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Aluminium alters mineral composition and polyphenol metabolism in leaves of tea plants (*Camellia sinensis*)

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Abstract

Tea plants (*Camellia sinensis*) can hyperaccumulate and tolerate high leaf concentrations of aluminium (Al). The quality of tealeaves and the positive health effects of their infusion depend on the leaf concentrations of both polyphenolic substances and mineral elements. This study explored the influence of Al supply on these leaf components under low and high phosphorus (P) availability. After 8 weeks exposure in hydroponics, multifactorial analysis revealed a negative influence of leaf Al on magnesium (Mg), P, boron (B), and manganese (Mn) leaf concentrations. Contrastingly, these essential mineral nutrients were positively related to leaf epigallocatechin. Galloylated catechins were positively related to leaf iron. After short-term exposure (24 and 96 days), qPCR analysis revealed upregulation of galloylation-related genes by substrate acidification both in old and young leaves. Only the extremely high Al accumulation in old leaves activated genes involved in biosynthesis of galloylated catechins, while in young leaves the lower Al leaf concentrations activated genes involved in anthocyanin accumulation. In conclusion, low pH and enhanced Al availability to tea plants have a strong influence on the polyphenolic pattern of tealeaves and therefore may alter both the leaves' antioxidant properties and their ability to bind Al and Fe in non-toxic form.

1. Introduction

Tea plants, *Camellia sinensis*, can hyperaccumulate and tolerate extremely high leaf aluminium (Al) concentrations [1,2]. Al-hyperaccumulation is present in different botanical families [3,4]. Therefore, this trait may have evolved several times along plant evolution. Al-hyperaccumulating species are almost exclusively either woody Angiosperms or ferns [3,5], growing on acid soils with low phosphorus and calcium availability, typical for tropical environments. Usually, Al-hyperaccumulators present high leaf concentrations of organic acids

(OA) and phenolic compounds [6]. Organic acids are strong Al-ligands involved in both the detoxification and transport of Al in plants. Al-excluder species commonly exudate malate and/or citrate to detoxify Al in the rhizosphere or inside root cells [7-9]. Common OAs in Al hyperaccumulating species are citrate and oxalate. Roots of buckwheat and tea exudate oxalate in response to Al [10,11]. Citrate seems to be the main ligand for the long-distance transport of Al in *Fagopyrum, Melastoma*, and tea. Storage forms in leaves are Al-oxalate in *Melastoma* and both Al oxalate and Al citrate in *Fagopyrum* [12-15]. The composition of phenolic compounds in leaves of Al hyperaccumulators is chemically more diverse and species specific. Some examples are: delphinidin (antocyanidin) in *Hydrangea* [16], quercetin and kaempferol in Rubiaceae [17], catechins, tannins, and antocyanidins in Melastomataceae [18], quercitin, isoquercitrin, quinic acid, and epicatechin in mistletoes infesting Al-hyperaccumulating trees [19], or catechins, theaflavins and hydrolysable tannates in tea plants [20].

In tea, polyphenolic compounds can play a role both in metal binding within the plant cells [21] and in defense against oxidative stress [22] due to their antioxidant properties. High interest in tea polyphenolic substances arises from the multiple beneficial health effects ascribed to these substances [23]. Green tea prepared by infusing dried leaves of *Camellia sinensis* is especially rich in bioactive compounds. Among the nutraceuticals in tea, catechins are the most active products. EGCG and ECG are highly abundant in hot water extracts and appear to be the major anti-tumorigenic and anti-oxidant substances [24].

The nutritional quality and beneficial health effects of green tea thus largely relay on the concentrations of catechins, especially EGCG and ECG in the tealeaves. The catechin composition and contents vary among tea cultivars [25]. However, comparative studies revealed that environmental factors, especially climate rather than variety, influence the leaf catechin contents [26]. Mineral composition also influences tea quality. Green teas from different geographical regions largely differ in their mineral contents [27]. Soil characteristics, variety, harvest season and leaf age have a large influence on the tealeaf mineral composition, including Al [28]. Under acid soil conditions (pH < 4.5), Al is solubilised into the trivalent cation, Al^{3+}, which is easily available to plants [8]. Aluminium accumulates to concentrations as high as 30,000 mg kg-1 in old and 600 mg kg-1 in young leaves of *C. sinensis* [1]. Aluminium accumulation in tealeaves is of concern as the Joint FAO/WHO Expert Committee on Food Additives [29] affirmed

Al as a food contaminant. According to Zhang et al [30] consumption of tea brewed from young leaves does not imply any risk, while infusions from mature leaves can have health risk indexes higher than unity due to high concentrations of both Al and Mn. These health risk indexes based on total leaf Al concentrations do not consider Al speciation.

Polyphenolics present in tealeaves can form highly stable complexes with Al. This may contribute to Al detoxification and hence to the high Al tolerance of tea plants. Moreover, complexation of Al to organic compounds in the tealeaves may also reduce toxicity to consumers. Nonetheless, under the acid pH conditions of the stomach, ligand exchange reaction can occur leading to more bioavailable compounds [31].

