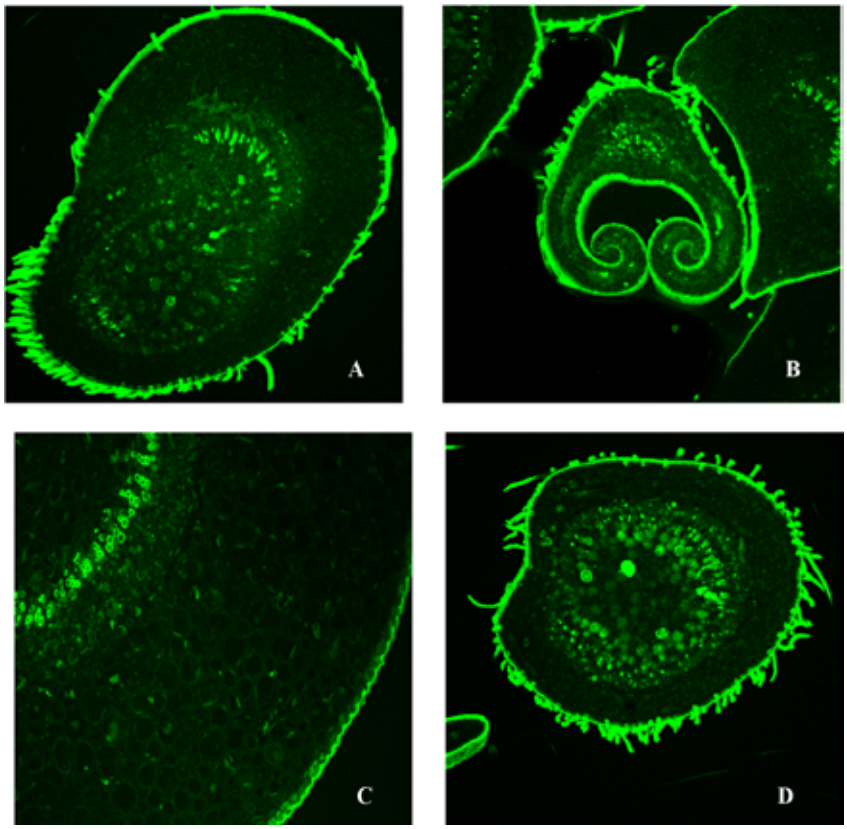

Fig. 11. Confocal laser scanning images of morin stained free-hand sections of shoot apical region (A), young leaf (B) old leaf (C) and petiole of the young leaf (D) in tea plants with Al treatment (×40).
Fig. 11. Imagen de microscopía confocal de láser de cortes a mano alzada teñidas con morin de la zona apical del tallo (A) hoja joven (B), hoja vieja (C) peciolo de hoja joven (D) de plantas de té tratadas con Al (×40).

