SUBCELLULAR DISTRIBUTION AND CHEMICAL FORMS OF FLUORIDE IN TEA TREE LEAVES (*CAMELLIA SINENSIS* L.) AND ITS CELL WALLS

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ABSTRACT: Tea tree (*Camellia sinensis* L.) is an hyper-accumulator of fluorine (F), especially in leaves. Here, the accumulation of different fluorine forms and their subcellular distribution in leaves and cell walls were analyzed in three tea cultivars (F-sensitive Wuniuzao, F-resistant Fudingdabai, and Fuyun No.6). Results indicated that the F content varied significantly in the three cultivars and increased with the maturity of leaves, with most F accumulated in cell walls. Specifically, the greatest amount of F was found in the water-soluble form, about 61.90–87.52% of the total fluorine, followed by the residual-fluorine, about 5.41–27.25%, the organic matter-bound fluorine, about 1.92–8.32%, and, with the lowest proportion, the iron-manganese-bound and exchangeable fluorine. Fluorine in the cell walls was mainly present in the water-soluble form, followed by residual-fluorine. There was a distinct difference between the three different cultivars in F concentration, subcellular distribution, and chemical forms. When compared with the two F-resistant cultivars, the F-sensitive Wuniuzao had higher water soluble F and less residual F in leaves and cell walls. These results suggest that mature leaves are the main F enrichment site, and the retention of F in the cell wall in a bound form might be an important way for tea plants to reduce F toxicity.

Keywords: *Camellia sinensis* L.; Chemical forms; Distribution; Fluorine.

Abbreviations: CW: cell wall; Ex-F: exchangeable fluorine; Fe/Mn-F: Fe/Mn combined fluorine; F: Fluorine; FD: Fudingdabai cultivar; FY No.6: Fuyun No.6 cultivar; Green: leaves from green stems; Grey: leaves from grey stems; MC: mitochondria fraction; NC: nucleus and chloroplast fraction; O.M.-F: organic matter bound fluorine; Red: leaves from red stems; Res-F: residual fluorine; SR: soluble fraction containing ribosome; WNZ: Wuniuzao cultivar; Ws-F: water soluble fluorine.

INTRODUCTION

Tea plant (*Camellia sinensis* L.), a hyper-accumulator of fluorine (F), contains an abundance of F when grown in acidic soils or exposed to additional airborne sources, although F is a non-essential element to plants.[1] For most plants, F is phytotoxic and can inhibit plant growth and development by affecting the activities of a series of enzymes and metabolism such as ATPases.[2] However, tea plants can accumulate more than 1,000 mg/kg F in leaf without obvious toxicity symptoms. F uptake from soil is the main pathway of F accumulation in tea plants, which is affected by pH and calcium content of the soil, and with pH increased from 4.3 to 6.5, F in the tea leaf decreased linearly.[3-4] Exogenous aluminum (Al) and ferrum (Fe) were also reported to promote F accumulation in leaves.[5-6] The amount of F accumulation was correlated with the form and concentration of fluorine in matrix as well as the duration of F exposure.[7-9] Besides, the breed variety of tea cultivars also has a great impact on the F accumulation ability.[10-11] Most of the fluoride is accumulated in tea leaves, especially in mature or old leaves, accounting for 90% of the fluoride accumulated in the whole tea plant.[12-13] When cultured in solution with a different concentration of fluorine, most of the fluoride in root, stem, and leaf of tea plant was distributed in cell wall (76.84%–91.58%) and soluble fraction (53.24%–80.35%). However, researchers varied in their views about the form of F in tea leaf. Negata and

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Liang considered that F is upwardly transported mainly as F-Al complex through xylem and finally accumulated in leaves,[14-15] while Morita maintained that F was mainly present in the form of free ion (F^-), with a small amount of F-Al complex stored in the leaf.[16] Currently, the F forms still remain to be elucidated in tea tree, and the mechanism for the tea tree as an F accumulator is also not clear. Some studies have shown that the toxicity of nonmetal ions or metal ions to plants is related to their biological activity, subcellular distribution, and chemical forms in plants.[17-18] The objective of the present work was to explore the possible mechanism for the F tolerance of tea plants by investigating the subcellular distribution of F in tea leaves and the F forms in tea leaves and cell walls from stems at three different maturity stages using three different F-sensitive cultivars.

MATERIALS AND METHODS

Tea leaves: Fresh leaves (*Camellia sinensis* L.) on green, red, and grey stems of the same branch were picked separately from Wuniuzao (WNZ), Fudingdabai (FD) and Fuyun No.6 (FY No.6) cultivars planted in the same tea garden of Huazhong Agricultural University, Wuhan, China, in 2015. Previous laboratory studies have shown that WNZ is an F-sensitive cultivar, while FY No.6 and FD are F-resistant cultivars.[19] After washing with distilled water and removing the surface moisture, one portion of the leaves was stored at –80°C and the rest were enzyme-deactivated with steam for 5 min, dried in a dryer at 55ºC, ground, and sieved through a 0.42 mm sieve.