In all case, a high concentration of polyphenolic compounds, especially EGCG, in combination with low Al and Mn contents is desirable to guarantee the beneficial effects of tea consumption and minimize potential health risk. What mineral nutrients and Al availability to tea plants affect the leaf polyphenol patterns and concentrations has only occasionally been analysed. Ammonium is the preferred nitrogen source of tea plants [32]. Ammonium supply enhances catechin leaf concentrations [33]. The tea plant is extraordinarily tolerant to P deficiency [34]. The response of catechin concentrations to P-deficiency is variety-dependent; either an increase of EGCG or no change was observed under P deficiency [35,36].

Chen et al [37] found an Al-induced increase of both total phenolics and ECG in tea leaves. The mechanisms behind these changes in catechin concentrations are still not clearly established. The dilucidation of the different metabolic pathways along with the characterization of the genes coding for the enzymes involved in the different biosynthesis steps of catechins in tea plants has made substantial progress in the recent years [26,38,39]. This allows now to address the question how changes in the nutrient supply modifies the production of bioactive compounds in tea plants.

Our aim here was to characterize the influence of Al, alone or in combination with different P supply, on catechin patterns and concentrations in tealeaves. For this purpose, we performed two types of experiments. The first was a pure hydroponic culture using a relatively low Al- burden for longer time (8 weeks) to assess the influence of Al and Al-induced mineral nutrient changes on catechin levels. The second implied a short (24 to 96 h), stronger Al treatment on plants growing in substrate-based hydroponics for characterizing the early influence of Al on the expression of key genes involved in catechin biosynthesis.

2. Materials and Methods

2.1. Hydroponic experiments

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 and germinated on perlite in the dark and moistened by distilled water and saturated $CaSO_4$ every day. After emergence of the primary leaves, seedlings were transferred to the light. One month-old seedlings were transferred to the 2 L pots containing nutrient solution (pH 4.0) [40]. After one week preculture, differential Al and P treatments were started. Plants received either 0 or 200 μM $AlCl_3$ (45 μM free Al^{3+} activity) in combination with the control P supply of 12.5 μM (-P) or extra P supply (+P) of 50 μM. So four different treatments were performed: -Al/-P,-Al/+P, +Al/-P, and +Al/+P. Nutrient solutions were replaced every 3 days. Plants were grown in a growth chamber 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 at a photon flux density of about 400 μmol $m^{-2}s^{-1}$.

2.2 Foliar analysis of Al and selected nutrients

Eight weeks after start of the treatment (13 weeks from sowing) young and old leaves were harvested and weighted. Part of the leaves were dried at 70 °C for 2 days and leaf dry weight was determined. For Al analysis, oven-dried samples were transferred to porcelain crucibles and wet-ashed with 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).

2.3 Extraction and analysis of soluble phenolics

Fresh harvested young and old leaves were frozen in liquid N_2 and stored at -80º C until processing. Extraction of soluble phenolics was performed according to the method of [41] with some modifications as previously described [42]. In brief, phenolics were extracted three times in

70% aqueous methanol at 4ºC in the dark. After centrifugation, the supernatant was dried and extracted with ethyl ether and after acidification and extraction with ethyl acetate, phenolics were determined after solubilising in MeOH.

All extracts were analysed with a HPLC-MS system equipped with an electrospray ionization source and a time-of-flight mass spectrometer (LC-ESI-QTOF) (LC 1200RR, Agilent; Micro-TOF-Q, Bruker Daltonics and an ESI Apollo 1/2 Bruker Daltonics).

2.4 Statistics and multifactorial analysis

If not otherwise stated, two-way ANOVA followed by Fisher's LSD test was used to assess differences among treatments. Data not normally distributed were log transformed prior to ANOVA. Data that were not normally distributed after log transformation were analysed by a non-parametric Kruskal-Wallis multiple comparison test. Means labelled with different letters are significantly different ($P < 0.05$). The results of leaf mineral elements and catechins were further analysed by multivariate exploratory techniques using a principal component method for factor extraction. Factors were extracted based on the observation of a scree plot of eigenvalues, selecting those factors included on the steep slope of the graph and excluding points shared with the rest of the factors on the shallow slope. All eigenvalues were >1. Factors with loading values equal or higher than 0.7 were considered. Factor coordinates of variables were represented two-dimensionally, with vectors pointing to the origin. All statistics were performed using Statistica version 6.0 (StatSoft Inc., St. Tulsa, OK).

2.5 Gene expression study in plants grown in substrate- based hydroponics

One month-old commercial tea plants (Plantfo, Uchacq, France) were pre-cultured on potting mix (4 L) enclosed in a plastic net and placed into pots irrigated to field capacity with nutrient solution (pH 4.8) with 50 µM P (+P) or 12.5 µM P (-P). After 4 weeks, the plants in the potting mix nets were transferred to pots containing 0.5 L of nutrient solution (pH 4.0) supplemented or not with Al (125 µM free Al^{3+} activity) in combination with the -P or the +P treatment. After 24 and 96 h, exposure to the Al/P treatments young and old leaves were harvested for analysis of both Al leaf concentrations, as described above, and gene expression.

For RNA extraction and cDNA synthesis, completely developed young leaves (around 1-2 weeks old) and old leaves (around 2 months) were harvested from three plants per treatment at two time-points (24h and 96h after Al treatment) and directly immersed into liquid nitrogen, homogenized to fine powder and stored at -80 °C until use. Total RNA of frozen pulverized leaves from around 100 mg was extracted using the Maxwell® RSC plant RNA kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Extracted RNA was quantified with a Nanodrop 2000 (Thermo Scientific, DE, USA) and quality was controlled using ratios 260/280 (around 2.0) and 260/230 (>2.0). The synthesis of cDNA (adjusted to 1 µg) was performed in a reverse-transcription reaction using the iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) according to manufacturer's instructions.