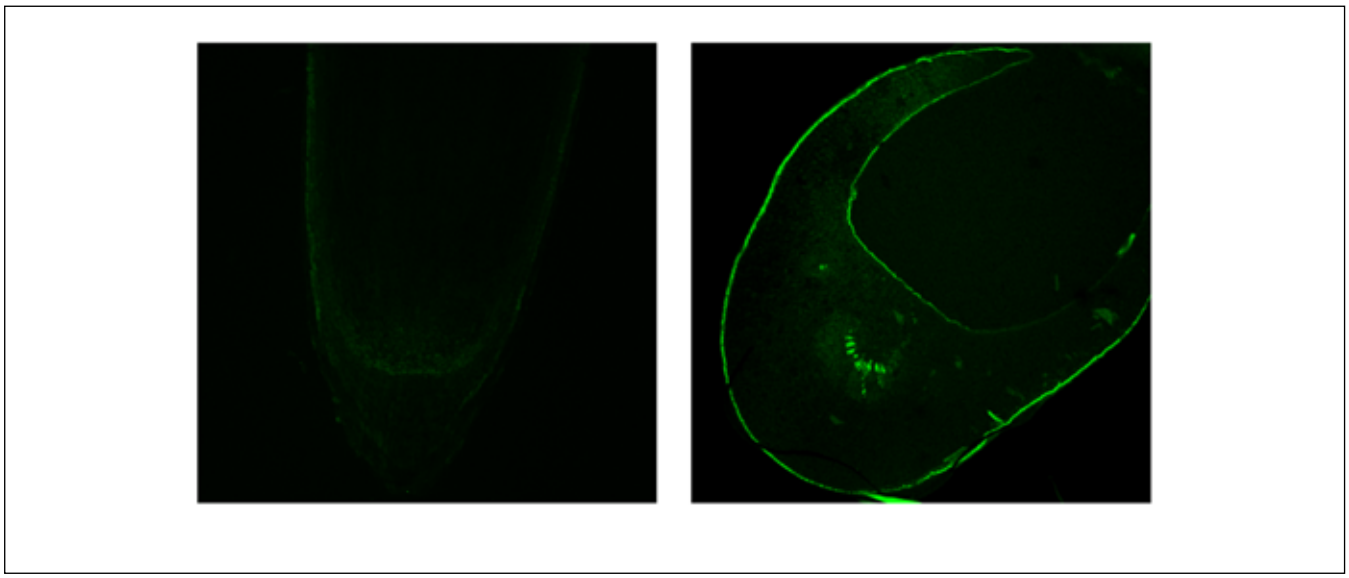

Fig. 12. Confocal laser scanning microscopy images of free-hand sections of root tip (A) and leaf (B) in Al-treated tea plants without morin staining (×40).
Fig. 12. Imagen de microscopía confocal de láser de cortes a mano alzada de ápice radicular (A) y hoja (B) de plantas de té tratadas con Al y sin teñir con morin (×40).

of the sections from old leaf (Fig. 11C and 11D) similar with that in conventional microscopy image (Fig. 9C), the signal was clearly limited to the outer face and partly radial CW of the leaf epidermis. It is likely that a similar pattern occurred in the young leaves. However, the higher intensity of fluorescence emission in comparison with the old leaves may have caused a considerable light scattering emitted from the whole epidermis of young leaves, and thus prevented spatial resolution.

We examined sections without morin staining for a possible auto-fluorescence of cell components within the excitation-emission wavelength of morin (420-510 nm). Fluorescence signal was observed from the epidermis and vascular bundles region, but with much lower intensity than the morin-stained sections (Fig. 12B). Surprisingly, root sections did not show any light signal (Fig. 12A) indicating that auto-fluorescence of cell structures is limited to particular cell types in the shoot.

DISCUSSION

Plants have evolved different strategies to cope with the enhanced Al availability in acid mineral soils (Poschenrieder et al., 2008). Most Al-resistant species exclude Al from both the Al-sensitive root tips, by the Al-induced exudation of organic acids, and the shoots, by avoiding root to shoot translocation of Al (Kochian et al., 2004; García-Oliveira et al., 2014). In this way, root elongation and photosynthesis can be maintained even when growing in soils with a high percent of Al saturation of their cation exchange capacity. Contrastingly, tea is an Al accumulating species with high Al burdens not only in roots, but also in shoots (Matsumoto et al., 1976; Morita et al., 2008). The tea plant not only is highly Al-tolerant, but its growth is even stimulated by Al. This hormetic effect (Poschenrieder et al., 2013) can be due to different mechanisms including stimulation of photosynthetic rate, reduction of lignification, enhancement of the activity of the antioxidant defense system (Hajiboland et al., 2013b), and alleviation of a latent Fe toxicity occurring in tea plants without Al supply (Hajiboland et al., 2013c). Here, vital staining of the root axis showed that roots of tea plants were not damaged even after a one-week treatment with Al concentrations as high as 100 μM (Fig. 1). Moreover, the Al-induced growth stimulation (Table 1) despite the accumulation of high Al concentrations in both roots and shoots (Table 2, Figs. 2-12), is consistent with the extraordinary high Al tolerance of tea.