F standard solution was purchased from Shanghai Metrology and Testing Technology Research Institute (Shanghai, China), dithioerythritol (DTE) from Beijing Suolaibao Science and Technology Co., Ltd. (Beijing, China), tris (hydroxy methyl) aminomethane (Tris) from Biosharp Co., Ltd. (USA), and cyclohexanediaminetetraacetic acid (CDTA) from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). All other reagents and solvents were purchased from China National Pharmaceutical Group Corporation (Beijing, China) and were of analytical grade. Purified water (18.2 MΩ) was prepared using a Millipore Mill-Q Ultrapure Water System (Billerica, MA, USA).

Subcellular separation method: The organelles were separated by differential centrifugation as previously described.[20] Briefly, 50 g of fresh leaves were placed in a mortar, followed by the addition of liquid nitrogen and grinding into powder. Next, 500 mL buffer (50 mmol/L Tris-HCl, 250 mmol/L sucrose, 1.0 mmol/L DTE, 5.0 mmol/L ascorbic acid, pH 7.5) was added into the mortar to grind the tissue into homogenate. After filtering through a 100-μm nylon gauze, the mixture was washed with 100 mL buffer, and the residue was the cell wall fraction (CW). The filtrate was collected and centrifuged for 15 min at 1500×g and 4ºC. The precipitated fraction was the nucleus and chloroplast fraction (NC). The supernatant was centrifuged for 35 min at 15000×g and 4ºC. The precipitated fraction was mitochondrial fraction (MC) and the supernatant was the soluble fraction containing ribosome (SR). Each fraction was freeze-dried.

Preparation of cell wall: Cell wall was prepared as previously reported.[20] Briefly, the fresh tea leaves were homogenized with the pre-cooled ethanol solution (75%) at the ratio of 1:10 (m/v), and after standing for 20 min, the mixture was centrifuged at 4000×g for 10 min at 4ºC. The precipitate was washed with pre-chilled acetone,

methanol-chloroform mixture (l: 1, v/v), and methanol in turn. The precipitate was lyophilized and ground in nitrogen as the cell wall sample.

Analysis of fluorine forms: Sample was continuously extracted as previously described.[21] Briefly, 5 g of tea powder (or cell wall) were added into a 500 mL stopper conical flask, followed by the addition of 200 mL ultra-pure water, incubation in a water bath at 70ºC for 30 min, shaking once per 5 min, and centrifugation at 4000×g for 20 min. The residue was extracted again as described above. The supernatant was the water soluble fluorine fraction. The precipitate was transferred to a 150 mL conical flask with 50 mL NaAc (1 mol/L, adjusted pH to 7.0 using acetic acid), incubated in a constant temperature oscillation incubator (200 r/min) at 25ººC for 2 hr, and centrifuged at 4000×g for 20 min. The supernatant was the exchangeable fluorine fraction. The precipitate was then extracted with 100 mL of hydroxylamine hydrochloride (0.04 mol/L, adjusted pH to 2.0 using HCl), warm bathed with shaking (200 r/min) at 25ºC for 2 hr, and centrifuged at 4000×g for 20 min. The supernatant was the Fe/Mn bound fluorine fraction. The precipitate was mixed with 6 mL 0.02 mol/L HNO_3, 20 mL 30% H_2O_2 (pH 2.0, adjusted with nitric acid), and bathed at 83± 3ºC for 2.5 hr. After cooling to room temperature, 5 mL ammonium acetate-nitric acid solution (3.2 mol /L) was added and diluted to 50 mL, and after standing at room temperature for 2 hr, the mixture was centrifuged at 4000×g for 20 min. The supernatant was organic matter-bound fluorine fraction. The residue was dried at 80ºC as the residue fluorine fraction.

Fluorine content analysis: Solid samples were pretreated as previously described.[9] Briefly, 0.05 g of samples was placed in a 50 mL nickel crucible, and wetted with a little ultrapure water, followed by the addition of 1.5 mL 8.375 mol/L NaOH, and incubation in an oven at 80ºC for 1hr, 150ºC for 1 hr, 300ºC for 30 min, and 600ºC for 2 hr. After cooling to room temperature, the samples were neutralized using HCl, filtered with a filter paper to 25 mL volumetric flasks, and brought to the volume of 25 mL with deionized water.

The analyte (10 mL) was mixed in a beaker with 10 mL TISAB buffer (3 mol/L sodium acetate and 0.75 mol/L sodium citrate for 1:1 mixing ratio), and the fluorine content of the samples was measured with a fluoride ion selective electrode.

Statistical analysis: The data are presented as the mean±SE of at least three repeated experiments. Significant differences were analyzed by one-way ANOVA, and LSD test was used for multiple comparisons (SPSS Statistics 17.0 software). Differences were considered to be significant at $p<0.05$.