2.6 RT-qPCR and gene expression analyses

The influence of the Al and P treatments, after 24 h and 96h, in young and old leaves was examined on the relative expression (RE) of selected genes from the general phenylpropanoid pathway and its derived pathways (anthocyanidins, catechins and epicatechins). Target genes from the common pathway were *PAL* (phenylalanine ammonia-lyase), *CHI* (chalcone isomerase), *DFR* (*dihydroflavonol* reductase) and *ANS* (anthocyanidin synthase) for the common branch of the pathway. Selected genes from the specific branches were *LAR* (*leucoanthocyanidin* reductase) from the catechin pathway, *ANR* (anthocyanidin reductase) from the epicatechin pathway and *3GT* (3-O-glucosyltransferase) from the anthocyanin pathway. Additionally, the RE of selected genes of the type 1A SCPL (serine carboxypeptidase-like acyltransferases) gene family were examined because they are tea-specific playing a critical role in galloylation of flavan-3-o [39]. The expression of target genes was normalized to the expression level of the reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) [39]. The constant expression of this gene in all treatments was confirmed.

The coding sequences of target genes were taken from the TPIA database (Tea Plant Information Archive, http://tpia.teaplant.org/). Gene functions were searched on the 'Tea Gene Annotation' option and genes from the list of given annotated loci were selected based on two criteria: 1) function specifically addressed to *Camellia* genus and 2) differential expression in young, mature and old leaves according to the browser information. Sense and antisense primer pairs for selected loci were designed with the primer-designing tool of GenBank (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Coding sequences from the rest of the used primers were obtained according to Wei *et al.* [39]. Amplified regions for each target gene were blast analysed by http://tpia.teaplant.org/Blast.html. All primer sequences used in this study are detailed in Supplementary Table S1. Amplification efficiencies, slope and R2 of each gene are reported in Supplementary Table S2.

The expression of the selected genes was analysed by reverse-transcription quantitative PCR (RT-qPCR). Real-time detection of fluorescence emission was performed on a CFX384 Real-Time System (Bio-Rad, CA, USA), and plates were edited using the CFX manager version 3.1 software. Treatment influence on relative gene expression was calculated by the ratio (Efficiency of target gene) $^{\Delta CT,\ \mathrm{target\ (calibrator\ -\ test)}}$ / (Efficiency of reference gene) $^{\mathbf{\Delta CT,\ reference\ (calibrator\ -\ test)}}$ [43]**.** Each sample was three-technically replicated on each reaction.

3. Results

3.1. Influence of Al and P supply on tealeaf mineral composition

Plants exposed to the different Al and P concentrations did not differ in growth neither in the long-term study nor in the 24 to 96 h treatment of the second experiment (data not shown). In the long-term study, however, a considerable influence on plant mineral nutrition and polyphenol patterns was observed, Figs. 1 - 3. Expectedly, Al supply enhanced leaf Al concentrations. Al accumulation was significantly influenced by leaf age, but not by P supply, Fig. 1. All three factors, leaf age and supply of Al and P significantly influenced leaf P concentrations. Old leaves of control plants had the highest P concentrations, while all young leaves and old leaves from Al-exposed plants had lower leaf P concentrations, Fig. 2a. Leaf age and the differential Al and P supply affected the leaf concentrations of other mineral elements in different ways, Figs. 2b-f. Statistically significant interactions between Al and leaf age as well as between Al and P supply were observed for leaf Fe, Fig. 2b, Ca, Fig. 2c, Mn, Fig. 2d , and Mg, Fig 2e concentrations. All three factors interacted on Fe, Mn and Mg, but not on Ca leaf concentrations. Aluminium supply had a strong negative influence on leaf B concentrations, Fig. 2f.

3.2. Influence of Al and P supply on leaf polyphenol substances

The leaf concentrations of analysed polyphenols increased in the order gallic acid (not shown) < catechin/epicatechin (C/EC) < epigallocatechin (EGC) < epigallocatechingallate

(EGCG) < Epicatechingallate (ECG), Figs. 3 a-d. The leaf concentrations of all analysed catechins were significantly influenced by the Al supply and leaf age, while P only influenced EGC and EGCG, Figs. 3 b and d. Leaves from Al-treated plants had lower concentrations of EGC, but enhanced levels of ECG, EGCG, and C/EC. Phosphorus increased EGC and EGCG levels. There were some notable differences between young and old leaves. The EGCG concentrations were considerably higher in the young leaves of both +P and +Al plants, Fig. 3d. ECG concentrations were not affected by the P supply, while young leaves exposed to Al showed a significant increase, Fig. 3c. Also, C/EC levels were higher in young leaves, regardless the P or Al treatment, Fig 3a. EGC showed a considerable increased in old leaves with extra P supply, while Al had a negative effect decreasing the EGC concentration to undetectable levels (Fig. 3b). In young leaves +P and +Al had positive effects. For all catechins, a positive effect of the +Al/+P treatment was observed in young leaves in comparison to the old ones.