How plants with a metal accumulation strategy protect their metal-sensitive processes, especially root elongation and photosynthesis, against the ion toxicity, and what are the evolutionary advantages of metal hyperaccumulation are questions that have attracted considerable research interest in the last two decades (Krämer, 2010; Poschenrieder et al., 2006). Binding of metals in non-toxic form and efficient tissular and sub-cellular compartmentation of metals are key factors in metal-tolerant accumulating species (Barceló & Poschenrieder, 2002; Krämer, 2010; George et al., 2012).

Organic acids and phenolics are major ligands for Al detoxification (Tolrà et al., 2005; Li et al., 2014), while cell walls and vacuoles seem to be major storage sites for Al ac-

cumulation in both accumulator and non-accumulator species (Vázquez et al., 1999; Tolrà et al., 2011; Li et al., 2014). However, different mechanisms seem to have evolved in different Al accumulating species (Maejima et al., 2014).

In this work, we observed that Al treatment altered both synthesis and partitioning of phenolics into the CW and soluble fractions. An increased activity of PAL, the key enzyme at the entrance of the phenylpropanoid pathway, implied that Al treatment caused activation of phenolics synthesis in the leaves (Hajiboland et al., 2013a). In addition, allocation of greater proportion of phenolics to the soluble fraction could be regarded a strategy for recruiting phenolic pools to the internal detoxification rather than to structural purposes (i.e., lignification of CW). This is in line with the observation of an Al-catechin complex in fresh tea leaves using ^{27}Al-NMR (Nagata et al., 1992). In contrast, in the soluble fraction of roots Al is likely present as complexes with oxalate (Morita et al., 2008).

Although for Al accumulators, restriction of Al entry into the cells does not seem to be critical because of highly specific internal detoxification mechanisms in these species; there are evidences for simultaneous occurrence of these two mechanisms in Al accumulator species (Jansen et al., 2002; Klug & Horst, 2010). For tea plants, binding to the CW for restriction of Al entry into the cell is expected to occur simultaneously with a detoxification-based mechanism (i.e., chelation by phenolics in the cytosol). A high Al signal was detected in the cell wall of the apical parts of the roots in this work (Fig. s 4B and 4C and 6B). Distribution of Al between cell compartments is still a matter of debate (Eticha et al., 2005) particularly when accumulator species are compared with non-accumulators. However, using various approaches and techniques in non-accumulator (Chang et al., 1999; Yang et al., 2011) and accumulator species (Carr et al., 2003; Gao et al., 2014) now there is little doubt that the majority of the cellular Al is located in the CW (Eticha et al., 2005). In the present work, fractionation of Al between CW and the remaining cell compartments revealed that about 40-50% of Al was partitioned into the CW-bound fraction. Published works on the quantitative distribution of Al between CW and soluble fractions in tissues of tea estimated about 70-80% of Al being in water-insoluble (Morita et al., 2008) or CW-rich fraction (Gao et al., 2014) obtained after centrifugation and filtration. In *Melastoma malabathricum* another Al accumulator species, approximately 50-70% of Al was found in the Tris-HCl (soluble) extract (Watanabe et al., 1998). In our study (Table 3), the lower ratio (50%) obtained by the fractionation procedure was likely due to using the whole root system and whole leaf lamina. Data for root were indeed an average of CW-bound Al in tissues with higher Al (root tips, root rhizodermal cells and root hairs; i.e., higher necessity for CW binding). Those root tissues without considerable Al accumulation consequently showed a lower ratio of Al in the CW. A steep Al concentration gradient has also been observed among midvein and interveinal regions in the leaves of some Al accumulators (Campos et al., 2014).