RESULTS

Fluorine content in tea leaves: The F contents of leaves from the same branch at three different maturity stages and three cultivars are shown in Table 1. The F content values ranged from 455.96 to 2061.75 mg/kg, with the lowest content in green stem leaves and the highest in grey stem leaves. The F content increased significantly with increasing leaf maturity. The F content varied obviously in the three cultivars, with the highest content in WNZ for the stem leaves with the same maturity.

Table 1. Fluorine content in tea leaves (mg/kg)

Cultivars	Green stem leaves	Red stem leaves	Grey stem leaves
WNZ	655.59±15.74Ac	1046.43±10.59Ab	2061.75±16.59 Aa
FD	455.96±18.42 Bc	684.56±13.13 Cb	2025.59±23.02 Ba
FY	476.11±7.50 Bc	876.80±26.86 Bb	1736.54±11.76 Ca

Note: WNZ:(Wuniuzao cultivar), FD: (Fudingdabai cultivar), FY No.6:(Fuyun No.6 cultivar). Data are expressed as mean±SE (n=3). Different capital letters (small letters) in the same column (line) mean significant differences at $p<0.05$.

Subcellular distribution of fluorine in tea leaves: Cell walls (CW), nuclear and chloroplast (NC), mitochondria (MC), and soluble fraction containing the ribosome (SR) were separated by differential centrifugation of fresh tea leaves. The F content was analyzed and shown in Figures 1A–1D.

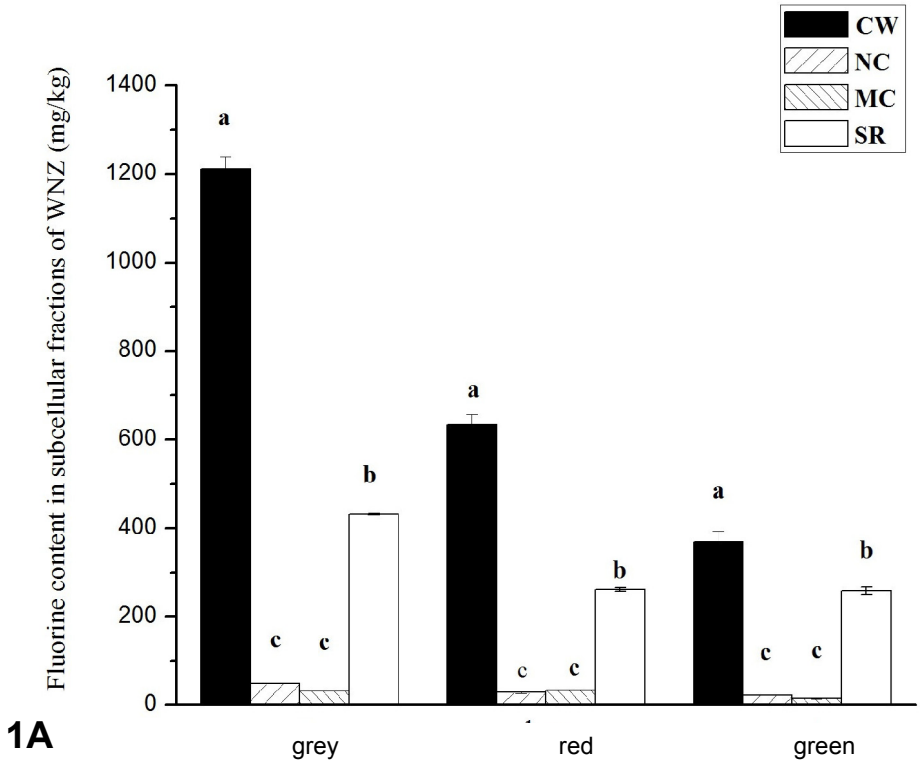


Figure 1A. Fluorine distribution in subcellular components of leaves of the WNZ (Wuniuzao cultivar) tea cultivar (mg/kg dry weight of leaves).
Note: CW: (cell wall), NC: (nucleus and chloroplast), MC: (mitochondria), SR: (soluble fraction containing ribosome). Green: (leaves form green stems), Red: (leaves from red stems), Grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