3.3. Multifactorial analysis

Multifactorial analysis was performed including mineral nutrient and catechin data (Fig 4). Based on Eigenvalues >1, three factors were extracted. Variables with negative loadings on factor 1 were leaf EGC and leaf Mg, P, B, and Mn concentrations, while leaf Al had a positive load on factor 1. The concentrations of the galloylated catechins, ECG and EGCG, loaded on factor 2 along with leaf Fe concentrations, while leaf Ec/C had a negative load on factor 3 (Fig 4). Figure 4 represents the projection of the variables with significant factor loadings on a two-dimensional plane. Factors 1 and 2 explained 47.66 and 26.3% of the total variation, respectively. The bidimensional graph clearly illustrates the opposite behaviour of Al regarding leaf EGC and the other significant mineral variables, excepting Fe.

3.4. Gene expression analysis

To further characterizing the influence of Al on catechin production in the leaves, the expression of genes coding for key enzymes in the catechin biosynthesis pathway (Figure 5) were analysed 24 h and 96 h after start of the Al supply in substrate-grown plants. The Al and P concentrations in young and old leaves after 24 h were similar to those achieved in the hydroponic experiment with lower Al concentrations and longer exposure times (Supplementary Fig. S1).

After 96 h, only the Al concentrations in the old leaves were substantially higher than in plants from the pure hydroponic experiment. Both lowering the pH (-Al treatments) and Al-exposure had a different influence in old and young leaves on the expression of genes involved in catechin and anthocyanidin biosynthesis, Fig. 6.

Relative expressions of phenyl alanine ammonium lyase (PAL) and chalcon isomerase (CHI), involved in the first steps of the common pathway were decreased by both acid and Al in old leaves, regardless the P treatment, Fig. 6a. Contrastingly, dihydroflavonol 4-reductase (DFR) expression was almost unaffected. Exposure to Al for 96 h under low P supply caused a 2.5-fold increase in antocyanidin synthase (ANS) expression and upregulated both *LAR* and *ANR* coding for leucoanthocyanidin reductase and anthocyanidin reductase, the enzymes leading to catechins and epicatechins, respectively. Lowering the pH also slightly enhanced the expressions of *LAR* and *ANR* in old leaves. After 96 h, low P supply furthermore enhanced the expression of genes involved in the galloylation of catechins; most enhanced was *SCPL1A,* Fig. 6c.

In young leaves, Fig.6 b, the most important changes in gene expression were observed for the 24 h +Al/+P treatment. Expressions of *PAL* and *CHI* were upregulated 2.5-fold and *DFR* about 3-fold. Most enhanced were the expressions of anthocyanidin synthase (ANS; 4-fold) and UDP-glucose:anthocyanidin/flavonol 3-O-glucosyltransferase (3GT; 3.8-fold). Surplus P supply stimulated the expression of DFR, ANS, LAR and ANR after 96 h exposure in plants without Al supply (Fig. 6b). In young leaves a 7-fold increase in the expression of serine carboxypeptidase (SCPL1A*)* was found in –Al/+P plants after 96 h treatment (Fig 6d). The expressions of other galloylation-related genes was also enhanced (2 to 3.5-fold) in the young leaves of these plants.

4. Discussion

Tealeaves' mineral composition and polyphenol contents strongly influence tea quality [44]. Here we found that even relatively low, non-toxic Al concentrations have a considerable influence on the mineral components of tealeaves. Al supply negatively affected P, Mg, Ca and Mn concentrations in old leaves. Contrastingly, in young leaves exposed to Al the concentrations of these essential elements were maintained or even increased, Fig. 2. Maintenance of Mg, Ca and Mn homeostasis in young leaves may have been favoured by the retranslocation of elements from old to young leaves. The antagonistic effects between Al and essential mineral nutrients are well established in other crop species [45]. Less information on tea plants is available. Most reported mineral nutrient analyses refer to commercial tealeaf samples and infusions [46]. Decreased P concentrations in young leaves were reported for tea plants exposed to progressive soil acidification leading to high Al availability [47]. Contrastingly, Mg and Ca concentrations remained unaffected, [48]. Al-induced enhancement of Ca and Mg uptake and a decline in Fe and Zn acquisition was found in hydroponically grown tea plants [49]. The ability to maintain adequate tissue concentrations of essential mineral nutrients, especially P, Ca and Mg seems to be a crucial feature of Al hyperaccumulators. Interestingly, Al accumulation in the hyperaccumulator *Melastoma malabathricum* was found to be independent of local Al soil availability, while the plants were apparently sensitive to local variations in availability of N, Ca and Mg [50]. Low P supply affected plant mineral nutrition less than Al. A direct influence of P supply was only found for leaf P and Ca, Fig. 2. However, significant interactions between Al and P supply on leaf concentrations of Fe, Mn, Ca, Mg, P and Al were observed. Such an interaction may be highly relevant in commercial tea plantations where P fertilization is a common management practice [51].