In the roots of non-accumulator species, Al in the CW is associated with pectin (Chang et al., 1999). However, detailed works are lacking on the Al binding form in the root CW of Al accumulators and/or any difference between accumulator and non-accumulator species in this regard. Although in a recent work on tea (Gao et al., 2014), lower Al binding capacity of pectin- and hemicellulose-free preparations of CW-rich fractions was considered as justification of Al binding to these CW components; the applied chemical treatments may readily remove also other CW components such as phenolic acids. For species such as tea plants with a high content of phenolic compounds in the CW, CW-bound phenolic acids are potential targets for Al. Plant CW phenolics consist of two groups of compounds: lignin (the polymer of monolignol units), and low molecular weight phenolic acids (that are bound to various CW components) (Wallace & Fry, 1994; Strack et al., 1988). The carboxylic groups of CW phenolic acids have high affinity for Al ions and the stability constants of Al complexes with these carboxylic groups are high (McDonald et al., 1996). Ruan & Wong (2004) attributed the increasing Al accumulation in a non-exchangeable Al fraction in tea roots to a substantial quantity of phenolic compounds in the endodermis layer that sequester a considerable amount of Al in the CW. In addition to roots, more detailed studies are needed to find out the binding form of Al in the CW of tea leaves. In contrast to Al sensitive species where many studies focused on the nature of Al binding molecules in the CW (for a review see Horst et al., 2010), the binding form of Al in the CW of Al accumulators in general, and tea in particular, has not been studied in detail to date.

This study revealed that Al is evenly distributed in the rhizodermal, cortical and central parts of the root tip. A high Al accumulation in the root hairs was also observed in this zone. Towards basal zones, coincident with differentiation of xylem vessels and endodermis, Al was obviously restricted to the epidermis and cortex and hindered to penetrate into the central cylinder. Accordingly, the endodermis, as a possible barrier for symplasmic Al influx (Klug & Horst, 2010), showed higher Al signals compared with adjacent cortex cells in this zone (Fig. 5D). In the more basal parts of root axis and lateral root initiation zone Al was not observed in the central cylinder (Figs. 5E-G). This may imply that xylem loading of Al did not occur in the basal parts of the root and that Al was transported symplastically from the apical to the basal parts, and then loaded in the newly differentiated xylem in this zone. Al signals in the second-order lateral roots (Figs. 7 and 8) in the basal parts, thus, are likely originated from Al transported symplastically from apical parts. A similar pattern of Al loading along the root axis has been observed in buckwheat, an Al accumulator species (Klug & Horst, 2010). In this species,

when Al was applied to the 11-20 mm zone (with differentiated xylem vessels) xylem loading was low, while Al taken up by the 0-10 mm root apex (without xylem vessels differentiation) was rapidly loaded into the xylem (Klug & Horst, 2010).

The intense fluorescence signal from the leaf epidermal cell layer (as well as stomatal cells) suggests accumulation of Al as a result of its transport with the transpiration stream. Preferential accumulation in these non-photosynthetic cells may contribute to avoid entrance of Al into the sensitive photosynthesizing mesophyll cells. As mentioned above, Al can be localized by morin staining when it is in association with ligands with lower binding affinity than morin, and thus formation of Al-morin complexes is a prerequisite for Al localization by fluorescence microscopy. Knowledge of the binding stages and stability constants of dye complexes is a prerequisite for qualitative and semi-quantitative statements about the Al distribution within plant tissues. Morin is not able to form a complex with Al in high stability complexes (Lian et al., 2003). Presence of organic acid anions such as oxalic, malic, or citric acid anions reduces partly the fluorescence intensity of Al-morin (Klug et al., 2011). Regarding oxalate as Al-chelating molecule in the tea root (Morita et al., 2008), an underestimation of Al accumulation in the morin-stained tea roots, is expected similarly to the effect of citrate in buckwheat (Klug et al., 2011). However, there is no information on the competitive inhibition of Al-morin complex by catechins or CW phenolic acids (see below).