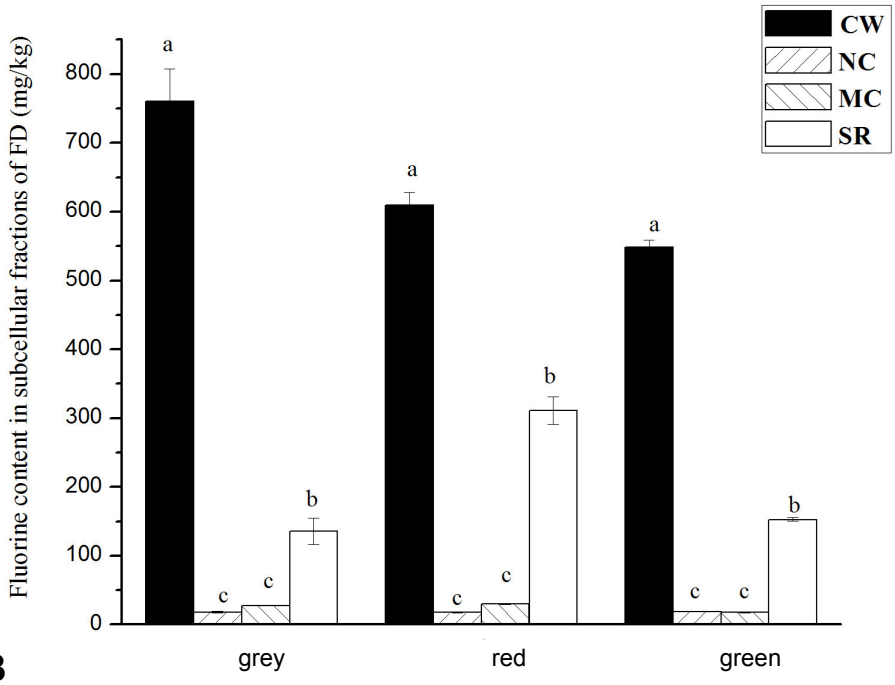


Figure 1B. Fluorine distribution in subcellular components of leaves of the FD (Fudingdafai cultivar) tea cultivar (mg/kg dry weight of leaves).
Note: CW: (cell wall), NC: (nucleus and chloroplast), MC: (mitochondria), SR: (soluble fraction containing ribosome). Green: (leaves form green stems), Red: (leaves from red stems), Grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

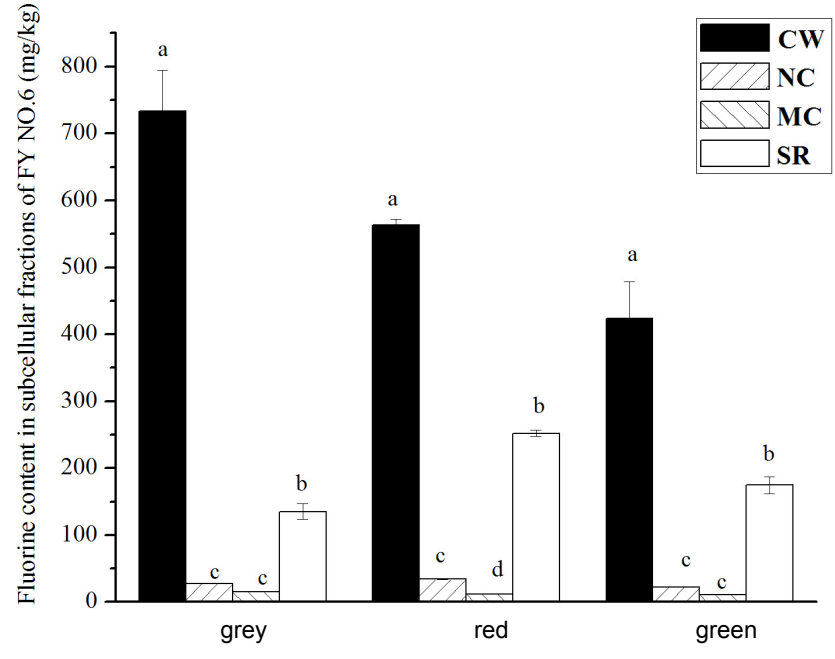
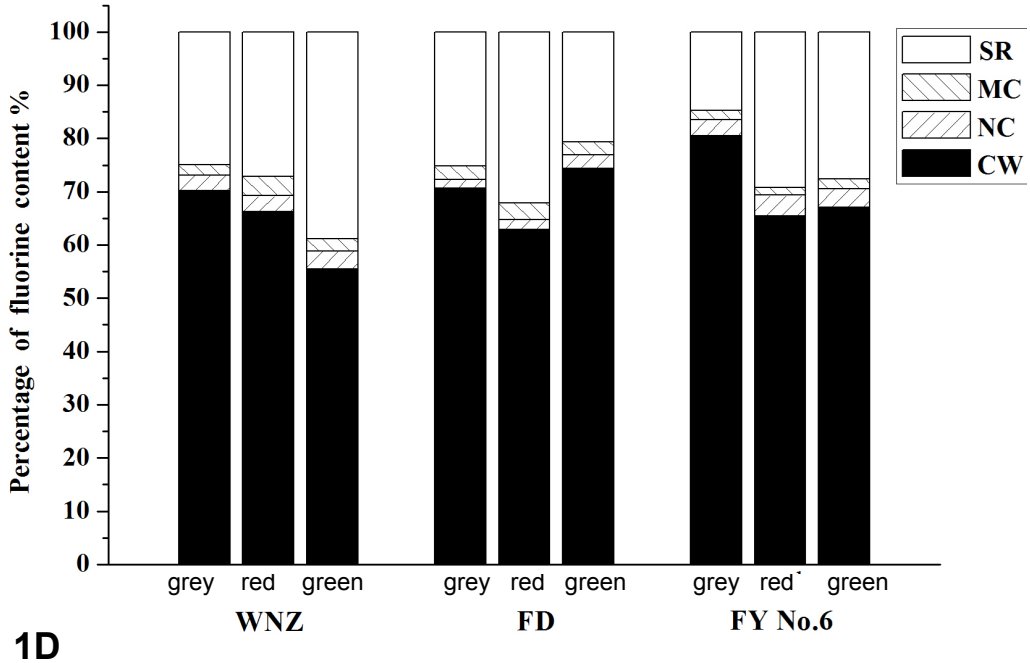