A direct negative interaction between Al and B tissue concentrations is clearly reflected in the PCA analysis, Fig.4. The strong interaction between Al and B is well described in both Al accumulator and Al-excluder plant species [52]. Despite the important Al-induced decrease of B in young leaves, no B deficiency symptoms were observed. According to current knowledge, the main function of B in plant performance is the cross linking of cis-diol groups in the cell walls. It has recently been proposed that the requirement for B is due to its ability to complex the polyphenolic substance in the plant cell walls so decreasing their toxicity [53]. Aluminium, instead of B, may assume such a role in the hyperaccumulator plants. In fact, according to different estimations between 50 and 80% of Al in tea leaves is located in the cell walls [54-56]. Mainly the cell-wall bound Al fraction increases in tealeaves under B deficiency [55]. There are different targets for Al binding in the cell walls including, among others, carboxyl groups of pectins and phenolic acids, cis-diol groups of sugars and polyphenolic compounds. Aluminium binding to cell wall pectins is a primary toxicity target causing cell wall stiffening and root growth inhibition in Al sensitive excluders [57]. Contrastingly, in hyperaccumulators a preferential binding of Al to cell wall polyphenols may favor root elongation by the inhibition of lignin formation [55]. Moreover, lignin formation may be reduced due to Al-induced decrease of cell wall bound phenolics, while the soluble fraction of leaf phenolics was increased by Al supply [55].

The influence of Al on the pattern of phenolics in tealeaves is not uniform. Our PCA analysis visualizes a negative influence of leaf Al concentrations on EGC, while the galloylated catechins ECG and EGCG were positively related to leaf Fe. Galloylated catechins like ECG and EGCG are mainly located in the vacuoles of tealeaves [58]. The serine carboxypeptidase like (SCPL) enzymes responsible for the transfer of the gallate group to the catechin moiety also operate in the vacuoles [59]. The abundant hydroxyl groups of galloyated catechins can strongly bind the excess of both Al and Fe that the cell wall has not retained, and store these potentially toxic elements safely in the vacuole.

In fact, after 96 h to either acid pH (-Al/-P and –Al/+P treatments) or Al (+Al/+P treatment), enhanced expression of galloylation-related genes of the SCPL1 was observed in old leaves (Fig 6). Activation of galloylation could help to detoxify the high concentrations of Al and Fe in the old leaves of the substrate-cultivated plants by both direct binding [60] and mitigation of oxidative stress [61]. Furthermore, Al supply in –P plants caused considerable activation of ANS, LAR and ANR in the old leaves, Fig. 6a, indicating enhanced biosynthesis of epicatechin and epigallocatechin, the substrates for galloylation to ECG and EGCG, respectively [39, 62].

In young leaves of +P plants, acid pH but not Al caused upregulation of genes of the SCPL family. Most enhanced was SCPL1A (7-fold) 96h after lowering the pH. DFR, ANS, LAR and ANR were also upregulated. This relatively large induction time suggests that the enhancement of galloylated catechins was not directly H^+-induced but may be an indirect consequence of the substrate acidification. This view is supported by the positive relation between leaf Fe concentrations and galloylated catechins in our PCA analysis (Fig 4).

In contrast to old leaves, the young ones showed an Al-induced enhancement in the relative expression of genes involved in the phenylpropanoid pathway (PAL and CHI); DFR and ANS from the flavonoid pathway were also enhanced, Fig. 6b. However, in contrast to old leaves the young ones did not show an Al-induced enhancement of LAR and ANR, while 3GT was upregulated, Fig. 6b. This gene coding for a glucosyl transferase is a key player in the control of anthocyanin accumulation [63]. Young leaves of purple tea varieties have enhanced 3GT expression. High levels of anthocyanin in young leaves relate to photoprotection [64]. Moreover, Al can strongly bind to anthocyanin causing a shift from purple to blue colour, especially visible in flowers like hydrangea [65]. In leaves, the green chlorophyll may mask the blue colour of the anthocyanin-Al complex, causing a dark green colouration of the leaves. This in fact was observed in our young tealeaves exposed to Al, Supplementary Fig. S2.

Taken together, our results show that acid pH and Al have a complex, age-dependent influence on the biosynthesis and accumulation of catechins in tealeaves. Under longer term, exposure, this in part is due to the strong negative influence of Al on the plant's mineral nutrition, affecting especially Mg, P, B and Mn leaf concentrations. In turn, these nutrients closely correlated to leaf EGC levels. Galloylated catechins show a positive relation with leaf Fe and galloylation related genes are upregulated by substrate acidification both in old and young leaves. Only the extremely high Al accumulation in old leaves activates genes involved in biosynthesis of galloylated catechins, while in young leaves the lower Al leaf concentrations activate genes involved in anthocyanin accumulation. This strong influence of low pH and enhanced Al availability on the polyphenolic pattern of tealeaves may alter both the leaves' antioxidant properties and their ability to bind Al and Fe in non-toxic form.

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Legends to figures

Fig. 1: Aluminium concentration (µg g^{-1} dry weight) in young and old leaves of tea plants grown for 8 weeks in nutrient solution without (-Al) or with (+Al) 200 Al µM $AlCl_3$ (45 µM free Al^{3+} activity) in combinationed with 12.5 µM P (-P) or 0.05mM P (+P) supply. Values followed by different letters a statistically different ($p < 0.05$). ANOVA, Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05.