Eticha et al. (2005) reported that Al can form fluorescent complexes with morin in the cytosol, but these authors were unable to detect Al tightly bound to the CW pectin. In contrast, many authors have successfully detected Al-morin complexes in the CW (Tice et al., 1992; Ahn et al., 2002; Wang et al., 2004; Zheng et al., 2005; Klug et al., 2011) implying that Al in the CW of these plant materials was still able to form complexes with morin. Apart from the Al binding form in the CW of tea plants (phenolic acids or pectins), fluorescence signal from the epidermal CW and that of vascular cylinder cells indicated that Al in these regions occurs as a free and/or loosely-bound form; thus, morin was able to replace these molecules. To our best knowledge, morin has not previously been used for localization of Al either in the leaves or roots of tea plants. Therefore, we cannot compare our results with others.

In addition to epidermal cells, vascular bundles of the leaves, and more likely the phloem tissue were the location of high fluorescence signals. This is in line with a previous study using the LEXRF technique, which showed that most of the Al in leaves is localized in the epidermis, followed by the vascular region. Within the vascular region, a slightly higher Al signal was seen to come from the phloem region (Tolrà et al., 2011).

According to the result of the fractionation experiment, at least half of the Al was associated with cellular components other than the CW. The Lack of any fluorescence signal from the cell lumen particularly in the CLSM images, may imply that chelation of Al by catechins prevents formation of Al-morin complexes. In addition, lower fluorescence signal from the CW of epidermis in the old compared with the younger leaves may also indicate that the binding form of Al in the CW is modified during leaf aging. Thereafter, CW-bound Al can hardly form complexes with morin in aged leaves. More detailed studies are needed for elucidating the Al binding form in the CW of leaves with different age.

An intense signal from the leaf trichomes revealed accumulation of Al in this organ. Two recently published works (Ezaki et al., 2013; Maejima et al., 2014) showed also the accumulation of Al in the leaf trichomes in other accumulator species. This emphasizes once again the presence of both avoidance and tolerance mechanisms at both the cellular and whole plant levels in tea. Accumulation of other heavy metals such as Ni was reported in the trichomes for various hyperaccumulators of this heavy metal (Psaras et al., 2000).

Several classes of phenolic compounds are strongly autofluorescent when irradiated with UV or blue light and thus are visible in plant tissues using fluorescent microscopy (Hutzler et al., 1998). Catechins, as the predominant phenolic compounds in tea leaves, have excitation wavelength of 325-331 nm, and emission wavelength of 455-550 nm (Nagaoka et al., 2002). Thus, an auto-fluorescence of catechins may contribute to the signals emitted particularly from the mesophyll region under conventional fluorescence microscopy. However, this is not the case in the images obtained by CLSM presented in this paper. Nevertheless, auto-fluorescence of phenolics other than catechins as well as other cell components could not be excluded within the excitation-emission wavelength range of morin (420-510 nm). Detection of even weak fluorescence signals from the leaf (but not root) sections under CLSM (Fig. 12) without morin staining revealed that (in contrast to the roots) morin cannot be used for a quantitative localization of Al in the leaves. A considerable Al concentration in mature leaves could be attributed to the Al derived from seeds (collected from acid soils with high Al availability). However, the presence of Al in the young leaves (Table 2) which developed in the absence of Al, indicates a high rate of phloem transport of Al from old to young leaves. Thus, the presence of Al in the leaves, even in the absence of Al treatment, indicates that morin staining is not a suitable approach for a quantitative comparison of various leaf tissues in tea plants.

ACKNOWLEDGEMENTS

This work was supported by Spanish MICIN (project BFU2010-14873/subprogramaBFI). Technical support from the Electron Microscopy Service of UAB is acknowledged. Research Council and the center of Excellence for Biodiversity, University of Tabriz are greatly acknowledged for financial supports.

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