Figure 1C. Fluorine distribution in subcellular components of leaves of the FY No.6 (Fuyun No.6 cultivar) tea cultivar (mg/kg dry weight of leaves).
Note: CW: (cell wall), NC: (nucleus and chloroplast), MC: (mitochondria), SR: (soluble fraction containing ribosome). Green: (leaves form green stems), Red: (leaves from red stems), Grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.



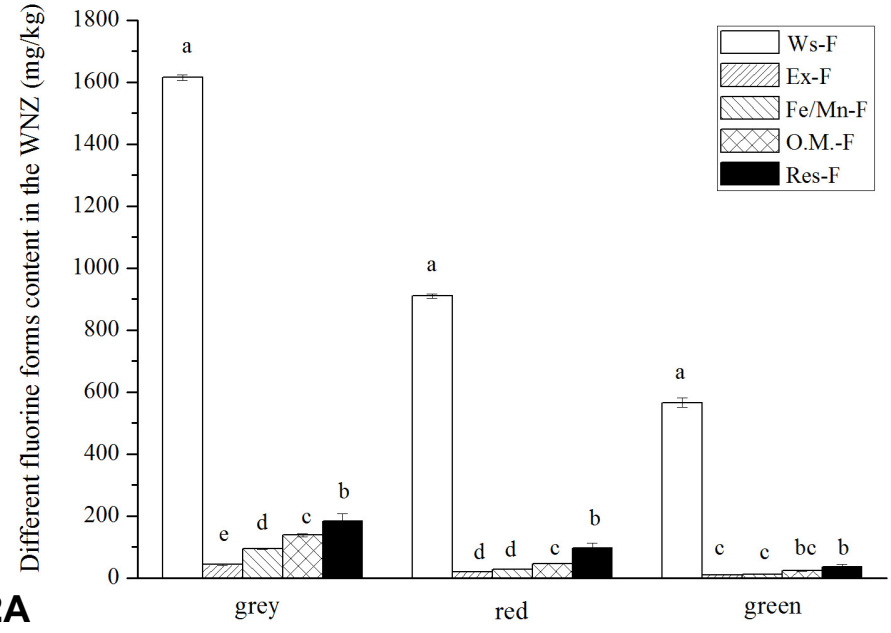
1D

Figure 1D. Fluorine distribution in subcellular components of leaves of three different tea cultivars. Percentage of fluorine content (%).
Note: CW: (cell wall), NC: (nucleus and chloroplast), MC: (mitochondria), SR: (soluble fraction containing ribosome). Green: (leaves form green stems), Red: (leaves from red stems), Grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

There was a significant difference in the F contents of the subcellular fractions, with the highest F content in CW, reaching 368.31 to 1,212.92 mg/kg dry weight of leaves, about 55.45–80.49% of the total, which was significantly higher than that of the other fractions. The second highest F content was found in SR fraction, covering 14.80–38.88% of the total. MC and NC contained the least amount of F, less than 4.0% of the total. The fluorine in WNZ, FD, and FY No.6 showed the same distribution tendency. In WNZ and FY cultivars, the rate of F content in CW increased in the older stems of leaves, while that in SR fraction increased in the tender stems of leaves. The F-sensitive Wuniuzao contained more F in SR and less F in the CW of the green stem leaf, when compared to the two F-resistant cultivars.