Fig. 2: Concentrations of selected mineral nutrients (µg g^{-1} dry weight) in young and old leaves of tea plants grown for 8 weeks in nutrient solution without (-Al) or with (+Al) 200 Al µM $AlCl_3$ (45 µM free Al^{3+} activity) in combinationed with 12.5 µM P (-P) or 0.05mM P (+P) supply. Values followed by different letters a statistically different ($p < 0.05$). a) Phosphorus concentrations; ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; b) iron conentrations; ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; c) calcium concentrations, ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; d) manganese concentrations, ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; d) magnesium concentrations, ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; e) boron concetrations, ANOVA: Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05;

Fig. 3: Leaf concentrations of polyphenols in tea leaves (mg g^{-1} fresh weight) in young and old leaves of tea plants grown for 8 weeks in nutrient solution without (-Al) or with (+Al) 200 Al µM $AlCl_3$ (45 µM free Al^{3+} activity) in combination with 12.5 µM P (-P) or 0.05mM P (+P) supply. Values followed by different letters a statistically different ($p < 0.05$). a) Catechin and epicatechin (C/EC), ANOVA, Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; b) epigallocatechin (EGC), ANOVA, Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; c) epicatechingallate (ECG), ANOVA, Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05; d) epigallocatechingallate (EGCG), ANOVA, Al <0.05, P < 0.05, age <0,05, Al/P/age < 0.05;

Fig. 4: Multifactorial analysis of data on leaf concentrations of Al, selected mineral nutrients and polyphenols. Principal components were used for factor extraction with Eigenvalues > 1. Factors with loadings ≥ 0.7, marked in red were used for bidimensional presentation.

Fig 5: Schematic presentation of metabolic pathways involved in polyphenol biosynthesis in tea. Genes coding for enzymes marked in blue were analysed by RT-qPCR.

Fig. 6: Relative expression of selected key genes of the phenylpropanoid/ flavonoid pathways (a, b) and catechin galloylation (c, d) in old (a, c) and young (b, d) tea leaves. Colour scale indicates down regulation (blue), no change (grey) and upregulation (red).

FIGURE 1

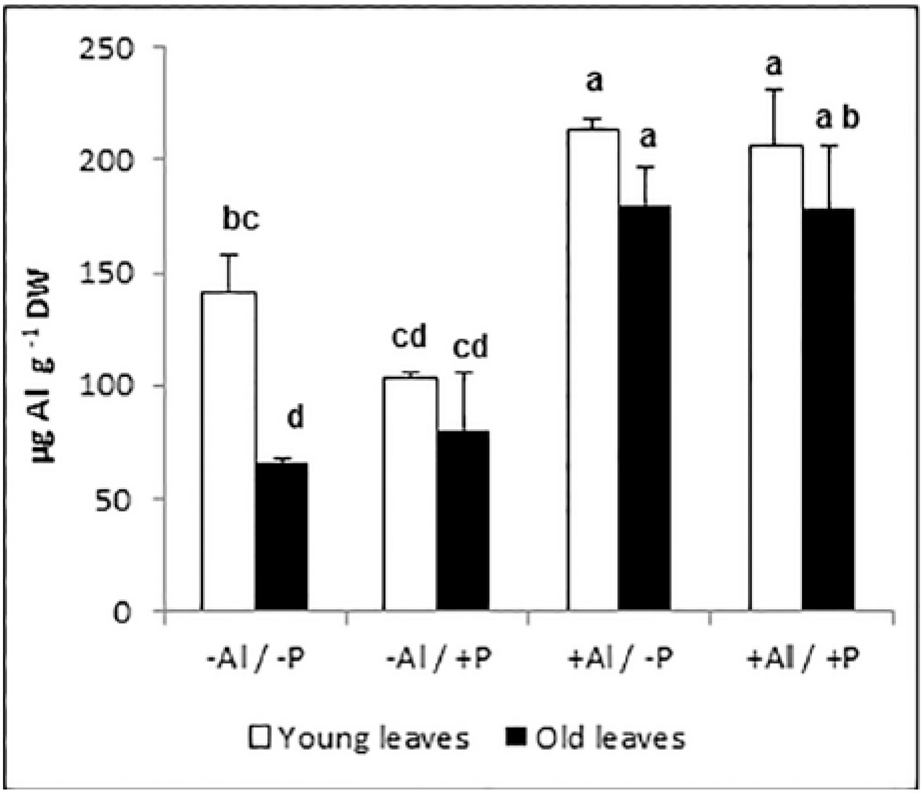
μg Al g -1 DW
250
200
150
100
50
0
bc
d
cd
cd
a
a
a
a b
-Al / -P
-Al / +P
+Al / -P
+Al / +P
☐ Young leaves
■ Old leaves

FIGURE 2

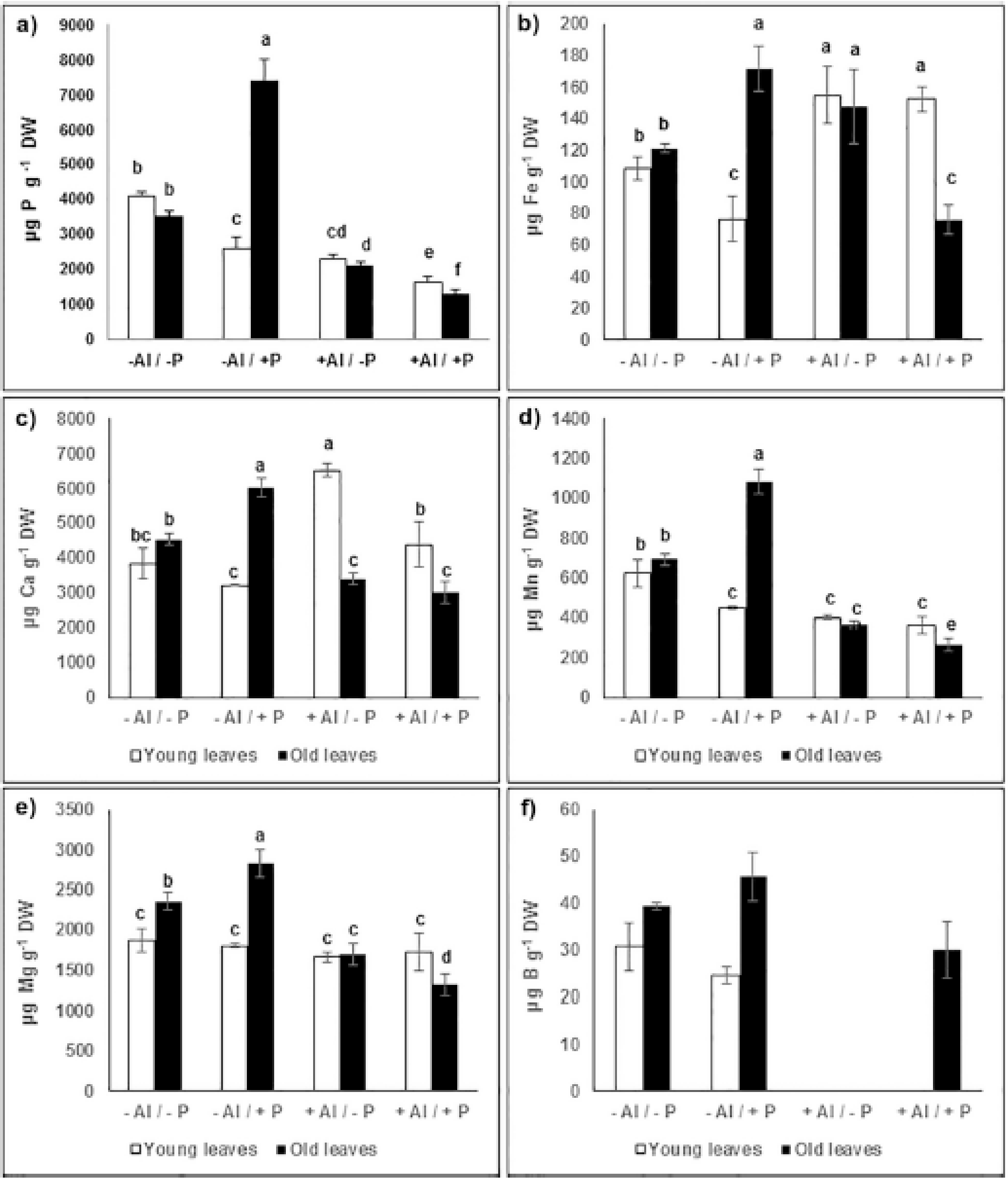
a)
μg P g-1 DW
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
b
b
c
a
cd
d
e
f
-Al / -P
-Al / +P
+Al / -P
+Al / +P
b)
μg Fe g-1 DW
200
180
160
140
120
100
80
60
40
20
0
b
b
c
a
a
a
a
c
- Al / - P
- Al / + P
+ Al / - P
+ Al / + P
c)
μg Ca g-1 DW
8000
7000
6000
5000
4000
3000
2000
1000
0
bc
b
c
a
a
c
b
c
- Al / - P
- Al / + P
+ Al / - P
+ Al / + P
□Young leaves
■Old leaves
d)
μg Mn g-1 DW
1400
1200
1000
800
600
400
200
0
b
b
c
a
c
c
c
e
- Al / - P
- Al / + P
+ Al / - P
+ Al / + P
□Young leaves
■Old leaves
e)
μg Mg g-1 DW
3500
3000
2500
2000
1500
1000
500
0
c
b
c
a
c
c
c
d
- Al / - P
- Al / + P
+ Al / - P
+ Al / + P
□Young leaves
■Old leaves
f)
μg B g-1 DW
60
50
40
30
20
10
0
- Al / - P
- Al / + P
+ Al / - P
+ Al / + P
□Young leaves
■Old leaves