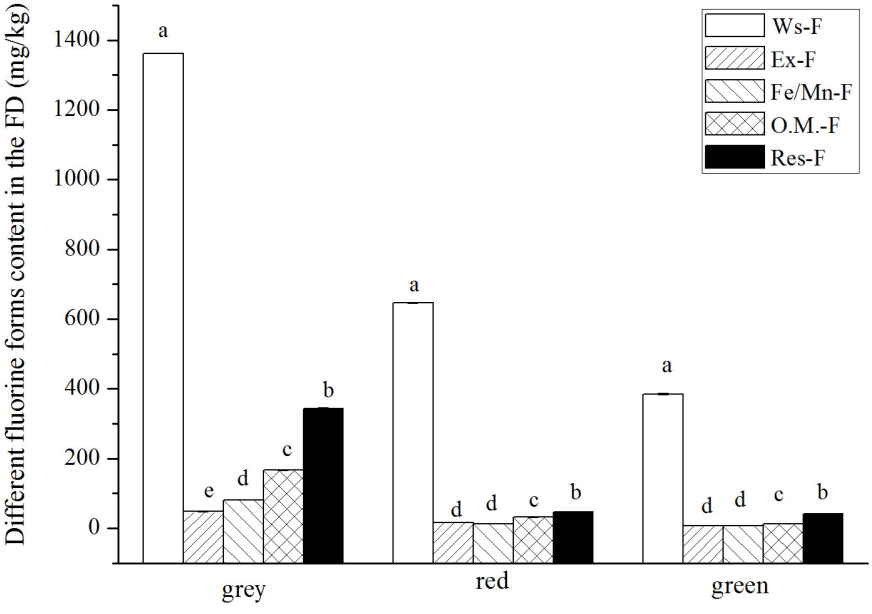
Fluorine forms in tea leaves: Using the analysis method of F forms in soil,[22] five F forms were obtained from the tea leaves, which consisted of water-soluble fluorine (Ws-F), exchangeable fluorine (Ex-F), Fe/Mn combined fluorine (Fe/Mn-F), organic matter-bound fluorine (O.M.-F) and residual fluorine (Res-F) (Figures 2A–2D). The main F form was Ws-F, about 385.17 to 565.94 mg/kg in green stem leaves and 1106.08 to 1615.77 mg/kg in grey stem leaves, accounting for 61.90–87.52% of the total in dry leaves, much higher than that of the other F forms. The second main F form was Res-F, about 34.95 to 54.05 mg/kg in green stem leaves and 183.15 to 486.91 mg/kg in grey stem leaves, occupying 5.41–27.25% of the total. Comparatively, Fe/Mn-F or Ex-F had the lowest proportion, covering less than 2.3% of the total, with 6.54 to 12.29 mg/kg in green stem leaves and 29.42 to 94.29 mg/kg in grey stem leaves. With the maturing of leaves, the proportion of Ws-F decreased significantly while the proportion of the other F forms increased significantly,

especially the Res-F in grey stem leaves, which means more Ws-F was transformed into Res-F. Among the three cultivars, the rate of each F form in leaves varied, and the F-sensitive Wuniuzao contained more Ws-F and less Res-F.



2A

Figure 2A. Different forms of fluorine in leaves of the WNZ (Wuniuzao cultivar) tea cultivar (mg/kg). Note: Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F: (residual fluorine). green: (leaves form green stems), red: (leaves from red stems), grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.



2B

Figure 2B. Different forms of fluorine in leaves of the FD (Fudingdafai cultivar) tea cultivar (mg/kg). Note: Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F: (residual fluorine). green: (leaves form green stems), red: (leaves from red stems), grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

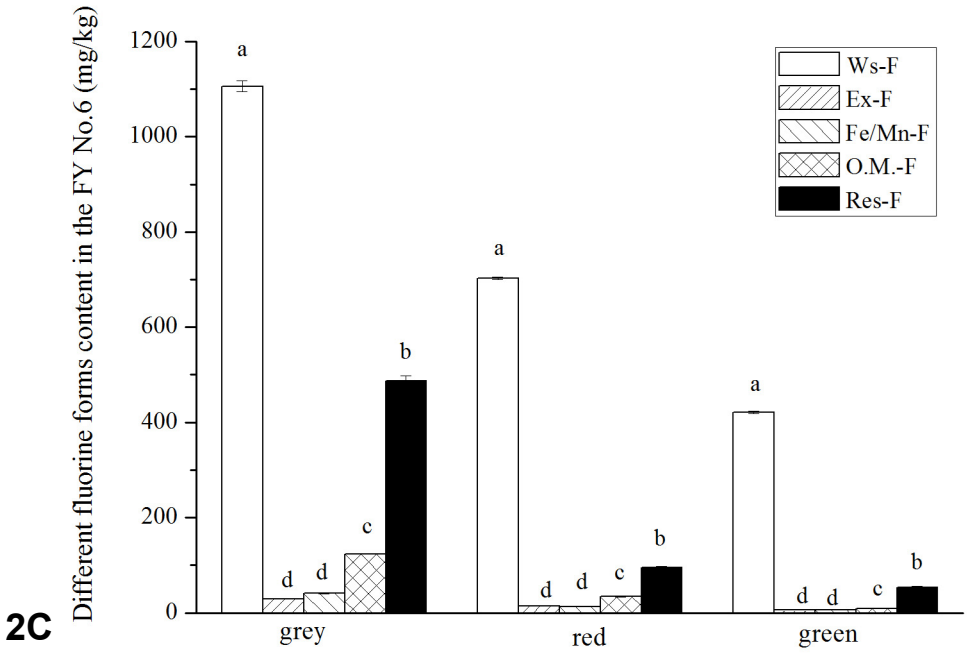


Figure 2C. Different forms of fluorine in leaves of the FY No.6 (Fuyun No.6 cultivar) tea cultivar (mg/kg). Note: Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F: (residual fluorine). green: (leaves form green stems), red: (leaves from red stems), grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

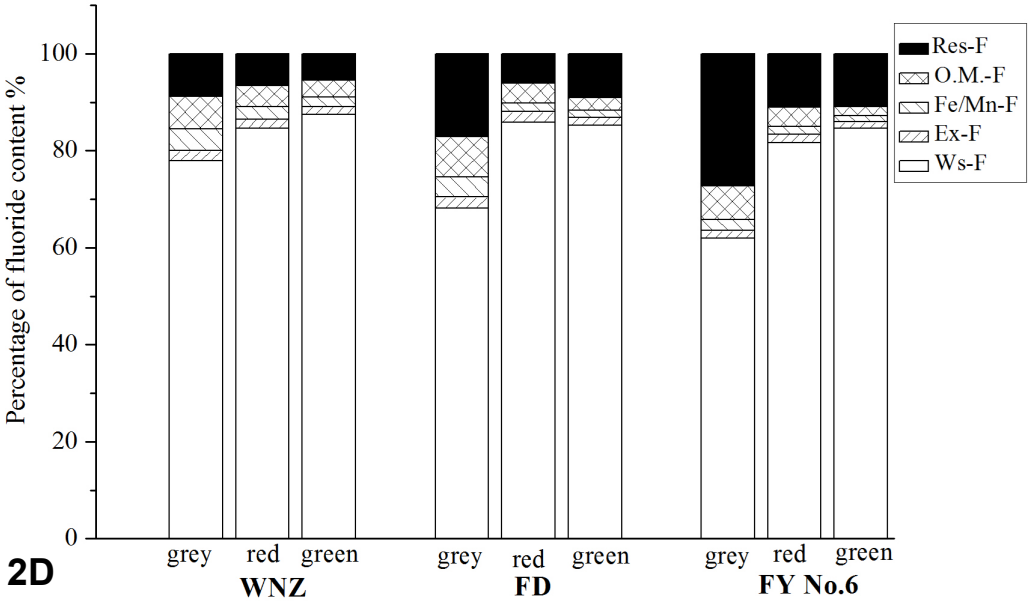


Figure 2D. Different forms of fluorine in leaves of three different tea cultivars. The proportion of fluorine forms (%). Note: Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F: (residual fluorine). green: (leaves form green stems), red: (leaves from red stems), grey: (leaves from grey stems). Data are expressed as mean±SE (n=3). Different small letters in the same leaf mean significant differences at $p<0.05$.

Fluorine forms in cell wall: As cell walls contained the most F of tea leaves, the F forms in cell walls were also analyzed and the results are shown in Table 2 and Figure 3. In the cell walls of tea leaves, the main F form was also Ws-F, accounting for 82.46–83.96% of the total in cell wall, which was significantly higher than that of the other F forms. Res-F was the second dominant F form, covering 6.82–8.94% of the total. The O.M.-F accounted for 1.50–1.90% of the total with the lowest proportion. Among the three cultivars, the F-sensitive Wuniuzao contained more Ws-F in cell wall than the other two cultivars, which was consistent with the result of leaves.

Table 2. Fluorine forms in cell wall

Fluoride forms	Fluorine content (mg/kg)		
	WNZ	FD	FY No.6
Ws-F	1721.70±14.22a	1623.97±11.45a	1354.90±42.48a
Ex-F	61.20±2.15d	67.23±1.12cd	49.74±3.49cd
Fe/Mn-F	86.40±2.98c	91.41±3.31c	66.90±3.71c
O.M.-F	31.80±3.83e	37.08±6.82de	24.62±1.61e
Res-F	149.51±9.83b	133.17±21.52b	146.88±12.66b

Note: Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F : (residual fluorine). WNZ : (Wuniuzao cultivar), FD: (Fudingdabai cultivar), FY No.6: (Fuyun No.6 cultivar). Data are expressed as mean±SE (n=3). Different small letters in the same column mean significant differences at $p<0.05$.