FIGURE 3

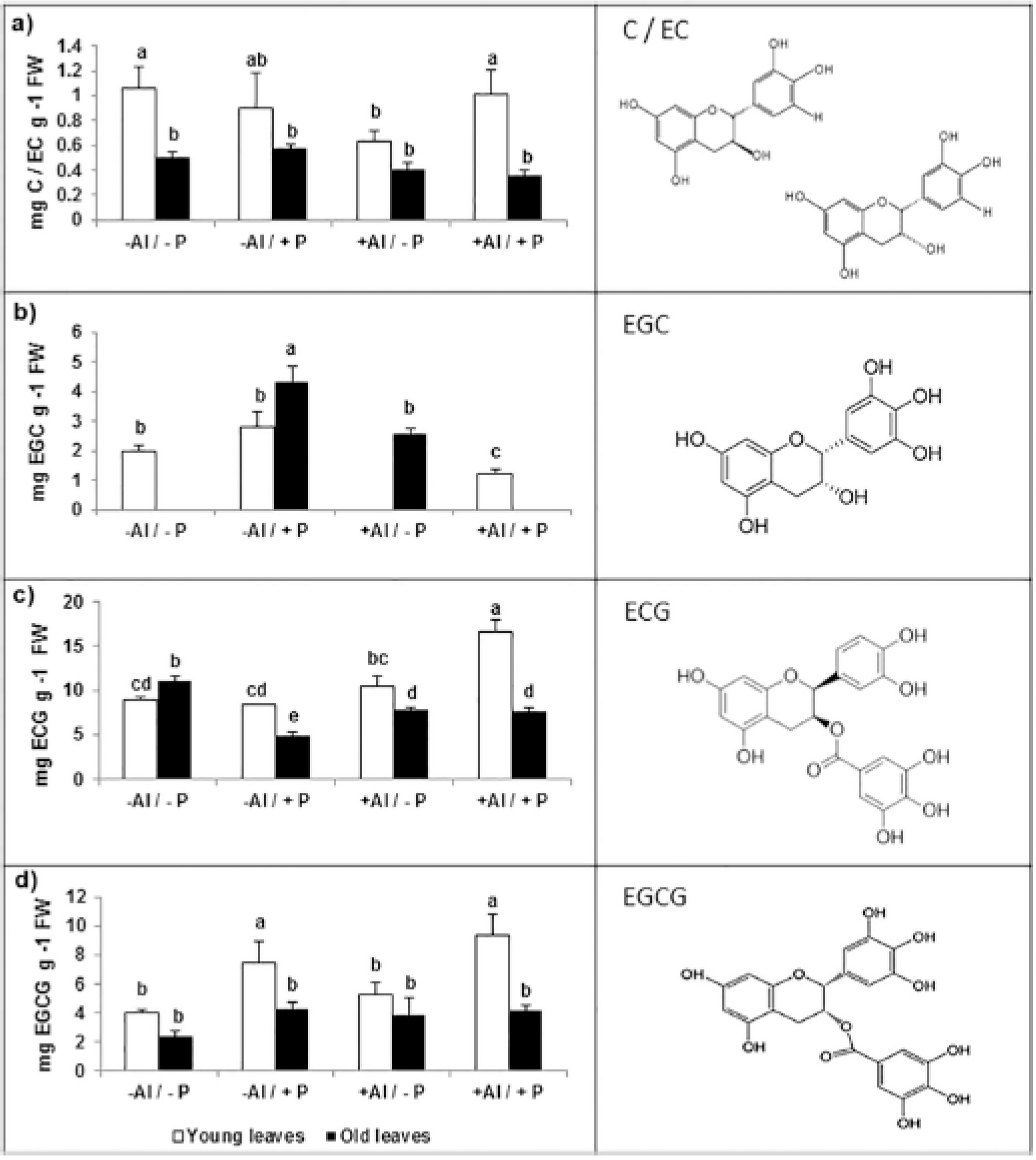
a)
mg C / EC g -1 FW
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
ab
b
b
b
a
b
-Al / - P
-Al / + P
+Al / - P
+Al / + P
C / EC
HO
OH
OH
OH
OH
H
b)
mg EGC g -1 FW
6
5
4
3
2
1
0
b
b
a
b
c
EGC
c)
mg ECG g -1 FW
20
15
10
5
0
cd
b
cd
e
bc
d
a
d
ECG
O
d)
mg EGCG g -1 FW
12
10
8
6
4
2
0
b
b
a
b
b
b
a
b
EGCG
□Young leaves ■Old leaves

FIGURE 4

FIGURE 5

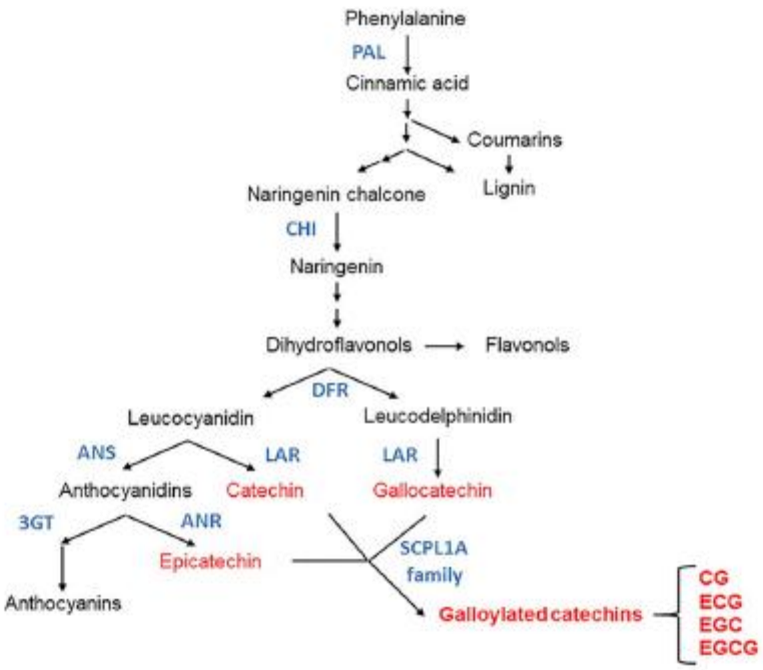

FIGURE 6