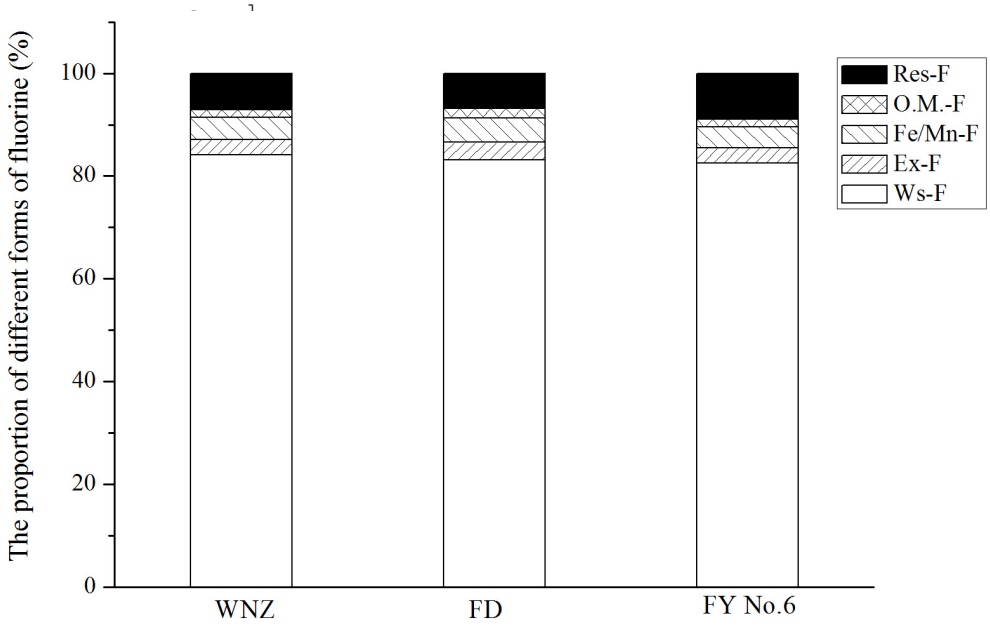


Figure 3. The proportion of different forms of fluorine in cell walls. Ws-F: (water soluble fluorine), Ex-F: (exchangeable fluorine), Fe/Mn-F: (Fe-Mn combined fluorine), O.M.-F: (organic matter bound fluorine), Res-F: (residual fluorine). WNZ: (Wuniuzao cultivar), FD: (Fudingdabai cultivar), FY No.6: (Fuyun No.6 cultivar).

DISCUSSION

It has been documented that there exists soil environment and genotypic differences in F accumulation.[23] In this study, genotypic difference was also observed in F accumulation of tea plants. F was mainly found to exist in mature leaf, suggesting the involvement of an F tolerance strategy based on F sequestration in the plant cells. Furthermore, F subcellular distribution was studied in three tea cultivars with a difference in F tolerance. It was noted that F was mainly accumulated in cell wall, then present in the form of soluble fraction, and the lowest in nucleus and chloroplast fraction or mitochondria fraction. The F content in cell wall fraction increased with increasing leaf maturity. The F content in green stem cell wall of F-sensitive cultivar (WNZ) was lower than that of F-resistant cultivars (FD or FY No.6), while the F content in SR fraction of WNZ was higher than that of the other two cultivars. The cell wall belongs to the non-physiological active region of plants. The accumulation of F in cell wall is helpful to reduce the toxicity of F in protoplast, the cell metabolism center, so as to maintain the normal physiological metabolism of the cells. Plant cell wall, a complex structure mainly consisting of polysaccharides, protein, and lignin, is widely involved in plant growth and development and stress response, which is the first barrier against the entry of heavy metal ions and other harmful elements into the cytoplasm.[22,24] Those macromolecules, including proteins, polysaccharides, and pectins in the cell wall, contain aldehyde, carboxyl, amino, and phosphate groups and can supply a large number of exchange sites for metal and non-metal ions to affect the combination of cell wall with ions.[20,22,25-27] Our previous studies have shown that polysaccharides in tea have the characteristics of F absorption, which is related to the composition and structure of polysaccharides and the contents of proteins, Mn^{2+}, Al^{3+}, and Fe^{3+} in it.[9,28] In addition, cell wall is the main calcium pool in plants, in which most of the calcium is combined with the pectin.[29-30] F in the cell wall might be combined with the metal ions such as Ca^{2+} and Mg^{2+} and is retained in the cell wall to reduce the F toxicity. Soluble fraction comes from the cytoplasm and vacuole. As an important organ for accumulation and storage of nutrients and metabolic products as well as a variety of proteins, organic acids, alkaloids, salts, and other substances, the vacuole can absorb and store the excess cytoplasm intermediates and reduce the enzyme and organelle damage.[31-32] Large amounts of F were retained in the cell wall and vacuole, which might be an important way for tea plants to reduce F toxicity. In this study, the organelles were separated by differential centrifugation, which is probably not a proper method for measuring the F content in organelles as it might cause the mixing of organelles with other substances, and the F content in each organelle was related to that of the total dry leaf. To obtain the exact content of F in organelles and its accumulation mechanism need to be further explored.

Several studies have shown that the toxicity of non-metal ions or metal ions to plants is related to the chemical forms of their biological activities in plants.[33-34] The fluoride in tea plants has many forms, and thus various bio-activities. Ws-F and Ex-F are strongly migratory and can be used by organisms as bio-available F, while Fe/Mn-F, O.M.-F and Res-F are relatively stable with lower bio-availability. In this study, the tender leaf contains less F but more water soluble F, while the mature leaf contains more F but less water soluble F, suggesting that F is not an essential element for tea plant, and the excessive F is transported to the mature leaves in cell wall or

vacuole in a bound form. Furthermore, F-sensitive Wuniuzao also retains more water soluble F and less residual F than the F-resistant cultivars, implying that the former F fixation capacity is weaker than that of the latter two and might be more vulnerable to F stress.

CONCLUSION

The F content in tea leaves increased with leaf maturity, and the F content in the subcellular fractions was highest in cell walls, followed by the soluble components of the ribosomes. Additionally, the F was mainly present in the water-soluble form in both tea leaves and cell walls. F-sensitive cultivar contained more soluble F than the two F-resistant cultivars in leaves and cell walls. Retaining F in cell walls and transforming water soluble F to bound forms in leaves might be important ways for tea plants to reduce F toxicity.

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